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**Semen-mediated enhancement of HIV-1 infection
markedly impairs the antiviral efficacy of microbicides**

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ABBREVIATIONS

aa	Amino acid
AIDS	Acquired Immune Deficiency Syndrome
β	Beta
β -gal	Beta-galactosidase
°C	Degree-Celsius
CCR5	C-C chemokine receptor type 5, CD195
CD	Cluster of differentiation
CXCR4	C-X-C chemokine receptor type 4
Da	Dalton
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DRK	German: Deutsches Rotes Kreuz (German Red Cross)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
<i>E. coli</i>	<i>Escherichia coli</i>
<i>et al.</i>	<i>et alia</i>
<i>e.g.</i>	<i>exempli gratia</i>
<i>Env</i>	Envelope
FCS	Fetal calf serum
FDA	Food and Drug Administration
Fig.	Figure
g	Gram
<i>g</i>	Gravitational force
<i>Gag</i>	Group specific antigen
GALT	Gut-associated lymphoid tissue
gp	Glycoprotein
gp120	Envelope glycoprotein of HIV, 120 kDa
gp41	Envelope glycoprotein of HIV, 41 kDa
h	Hour(s)
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus

HRP	Horseradish peroxidase
<i>i.e.</i>	<i>id est</i>
IL-2	Interleukin-2
IN	Integrase
INI	Integrase inhibitor
kb	Kilo base pairs
kDa	Kilodalton
LTR	Long-terminal-repeat
mAB	Monoclonal antibody
min	Minute(s)
MOI	Multiplicity of infection
mRNA	Messenger RNA
nAb	Neutralising antibody
<i>Nef</i>	Negative factor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide analogue reverse transcriptase inhibitor
p24	Capsid protein of HIV, 24 kDa
pAB	Polyclonal antibody
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pH	<i>Potentia hydrogenii</i>
PHA	Phytohaemagglutinin
PR	Protease
PI	Protease inhibitor
<i>Pol</i>	Polymerase
<i>Rev</i>	Regulator of expression of virion proteins
RFU	Relative fluorescence units
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
Rpm	Revolutions per minute

RT	Room temperature, 20-25 °C
RT	Reverse transcriptase
RTI	Reverse transcriptase inhibitor
s	Seconds
SE	Semen
SEVI	Semen-derived Enhancer of Virus Infection
SOC	Super optimal broth
SP	Seminal plasma
TAE	Tris base, acetic acid, EDTA
Tat	Transactivator of transcription
Tris	Tris(hydroxymethyl)aminomethane
<i>Vif</i>	Viral infectivity factor
<i>Vpr</i>	Viral protein R
<i>Vpu</i>	Viral protein unknown
v/v	Volume per volume

Amino acid code:

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

1 INTRODUCTION

The AIDS Pandemic is still thriving despite medical breakthroughs and an improved accessibility of antiretroviral medication. In the early 1980s, several cases of an aggressive form of Kaposi's sarcoma, a cancer caused by herpesvirus 8, was detected amongst young homosexual men in the US [50, 63]. In 1983, Human Immunodeficiency Virus type 1 (HIV-1) was identified and associated with the increased incidence of Kaposi's sarcoma [17]. In the last 30 years, more than 35 million people have died from Acquired immunodeficiency syndrome (AIDS), the result of HIV-1 infection [1]. In 2013, with over 2.1 million new HIV-1 infections and 1.5 million AIDS deaths recorded, the AIDS pandemic persists. [1]. Indeed, it is estimated that 35 million people are currently living with HIV-1. Although the number of new infections are on the decline, the number of infected people is increasing, since a greater number of people with access to antiretroviral treatments are living longer [1].

The enveloped HIV-1 *lentivirus* (a subgroup of the *retroviridae* family) harbours an icosahedral nucleocapsid with a cone-shaped core structure housing two copies of single-stranded RNA (Fig. 1) [140]. The approximately 10 kb genome contains nine genes: *gag*, *env*, *pol*, *vif*, *vpr*, *vpu*, *nef*, *tat*, and *rev*. These genes encode 15 proteins, defined by three groups: structural, regulatory or accessory. The three structural genes include *gag*, *env* and *pol*. *Gag* produces the viral capsid whereas *env* produces the envelope protein gp160, which is cleaved into the surface protein gp120 and the transmembrane glycoprotein gp41. *Pol* produces the reverse transcriptase, protease and integrase protein. The accessory genes *vif*, *vpr*, *vpu* and *nef* encode for multifunctional proteins that counteract host-cell defence factors, enabling retroviral infection and replication. The regulatory genes *tat* and *rev* produce early proteins that support the efficiency of viral transcription [140].

1.1 HIV life cycle and antiretroviral drugs

The earliest treatments of HIV infection consisted of symptomatic therapy against opportunistic diseases [78]. However, by 1987, the first antiretroviral drug, Zidovudine (AZT) – a reverse-transcriptase inhibitor (RTI) - was approved by the FDA [40, 88].

In the 27 years since AZT was introduced, 25 antiretroviral drugs have been approved for clinical use in treating HIV-1 infection [27]. Antiretroviral drugs target key stages in HIV replication [140]: viral entry, including binding and fusion to the host-cell, reverse transcription of viral RNA into DNA, integration of the viral DNA into the host-cell's genome, and viral protein expression, assembly, budding, and maturation (Fig. 1 and 2). In the following paragraphs, I will discuss each phase of the HIV-1 life-cycle and the five stage-specific drug classes currently in use to treat HIV-1.

Fig. 1. HIV life cycle. Reproduced with permission from R. J. Shattock & Z. Rosenberg, *Microbicides: Topical Prevention against HIV*, Cold Spring Harb Perspect Med. 2012, page 4. Copyright © by Cold Spring Harbor Laboratory Press [125].

HIV entry & fusion

HIV gains entry into target T helper cells by binding to the CD4 co-receptor protein as well as non-specific interactions with the cell surface. Attachment is the rate-limiting step for HIV-1 infection, since the negatively charged surfaces of both virions and target cells cause repulsive forces [44, 95]. The binding of the gp120 envelope protein to CD4 initiates the entry process and exposes gp120's co-receptor binding site. Subsequent binding of gp120 to

the CXCR4 or CCR5 co-receptors results in further conformational changes, allowing insertion of the hydrophobic fusion peptide gp41 into the target cell membrane. Further rearrangement of gp41 draws the virus and host-cell closer together, ultimately resulting in membrane fusion. Inhibitors for the entry and fusion process, thus blocking the early steps of the viral life cycle, have been approved for clinical use (Fig. 2). Specifically, Maraviroc (MVC), a CCR5-antagonist, blocks viral entry by binding to the CCR5 co-receptor [35], and Enfuvirtide (also known as T20) blocks fusion by directly binding to gp41 [68].

Reverse transcription & integration

After fusion with the host-cell, the viral core is released into the cytoplasm. This is followed by the synthesis of double-stranded viral DNA – the provirus – via reverse transcription. This process is targeted by the before mentioned RTI class of drugs [40]. RTIs consist of two sub-groups, defined by their method-of-action: nucleoside analog reverse-transcriptase inhibitors (NRTIs), and non-nucleosid reverse transcriptase inhibitors (NNRTIs). NRTIs, such as the original AZT and the popular Tenofovir, block reverse transcription by introducing non-natural triphosphates, thereby interrupting transcript elongation [26]. NNRTIs, such as Nevirapine, block reverse transcription by directly binding to the reverse transcriptase protein. The provirus is subsequently integrated into the host-cell genome by the HIV integrase protein. The newest class of approved antiretroviral drugs are integrase inhibitors (INI), such as Elvitegravir, which block strand transfer and thus integration of HIV DNA into the host genome [123].

Protein expression, assembly, budding & maturation

Viral protein production, carried out by the host-cell, is followed by the assembly and budding of progeny virions. Virions become infectious after proteolytic cleavage of Gag and Pol proteins by the HIV protease [140]. Another class of drugs, called protease inhibitors (PIs), such as Indinavir, directly bind to the enzyme and block this process [104, 136].

Introduction of the highly active anti-retroviral therapy (HAART) regime revolutionised the treatment and prognosis of HIV/AIDS [42, 56]. HAART relies on the combination of several classes of antiretroviral drugs, such as two NRTIs, an INI, or a PI. The therapy resulted in a marked suppression of viral replication and reduced the plasma viral load to levels below the detection limit [11, 71, 74]. Notably, this led to a drastically increased lifespan of HIV-infected patients [121].

Fig. 2. Antiretroviral drugs targeting the HIV life cycle. Reproduced with permission from R. J. Shattock & Z. Rosenberg, *Microbicides: Topical Prevention against HIV*, Cold Spring Harb Perspect Med. 2012, page 5. Copyright © by Cold Spring Harbor Laboratory Press [125].

1.2 HIV Transmission

Although HIV-1 can be transmitted from mother-to-child or parenteral exposure, such as needle sharing among drug users, unprotected sexual intercourse is the most common route of HIV transmission [100, 126]. In 2013 alone, 1.5 million new HIV-1 infections occurred by sexual transmission, representing 70% of new HIV-1 infections worldwide. For transmission, HIV requires direct contact between the bloodstream or mucosal surfaces with infected blood or other bodily fluids, such as semen, vaginal fluid or breast milk [1, 100, 126]. Since HIV-1 transmission mainly occurs via unprotected sexual intercourse, semen represents the main transmission vector containing HIV-1 particles and infected leukocytes that are deposited on mucosal surfaces of the anogenital tract [100, 126]. In the first step of HIV-1 transmission, the virus needs to cross the mucosal epithelium to reach its target cells. This is facilitated by small lesions that occur via sexual intercourse or sexually transmitted diseases that affect the epithelial integrity [10]. After crossing the mucosal epithelium, the virus interacts with the major targets of HIV-1 infection: CD4⁺ T lymphocytes, macrophages and dendritic cells (DCs) that express the main receptor CD4 and the co-receptors CXCR4 and CCR5. The virus remains in the mucosa for about a week and propagates a local infection. This is referred to as the “eclipse phase”. After the eclipse phase, HIV-1 spreads

to lymph nodes and gut-associated lymphoid tissues (GALT). At this point HIV-1 replication increases exponentially and leads to the systemic dissemination of the virus throughout the body [126].

The major barrier during sexual transmission of HIV-1 is the limited ability of the virus to cross the mucosal barrier and to reach target cells [105]. The intact mucosal epithelium provides a strong barrier to HIV-1 infection. For example, the female genital tract harbours *lactobacilli* that produce hydrogen peroxide, with antiviral properties [70]. Furthermore, cationic polypeptides, such as defensins, that are secreted by mucosal epithelium, exhibit antibacterial and anti-HIV-1 activity [81]. Viral particles are inactivated at acidic environments *in vitro*, and thus the acidic environment of the vaginal tract might provide a protective mechanism against the sexual transmission of HIV-1. Despite these natural barriers, unprotected sexual intercourse is still the major route of HIV-1 transmission.

1.3 Factors in semen modulating HIV-1 infection

For many years, it has been suggested that semen is simply a passive carrier of HIV-1. However, semen contains factors that may inhibit or enhance HIV-1 infection [34]. There is some evidence that semen contains inhibitory factors, such as reactive oxygen species (ROS) or cationic polypeptides [34, 81]. Indeed, the HIV-1 envelope is highly sensitive to ROS. Semen may produce enough ROS to impair the infectivity of viral particles. Similarly, seminal cationic polypeptides display antiviral activity *in vitro* [34]. Despite these inhibitory factors, there are several variables that actually facilitate HIV-1 transmission. For example, semen induces a strong inflammatory response in the female genital tract, which may increase the transmission of HIV-1 by disrupting the mucosal epithelium and recruiting CD4⁺ T cells to the site of infection [34]. Additionally, semen may also counteract the acidic environment of the vaginal tract by increasing its pH after sexual intercourse [120]. Recent studies have also identified specific seminal factors enhancing HIV-1 infection [8, 90, 110]. Peptide fragments derived from the abundant semen protein prostatic acid phosphatase (PAP), specifically PAP248-286 and PAP85-120, and from semenogelin 1 and 2 (SEMs), the major components of the semen coagulum, form fibrils that greatly enhance viral infectivity at low viral titres [8, 90, 110, 111]. Notably, semen itself greatly enhances HIV-1 infection when tested under conditions that minimise or preclude its cytotoxic effects [5, 39, 67, 69, 90, 96, 137, 144], and that semen-mediated infection enhancement correlates with the levels of PAP248-286, SEM1 and SEM2 fibrils [69, 111]. Preliminary animal studies

suggest that semen and seminal fibrils may facilitate HIV-1 infection after vaginal exposure to low viral doses that reflect the *in vivo* situation [87, 91]. To summarise, semen is no longer viewed as a passive carrier of HIV-1, but rather as an active enhancer of HIV-1 infection.

1.4 Mechanism of infection enhancement by seminal amyloids

The link between PAP248-286 and HIV-1 was identified by Münch *et al.* (2007). They discovered that PAP248-286 forms amyloid fibrils which drastically enhance HIV-1 infection (Fig. 3) [90]. Amyloid fibrils are insoluble, fibrillar aggregates formed by misfolded cellular proteins [132]. Common features of these fibrils are cross- β sheet structures and positive staining by Thioflavin T or Congo Red dyes [19, 37]. Detection is based on fluorescence-enhancement, following binding to amyloid fibrils.

Further analysis of these amyloid fibrils, referred to as Semen-derived Enhancer of Viral Infection (SEVI), revealed that their respective monomeric peptides did not affect infection at all. Subsequent studies showed that SEVI displays a positively charged surface, which binds directly to both virions and target cells. This decreases the charge-charge repulsions between them and promotes virion attachment and fusion, thereby boosting viral infectivity and very likely transmission as well [8, 90, 112, 113]. The discovery of infectivity-enhancing amyloid opens novel strategies to affect HIV-1 transmission.

Fig. 3. PAP248-286 forms amyloid fibrils that drastically enhance HIV-1 infection. (left) Electron microscopy image of SEVI (scale bar 200 nm). **(right)** HIV-1 infection of CEMx-M7 cells in the absence or presence of increasing concentrations of SEVI (here detected as GFP expression). Münch *et al.*, 2007, page 1061 [90].

1.5 Counteracting amyloid-mediated enhancement of HIV-1 infection

Counteracting amyloid activity in semen represents a novel strategy to reduce HIV-1 transmission rates via sexual intercourse. To this end, several compounds have been

identified that block SEVI-mediated enhancement of HIV-1 infection. One such molecular is surfen, a heparin sulfate proteoglycan (HSPG) antagonist. HSPG resides on the surface of target cells. Not only does surfen inhibit the binding of SEVI to HSPG, but also diminishes interaction with virions [113]. BTA-EG(6), a derivative of Thioflavin T, functions in a similar way [97]. Furthermore, Epigallocatechin-3-gallate (EGCG), an antioxidant in green tea, disaggregates preformed fibrils [59] but was only modestly active against semen-mediated infectivity enhancement [57]. In a recent study, seminal zinc and copper delayed SEVI formation by binding to histidine residues [127]. Although these studies have focused on interfering with SEVI-mediated enhancement of HIV-1 infection, this is not the only process we need to consider. Another PAP fragment, PAP85-120, and fragments of SEMs also form fibrils which drastically enhance HIV-1 infection [8, 110, 111]. Anti-amyloid strategies have to be tested against all known seminal amyloid and in particular against semen-mediated enhancement of HIV-1 infection. Thus, blocking the enhancing activity of all semen-derived amyloid fibrils is necessary for preventive measures that can reduce HIV-1 transmission.

1.6 Structure-based peptide inhibitors of amyloid formation

In addition to general agents which block semen-derived amyloid fibrils, a novel approach to counteract amyloid activity is to computationally design highly-specific structure-based peptide inhibitors [93, 122, 154]. This procedure is based on an algorithm, termed “3D profile”, which predicts segments within a peptide sequence that have a high probability of forming fibrils *per se* (Fig. 4) [93, 139]. This algorithm can be applied to any peptide sequence of interest. Possible side-chain conformations are scanned between a template and all amino acids. Each segment is evaluated for its favourability by an energy function, whereby the segment with the lowest energy function has the highest probability to form fibrils. The crystal structure of this peptide segment is then used by RosettaDesign as a template for designing fibrillation inhibitors. The designed peptides are then optimised by improving hydrogen bonding and other intermolecular forces. This allows the design of fibrillation inhibitors that might form a strong contact with the underlying template, cap the growing end of the fibril, thereby blocking further elongation and hence formation of mature fibrils [130].

Fig. 4. Schematic representation of the “3D profile” method with RosettaDesign for detecting fibril-forming segments. Reproduced with permission from M. J. Thompson *et al.*, The 3D profile method for identifying fibril-forming segments of proteins, Proc Natl Acad Sci USA 2006, page 4075. Copyright © by the National Academy of Sciences [139].

1.7 Microbicides

Microbicides are a female-specific method to inhibit sexual transmission of HIV-1 and other sexually transmitted pathogens (Fig. 5). These products are designed as topical pre-exposure prophylaxes that are administered prior to sexual intercourse as gel, cream, film, tablet, or vaginal ring [48, 52].

Fig. 5. Possible actions of topical microbicides to prevent HIV-1 infection. Reproduced with permission from J. P. Moore, Topical microbicides become topical, N. Engl. J. Med.. 2005, page 299. Copyright © by the Massachusetts Medical Society [89].

“First-generation” microbicides were based on surfactants, buffers, or polyanions with a broad, non-specific antiviral activity against various sexually-transmitted pathogens (Table 1). For example, the early surfactant-based microbicide nonoxynol-9 disrupted the cell membrane of both viral and bacterial pathogens *in vitro* [146]. Other strategies were based on the inactivation of HIV. Since low pH inactivates viral particles [82], researchers proposed to lower the vaginal pH by using acid-buffering gels [109]. Long chain polyanionic agents held promise as microbicides as they prevented HIV-1 infection *in vitro* by inhibition of viral binding to host-cells [3, 46, 83, 145]. Despite their potent *in vitro* activity, all topical microbicides that were tested in clinical trials were either inactive or even increased the risk of HIV acquisition due to a lack of adherence, induction of inflammation and cytotoxic effects [38, 52, 55, 117, 133, 145]. The injury to the mucosa can increase the passage of HIV across the mucosal barrier, where it has access to numerous target cells. This inflammatory response can facilitate HIV transmission by recruiting target cells and increasing viral gene transcription [150].

Table 1. “First-generation” microbicides

Microbicide	Trial name	Substance	Results
Surfactant	COL-1492	Nonoxynol-9	Phase II/III: Enhanced HIV infection [146]
Surfactant	Savvy®	C31G	Phase III: Failed (adverse events) [38]
Polyanion	MDP 301	PRO 2000 (Sodium naphthalene sulfonate)	Phase III: Failed (no effect) [83]
Acid buffer and Polyanion	HPTN 035	Carbopol974P (BufferGel®) and PRO2000	Phase II/IIb: 30% reduction (<i>ns</i> significant) [3]
Polyanion	Ushercell	Cellulose sulfate	Phase III: Enhanced HIV infection [145]
Polyanion	CAP	Cellulose acetate phthalate	Phase I: Failed (adverse events)[73]
Polyanion	Carraguard®	Carrageenan	Phase III: Failed (no effect)[133]
Dendrimer	MTN 004	SPL7013	Phase I: Failed (adverse events)[84]

Because of the devastating results obtained with polyanions and related substances, ‘next-generation’ microbicides were developed that are based on drugs used in standard antiretroviral therapy (see section 1.1). A vaginal gel containing Tenofovir (NRTI) showed protection rates of up to 54% in one trial [2]. However, a subsequent larger phase III trial testing the same formula failed to confirm these protective effects [80]. Tenofovir is still

under investigation as a vaginal or rectal gel, as well as in combination with the CCR5 antagonist Maraviroc. There are many other clinical trials in progress, such as a vaginal ring releasing Dapivirine (NNRTI) alone, or in combination with Maraviroc (MTN 013/IPM 026).

1.8 Scientific aim

AIDS is a sexually transmitted disease and semen represents the main vehicle for the dissemination of HIV-1. Far from being a passive carrier for the virus, semen markedly enhances the infectiousness of HIV-1. This activity can be attributed to amyloid fibrils that form by self-assembly of peptides derived from the abundant semen markers PAP and SEMs. Counteracting the viral enhancing activity of amyloid fibrils in semen provides a promising novel strategy to reduce or block sexual HIV-1 transmission. One goal of this thesis is to identify compounds that prevent the formation of seminal amyloid fibrils. For this, peptides were designed to inhibit formation of SEVI fibrils, the best characterised of the seminal amyloids. The ability of these peptides to block SEVI formation, and thus antagonise the HIV enhancing activity of semen, are described herein.

The second and main aim of my thesis is to provide an explanation for why microbicides failed to protect against HIV-1 transmission in clinical trials, despite their potent antiviral activity *in vitro*. I hypothesised that the ability of semen to enhance HIV-1 infection may impair the antiviral efficacy of microbicides. To test this, I set out to determine the antiviral activity of microbicides targeting different steps in the viral life cycle in the presence and absence of semen and amyloids. These studies may not only explain the clinical failure of current microbicides but may also allow for the development of more efficient measures to halt the sexual transmission of HIV.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Eukaryotic cells

HEK293T A Human cell line originally derived from embryonic kidney. Transformed by adenovirus type 5 expressing SV40 (simian virus 40) large T-antigen [51]. HEK293T cells are competent to replicate vectors carrying the SV40 region of replication, and widely used for retroviral production. The cells were obtained from the American Type Culture Collection.

TZM-bl A HeLa-derived adherent cell line expressing CD4, CXCR4, CCR5, and stably transfected with an LTR- β -galactosidase cassette [151]. Upon infection with HIV-1, the viral transactivator protein Tat is expressed and initiates transcription of the LTR-controlled reporter gene β -galactosidase. The cells were obtained from the NIH repository.

CEMx-M7 A suspension T-cell line expressing CD4, CXCR4, CCR5, and stably transfected with an LTR-luciferase and LTR-green fluorescent protein cassette [61]. Upon infection with HIV-1, the viral transactivator protein Tat is expressed and initiates transcription of the LTR-controlled reporter gene luciferase. The cells were kindly provided by N. Landau (New York, NY, USA).

CaSki A Human cervical epithelial cell line that was obtained from the American Type Culture Collection [101].

2.1.2 Primary cells

Human PBMC (Peripheral blood mononuclear cell)

The cells were isolated from a buffy coat that was provided by the DRK blood bank, Ulm.

2.1.3 Bacteria

E. coli XL-2 blue™ *recA1, endA1, gyrA96, thi-1*
hsdR17, supE44, relA1,
lac [F' proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr]

Agilent Technologies, Santa Clara, CA, USA

2.1.4 Nucleic acids

Plasmid DNAs

pBRNL4-3_92TH014.12 The plasmid encodes the HIV-1 NL4-3 provirus. The V3-loop region has been exchanged by the V3-loop of the R5-tropic 92th014.12 isolate [99].

pBRNL4-3_92TH014.12(R5)*nef*⁺*_IRES_g-Luc* The plasmid encodes the pBRNL4-3_92TH014.12 provirus and a Gaussia-luciferase cassette in place of *nef*.

Molecular weight size marker

1 kb Plus DNA Ladder Life Technologies GmbH, Darmstadt

2.1.5 Proteins

Antibodies

Monoclonal mouse anti-p24 (MAK183) antibody EXBIO, Praha, Czech Republic

Polyclonal rabbit anti-p24 antibody Eurogentec GmbH, Köln

Goat anti-Rabbit IgG, Fc-HRPO Dianova GmbH, Hamburg

Enzymes

Restriction endonucleases New England Biolabs, Ipswich, MA, USA

2.1.6 Peptides

HIV-1 p24 Abcam, Cambridge, UK

PAP248-286 The peptide was synthesised and lyophilised at a purity of >90% by Celtek peptides (Franklin, TN, USA). Peptides were dissolved in PBS at a concentration of 10 mg/ml and agitated overnight at 37 °C and 179 g in a thermomixer to promote amyloid fibril formation.

Sequence

PAP248-286 GIHKQKEKSRLQGGVLVNEILNHMKRATQIPSYKKLIMY

Semen was obtained from the ‘Kinderwunschzentrum’ (Göttingen) and collected from healthy individuals with informed consent. Pooled semen was generated from semen samples derived from >20 individual donors. All ejaculates were allowed to liquefy for 30 minutes and semen samples were stored in 1 ml aliquots at -80 °C. Seminal plasma represents the cell free supernatant of semen pelleted for 5 minutes at 10,621 g. In all experiments, aliquots were rapidly thawed, analysed immediately and the remainder discarded. Seminal plasma samples from de-identified EDO patients were provided by Ole Sorensen (Lund University, Sweden).

2.1.7 Antiviral agents

Cellulose sulfate (CS), Polystyrene acid (PSA) and Polynaphthalene sulfonate (PNS)

Thermo Fisher Scientific, Marietta, OH, USA

Polysciences, Warrington, PA, USA

Indinavir (IDV)

NIH AIDS Reagent Program, Germantown, MD, USA

Maraviroc (MVC)

Pfizer, New York City, NY, USA

Monoclonal Antibodies (2F5, 2G12)

Polymun Scientific Immunbiologische Forschung GmbH, Klosterneuburg, Austria

SPL7013 (Vivagel®)

Starpharma, Melbourne, Australia

Tenofovir disoproxil fumarate (TDF), Elvitegravir (ELV)

Selleckchem, Houston, TX, USA

2.1.8 Chemical reagents

Agarose	Carl Roth GmbH + Co. KG, Karlsruhe
Ampicillin	Ratiopharm GmbH, Ulm
Bacto tryptone	Becton Dickinson, Franklin Lakes, NJ, USA
Bacto yeast extract	Becton Dickinson, Franklin Lakes, NJ, USA
Buffers for restriction digestion	New England Biolabs, Ipswich, MA, USA
Calcium chloride dihydrate ($\text{CaCl}_2 \times 2\text{H}_2\text{O}$)	AppliChem GmbH, Darmstadt
Coelenterazine	Avidity, LLC, Aurora, CO, USA
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, St. Louis, MO, USA
Disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$)	AppliChem GmbH, Darmstadt
DMEM	Life Technologies GmbH, Darmstadt
Ethanol	Sigma Aldrich, St. Louis, MO, USA
Ethidium bromide	AppliChem GmbH, Darmstadt
Fetal calf serum (FCS)	Life Technologies GmbH, Darmstadt
Ficoll separation solution	Merck KGaA, Darmstadt
Gentamicin	Life Technologies GmbH, Darmstadt
Glucose	Sigma Aldrich, St. Louis, MO, USA
L-Glutamine	Life Technologies GmbH, Darmstadt
Hepes ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$)	Sigma Aldrich, St. Louis, MO, USA
HPLC water	VWR International GmbH, Darmstadt
Hydrogen chloride (HCl)	VWR International GmbH, Darmstadt
Interleukin-2 (IL-2)	Miltenyi Biotec GmbH, Bergisch Gladbach
Isopropanol	Merck KGaA, Darmstadt
Methanol	Merck KGaA, Darmstadt
MTT (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide)	Sigma Aldrich, St. Louis, MO, USA
Penicillin/Streptomycin (Pen/S)	Life Technologies GmbH, Darmstadt
Phosphate buffered saline (PBS)	Life Technologies GmbH, Darmstadt
PHA (Phytohaemagglutinin)	Thermo Fisher Scientific, Marietta, OH, USA
Poly-L-lysine hydrobromide	Sigma Aldrich, St. Louis, MO, USA
Potassium chloride (KCl)	Merck KGaA, Darmstadt
Roti®-Load DNA	Carl Roth GmbH + Co. KG, Karlsruhe
RPMI-1640 medium	Life Technologies GmbH, Darmstadt

SOC medium	Life Technologies GmbH, Darmstadt
Sodium ascorbate (C ₆ H ₇ NaO ₆)	Sigma Aldrich, St. Louis, MO, USA
Sodium chloride (NaCl)	Merck KGaA, Darmstadt
Sodium hydroxide (NaOH)	Merck KGaA, Darmstadt
Sulfuric acid (H ₂ SO ₄)	Sigma Aldrich, St. Louis, MO, USA
SureBlue™ TMB Microwell Peroxidase Substrate	KPL, Gaithersburg, MD, USA
TAE buffer (50x)	5 PRIME, Gaithersburg, MD, USA
Tris	VWR International GmbH, Darmstadt
Tryptone	Becton Dickinson, Franklin Lakes, NJ, USA
Trypsin / EDTA, 0.05%	Life Technologies GmbH, Darmstadt
Triton X-100	Sigma Aldrich, St. Louis, MO, USA
Tween 20	Sigma Aldrich, St. Louis, MO, USA

2.1.9 Consumables

8-channel manifold for QuikSip™ BT-Aspirator	BRAND GMBH + CO KG, Wertheim
Cell culture flasks	Sarstedt, Nümbrecht
Cell culture well plates	Greiner Bio-One GmbH, Frickenhausen
Falcons (15 ml and 50 ml)	Sarstedt, Nümbrecht
Micro tubes, 1.5 and 2.0 ml, PP, with attached PP cap	Sarstedt, Nümbrecht
Millex-HA Filter Unit 0.45 µm	Merck KGaA, Darmstadt
Polystyrene microplates	Corning, Corning, NY, USA
MaxiSorp™	Thermo Scientific, Waltham, MA, USA
White plates	Thermo Scientific, Waltham, MA, USA
Serological pipettes (5 ml, 10 ml and 25 ml)	Sarstedt, Nümbrecht
Syringe	Becton Dickinson, Franklin Lakes, NJ, USA
Tips without filter, Type A/B/E (10, 200, 1,000 µl)	Sarstedt, Nümbrecht

2.1.10 Technical equipment

Centrifuge 5417 R (Rotor: F-45-30-11)	Eppendorf AG, Hamburg
Centrifuge 5810 R (Rotor: A-4-81)	Eppendorf AG, Hamburg
Multipette plus	Eppendorf AG, Hamburg
Thermomixer comfort	Eppendorf AG, Hamburg
MR Hei-Standard (stirrer)	Heidolph Instruments GmbH & Co. KG, Schwabach
Titramax 100 (plate shaker)	Heidolph Instruments GmbH & Co. KG, Schwabach
Pipetus®	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt
DISCOVERY Comfort Multichannel Pipette	HTL Lab Solutions Warsaw, Poland
FE20–FiveEasy™ pH-meter	Mettler-Toledo, Columbus, OH, USA
VMax Kinetic ELISA Microplate Reader	Molecular Devices, Sunnyvale, CA, USA
SoftMax Pro 5.3	
HERAsafe® KSP 18	Thermo Fisher Scientific, Marietta, OH, USA
Steri-Cult CO2-Inkubator Modell 3311	Thermo Fisher Scientific, Marietta, OH, USA
Vortex-Genie®	VWR International GmbH, Darmstadt
Orion II Microplate Luminometer	Zylux Corporation, Pforzheim
Software Simplicity	

2.1.11 Kits

Tropix® Gal-Screen® assay	Applied Biosystems, Foster City, CA, USA
Luciferase Assay System (E1501)	Promega, Madison, WI, USA
Wizard® Plus Midipreps DNA Purification System	Promega, Madison, WI, USA

2.1.12 Buffers and solutions

2 M CaCl_2

Gaussia substrate dilution buffer: 0.1 M Tris, 0.3 M sodium ascorbate, adjust pH to 7.4

10x HBS

8.18 g NaCl

5.94 g Hepes

0.25 g $\text{Na}_2\text{HPO}_4 \times 2 \text{ H}_2\text{O}$

dissolved in 100 ml H_2O , adjusted to pH 7.12

2.1.13 Media

Bacteria culture media

LB medium

10 g/l bacto tryptone, 5 g/l bacto yeast extract,
8 g/l NaCl, 1 g/l glucose, add 100 mg/l ampicillin
prior to use

LB / AMP agar

add 15 g/l agar and 100 mg/l ampicillin to LB
medium

Cell culture media

DMEM supplemented with:

350 $\mu\text{g}/\text{ml}$ L-glutamine

120 $\mu\text{g}/\text{ml}$ penicillin, 120 $\mu\text{g}/\text{ml}$ streptomycin

10% (v/v) heat-inactivated FCS

add gentamicin (in presence of SE or SP)

used for TZM-bl, HEK293T and CaSki cells

RPMI supplemented with:

350 $\mu\text{g}/\text{ml}$ L-glutamine

120 $\mu\text{g}/\text{ml}$ penicillin, 120 $\mu\text{g}/\text{ml}$ streptomycin

10% (v/v) heat-inactivated FCS

add gentamicin (in presence of SE or SP)

(add 10 ng/ml IL-2, 1 $\mu\text{g}/\text{ml}$ PHA to stimulate
PBMCs)

used for CEMx-M7 cells and PBMCs

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Adherent and suspension cell culture

DMEM culture medium was used to cultivate adherent cell lines. The cells were incubated at 37 °C, 90% humidity, and 5% CO₂ in a cell culture incubator and passaged 2-3 times a week. The adherent cells were detached from the culture flask at 90-100% confluence by 0.05% Trypsin / EDTA, resuspended in the respective culture medium and passaged 1:5 or 1:10. Suspension cell lines were cultivated in the same way using RPMI culture medium.

2.2.1.2 Isolation of primary blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat (lymphocyte concentrate from 500 ml whole blood). First, the buffy coat was diluted 1:3 in PBS and overlaid with Ficoll separating solution. After centrifugation at 515 g for 20 minutes without brakes the white PBMC interphase between plasma and Ficoll solution was removed and transferred into a fresh tube. PBMCs were washed twice in PBS and cultured in supplemented RPMI culture medium at a density of 1×10^6 cells/ml and stimulated with 10 ng/ml IL-2 and 1 µg/ml PHA for 3 days.

2.2.2 Bacterial methods

2.2.2.1 Transformation of bacteria

Plasmids were amplified by transforming *Escherichia coli* XL2 Blue™. 1 µg of plasmid DNA was mixed with 5 µl bacteria and incubated for 20 minutes on ice. Then, a 30 seconds heat-shock at 42 °C and 2.5 minutes cooling on ice followed. To promote bacterial growth, 200 µl of SOC medium was added followed by incubation at 37 °C and 13 g for 40 minutes on a thermomixer. Finally, 70 µl of transformed bacteria were distributed on LB agar plates supplemented with respective antibiotics for selection. The plates were incubated at 37 °C in a compartment drier over night.

2.2.2.2 Culture of bacteria

Plasmid DNA was prepared by inoculation of 120 ml LB medium (supplemented with 100 mg/l of respective antibiotics) with single colonies of bacteria and incubated at 37 °C on a shaker for approximately 16 hours.

2.2.3 DNA methods

2.2.3.1 Plasmid DNA preparation

Plasmid DNA preparations were performed using Wizard® Plus Midiprep Kit from Promega as recommended by the manufacturer. Plasmid DNA concentration and purity were determined using the Nano Drop 2000 spectrophotometer.

2.2.3.2 Restriction digest

Plasmid DNA was checked by incubating 1 µg of DNA with 0.5 µl of appropriate restriction endonucleases and 2 µl of the recommended 10x NEB restriction buffer at 37 °C for 1 hour on a thermomixer. The final volume was 20 µl.

2.2.3.3 Agarose gel electrophoresis

The restricted plasmid DNA was separated by an agarose gel electrophoresis. 5 µl loading dye was mixed with 20 µl of the digested DNA. The samples were analysed in a 0.7% agarose gel in 1x TAE buffer at 120 V for 30 minutes using the Voltage PowerPAC Basic Power Supply. DNA ladder was run in parallel with the samples. To visualise DNA bands, ethidium bromide was added to the gel.

2.2.4 Viral methods

2.2.4.1 Generation of HEK293T cells derived virus stocks

Infectious virus was produced by the transiently transfection of HEK293T cells using calcium phosphate precipitation. HEK293T cells were seeded in 6 well plates and cultured over night to obtain a cell density of 60-80% the next day. Prior to transfection, medium was replaced with 2 ml of fresh medium per well. Then, 5 µg of proviral DNA per well were diluted with 13 µl 2 M CaCl₂. Sterile water was added to a final volume of 100 µl per well. The mixture was added to an equal volume of 2x HBS followed by 10 seconds of vortexing. Then, the DNA precipitate was added dropwise to the cells. To reduce Cytotoxicity, 6 to 16 hours later medium was changed and fresh DMEM (2.5% (v/v) FCS) was added. Virus was harvested when the supernatant showed an orange colour (24-40 hours post transfection). To remove cellular debris, virus stocks were centrifuged (5 minutes, 805 g) and stored at -80 °C for long-term storage.

2.2.4.2 HIV-1 p24 antigen ELISA

Quantification of virus stocks was based on p24 antigen content and performed by an in-house HIV-1 p24 capsid antigen ELISA [72]. The monoclonal antibody MAK183 was purchased from ExBIO (Praha, Czech Republic). 96-well MaxiSorp™ microplates were coated with 500 ng/ml anti-HIV-1 p24 (100 µl/well) and incubated in a wet chamber at RT over night. The next day, the plate was washed 3 times with 350 µl PBS-T (PBS and 0.05% Tween 20). Then, 150 µl blocking solution (PBS and 10% (v/v) FCS) was added and the plate was incubated at 37 °C. To perform this sandwich ELISA, virus stocks (supernatant from transfected HEK293T cells) were lysed with 1% (v/v) Triton X-100. After 2 hours, the plate was washed again 3 times. 100 µl serially diluted HIV-1 p24 protein as standard and dilutions of virus stocks were transferred. The plate was incubated in a wet chamber at RT over night. The next day, the plate was washed to remove unbound capsid proteins. The polyclonal rabbit antiserum against p24 antigen was generated by Eurogentec (Köln). Next, the polyclonal antibody was diluted (1:1,000) in PBS-T (PBS, 0.05% Tween 20 and 10% (v/v) FBS) and added (100 µl/well) for 1 hour at 37 °C. Following another washing step, 100 µl of a goat anti-rabbit HRP-coupled antibody (1:2,000) was added and incubated for 1 hour at 37 °C. Finally, the plate was washed 3 times and 100 µl SureBlue TMB 1-Component Microwell Peroxidase Substrate was added. After 20 minutes shaking at 450 rpm and RT, the reaction was stopped with 0.5 M H₂SO₄ (100 µl/well). The optic density, proportional to the p24 capsid antigen amount was determined by comparing with a standard curve. The optical density was measured at 450 nm and 650 nm with an ELISA microplate reader.

2.2.4.3 Effect of peptides on SEVI-mediated enhancement of HIV-1 infection

40 µl R5-tropic HIV-1 containing 0.1 ng p24 antigen was incubated with 40 µl dilutions of PAP248-286/peptides mixtures, containing 2-fold molar excess of inhibitory peptides, that were either freshly prepared (t=0) or had been agitated for 5 (t=5), 20 (t=20) or 23 (t=23) hours at 37 °C and 340 g. Peptide concentrations and experimental conditions during agitation were similar to those used in the fibrillation assays [130]. Thereafter, 20 µl of the mixtures were used to infect 180 µl TZM-bl cells seeded the day before (1 x 10⁴/well). 2 or 3 days later infection rates were determined using the Tropix® Gal-Screen® assay.

2.2.4.4 Effect of peptides on HIV-1 infection in the absence of SEVI

40 µl peptides were incubated with equal volumes HIV-1 that contains 0.1 ng of p24 antigen for 5 minutes at RT. Peptide concentrations were 150, 30, 6, 1.2 and 0 µg/ml during

preincubation with virus. Thereafter, 20 μ l of mixtures were added to 180 μ l TZM-bl cells (1×10^4 /well). Infection rates were determined 2 days later by using the Tropix® Gal-Screen® assay. These concentrations are comparable to its molar concentrations in the inhibition assay with PAP248-286.

2.2.4.5 Effect of WW61 on poly-L lysine mediated enhancement of HIV-1 infection

50 μ l of poly-L lysine was mixed with equal volumes of WW61. Then, 35 μ l of 5-fold dilutions of poly-L lysine/WW61 mixtures or poly-L lysine alone were incubated with the same volumes of R5-tropic HIV-1 (0.1 ng p24) and incubated for 5 minutes at RT. Poly-L lysine and WW61 concentrations were 100, 20, 4, 0.8, 0.16, 0.064, 0.032 and 0 μ g/ml during preincubation with virus stocks. Thereafter, 20 μ l of mixtures were added to 180 μ l TZM-bl cells (1×10^4 /well). Infection rates were determined 2 days later by using the Tropix® Gal-Screen® assay.

2.2.4.6 Effect of inhibitory peptides on semen-mediated enhancement of HIV-1 infection

40 μ l R5-tropic HIV-1 containing 0.1 ng p24 antigen was incubated with 40 μ l 10% semen that had been incubated with 300 μ M inhibitory peptides for 2 hours at RT. Thereafter, 20 μ l of the mixtures were used to infect 280 μ l TZM-bl cells seeded the day before (1×10^4 /well). After 2 hours of incubation, supernatants were removed and cells were further cultivated in 200 μ l fresh media. 3 days later, infection rates were measured using the Tropix® Gal-Screen® assay.

2.2.4.7 Infection of TZM-bl cells by mock- or semen-treated HIV-1 in the presence of microbicides

To examine the activity of candidate microbicides in the presence of semen, 100 μ l 5×10^3 TZM-bl cells were seeded into 96 well flat-bottom plates. The next day, when cells were ~70% confluent, the medium was replaced by 250 μ l fresh medium in the presence of 30 μ l antivirals. After 1 hour incubation, semen was mixed 1:1 (v/v) with R5-tropic HIV-1 (0.05 and 0.5 ng p24) in order to obtain semen concentrations of 10% during virion treatment. After incubating the semen/virus mixture for 5 minutes, 20 μ l of these mixtures (or PBS-virus control) were then added to 280 μ l of compound-treated cells. Final cell culture concentrations of semen were 0.67 or 0%. I used a viral multiplicity of infection (MOI) of ~ 0.05, which gives a low, but consistently detectable level of infection in the absence of semen. This MOI allowed us to detect an enhancing effect of semen, as assessed

by an increase in the numbers of infected cells. Furthermore, I used a MOI ~ 0.5 , which gives the same detectable level of background infection as a low MOI in the presence of semen. A high MOI is necessary to compare the inhibitory effect of the microbicides in presence and absence of semen, as assessed by a decrease in the numbers of infected cells. After 2 hours of incubation, supernatants were removed and cells were further cultivated in 200 μ l fresh media supplemented with the microbicides. 3 days later, infection rates were determined by quantifying β -galactosidase activities in cellular lysates using the Tropix® Gal-Screen® assay as recommended by the manufacturer. Luminescence was recorded on an Orion microplate luminometer as relative light units per second (RLU/s).

2.2.4.8 Infection of PBMCs by mock- or semen-treated HIV-1 in the presence of microbicides

2×10^5 activated PBMCs were preincubated with 30 μ l compounds in 250 μ l RPMI medium for 1 hour. Afterwards, semen was mixed 1:1 (v/v) with R5-tropic HIV-1 encoding gaussia-luciferase (5 and 50 ng p24) and incubated for 5 minutes at RT. The cells were infected with 20 μ l of the semen/virus mixtures for another 2 hours. After infection, the cells were transferred to V-shaped microtiter plates and pelleted at 453 g for 5 minutes. Supernatants were removed, and cells were resuspended in 180 μ l fresh medium with 20 μ l of the microbicides. At 3 days post-infection, infectivity was analysed by detecting gaussia-luciferase activity in cell supernatants.

2.2.4.9 Infection of PBMCs by mock- or semen-treated HIV-1 in the presence of a protease inhibitor

2×10^5 activated PBMCs were preincubated with 30 μ l Indinavir in 250 μ l RPMI medium for 1 hour. Afterwards, semen was mixed 1:1 (v/v) with R5-tropic HIV-1 encoding gaussia-luciferase (5 and 50 ng p24) and incubated for 5 minutes at RT. The cells were infected with 20 μ l of the semen/virus mixtures for another 2 hours. After infection, the cells were transferred to V-shaped microtiter plates and pelleted at 453 g for 5 minutes. Supernatants were removed, and cells were resuspended in 180 μ l fresh medium with 20 μ l of the microbicides. 2 days post-infection, cells were pelleted and further cultivated in 200 μ l fresh media supplemented with the protease inhibitor. 4 days later, infection rates were measured using the Luciferase assay system as recommended by the manufacturer.

2.2.4.10 Infection of CEMx-M7 cells by mock- or semen-treated HIV-1 in the presence of a protease inhibitor

To examine the activity of Indinavir in the presence of semen, 2×10^5 CEMx-M7 cells were seeded in 250 μ l into 96 well V-shape plates. Then, 30 μ l of antivirals were added and incubated for 1 hour. Semen was mixed 1:1 (v/v) with R5-tropic HIV-1 (0.05 and 0.5 ng p24) in order to obtain semen concentrations of 10% during virion treatment. After incubating the semen/virus mixture for 5 minutes, 20 μ l of these mixtures (or PBS-virus control) were then added to compound-treated cells. Final cell culture concentrations of semen were 0.67 or 0%. After 2 hours, the cells were transferred to V-shaped microtiter plates and pelleted at 453 g for 5 minutes. Supernatants were removed, and cells were resuspended in 180 μ l fresh medium with 20 μ l of the microbicides. 2 days post-infection, cells were pelleted and further cultivated in 200 μ l fresh media supplemented with the microbicides. 4 days later, infection rates were measured using the Luciferase assay system as recommended by the manufacturer.

2.2.4.11 Infection of TZM-bl cells by mock- or SEVI-treated HIV-1 in the presence of microbicides

SEVI was generated as described in 2.1.6. To analyze the activity of candidate microbicides in the presence of SEVI, 5×10^3 TZM-bl cells were seeded in 100 μ l into 96-well flat-bottom plates. The next day, when cells were ~70% confluent, the medium was replaced by 160 μ l fresh medium in the presence of 20 μ l antivirals. After 1 hour incubation, SEVI was mixed 1:1 (v/v) with R5-tropic HIV-1 (0.05 and 0.5 ng p24) in order to obtain SEVI concentrations of 35 μ g/ml during virion treatment. After incubating the SEVI/virus mixture for 5 minutes, 20 μ l were then added to compound-treated cells. 3 days later, infection rates were measured using the Tropix® Gal-Screen® assay.

2.2.4.12 Infection of CEMx-M7 cells by mock- or SEVI-treated HIV-1 in the presence of microbicides

To examine the activity of candidate microbicides in the presence of SEVI, 2×10^5 CEMx-M7 cells were seeded in 160 μ l into 96 well V-shape plates. Then, 20 μ l of Indinavir was added and incubated for 1 hour. SEVI was mixed 1:1 (v/v) with R5-tropic HIV-1 (0.05 and 0.5 ng p24) in order to obtain SEVI concentrations of 35 μ g/ml during virion treatment. After incubating the SEVI/virus mixture for 5 minutes, 20 μ l of the SEVI-virus (or PBS-virus control) were then added to compound-treated cells. 2 days post-infection, cells were

pelleted and further cultivated in 200 µl fresh media supplemented with the microbicides. 4 days later, infection rates were determined using the Luciferase assay system.

2.2.4.13 Gaussia-luciferase assay

To measure infection of cells that do not express a reporter-gene under control of an HIV-1 promoter, a gaussia-luciferase reporter virus (NL4-3_R5_G-Luc encoding the secretable *Gaussia* princeps luciferase in place of *nef*) was produced by the calcium phosphate precipitation method and used for infection. 2 hours post-infection, the inoculum was removed and wells were washed twice with 200 µl PBS per well. Fresh medium was added and immediately 50 µl were collected and stored at -20 °C as a washing control. The background signal was later subtracted in the analysis to exclude the possibility of measuring residual virus inoculum. Supernatants were further collected and replaced with fresh medium every 2 days. To measure gaussia-luciferase activity, the substrate was prepared by adding 1 mg coelenterazine to acidified methanol (1 drop concentrated HCl to 10 ml methanol) and stored at -80 °C. The substrate was diluted 1:170 in dilution buffer (0.1 M Tris, 0.3 M sodium ascorbate, pH 7.4). 20 µl of supernatant were mixed with 80 µl of gaussia substrate and luminescence was recorded on an Orion microplate luminometer as relative light units per second (RLU/s).

2.2.4.14 Cell-to-cell transmission

1 x 10⁴ CaSki cells were seeded in 250 µl medium and incubated overnight. The next day, the cells were preincubated with 30 µl of microbicides for 1 hour and then inoculated with 20 µl of semen-treated R5-tropic HIV-1 expressing gaussia-luciferase (1 ng p24) for 2 hours. Inoculum was then removed, and 5 x 10⁴ CEMx-M7 cells were added onto the CaSki cells in the presence of microbicides. 3 days post-infection, the antiviral activity of microbicides was determined by measuring gaussia-luciferase activity of cell supernatants.

2.2.4.15 Cell Viability

The effect of semen on cell viability was analysed using the MTT-assay under experimental conditions corresponding to those used in the respective infection assays. To assess cell viability for each compound, 1 x 10⁴ TZM-bl or 2 x 10⁵ CEMx-M7 cells (in 180 µl) were incubated with 20 µl of the indicated compounds to achieve the indicated final concentration. After 3 days of incubation, 20 µl of a 5 mg/ml MTT (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide solution was added to cells. After 3 hours, the cell-free supernatant was discarded, formazan crystals were dissolved in 100 µl DMSO-ethanol

(1:1 v/v) and optical density was measured at 450 nm and 650 nm with an ELISA microplate reader. The % viability rates were calculated relative to NAD(P)H-dependent cellular oxidoreductase activity in PBS-treated cells (100%).

3 RESULTS

3.1 Development of PAP248-286 fibrillation inhibitors

Semen contains amyloid fibrils that enhance HIV-1 infection [8, 90, 110, 111]. The best characterised fibril-forming peptide is the C-terminal fragment of the prostatic acid phosphatase protein (PAP248-286) that forms fibrils termed SEVI [90]. Since only the assembled but not the monomeric peptide increases viral infection, compounds that inhibit SEVI formation might also abrogate the HIV enhancing activity of semen. In a collaborative approach together with the group from David Eisenberg (UCLA, USA), we aimed at developing structure-based peptides that counteract peptide self-assembly and hence the formation of infection-enhancing amyloids. For this, the “3D profile” method was applied to PAP248-286 to identify regions capable of driving fibril formation by using the RosettaDesign software (Fig. 6) [139]. The implemented algorithm evaluates the favourability of a given sequence to form fibrils using an energy function [76]. This prediction method identified 2 regions using the lowest energy function: the GGVLVN motif (PAP260-265) and the KLIMY motif (PAP282-286) (Fig. 6).

Fig. 6. Application of the “3D profile” method with RosettaDesign for detecting fibril-forming segments of PAP248-286. The vertical bars represent the energy predictions for each hexapeptide. The red bars indicate the segments of the protein PAP248-286 that may form fibrils itself, and these regions contain the segments with the lowest energies. Missing bars represent positions without experimental data. Black dashed horizontal line indicates the energy threshold.

Crystal structures of the two peptides GGVLVN and KLIMY were determined and used as templates to design peptides that form a strong contact with GGVLVN and KLIMY, and thus blocking its further elongation. Five peptides were designed which were predicted to bind to the 2 identified segments of PAP248-286 (Table 2, see page 32). Peptides HM6 (HYRLYM), WI6 (WHKLWI) and WI62 (WHRLYI) target the GGVLVN motif and HW5 (HYKWW) and YW5 (YTWKW) target the KLIMY motif. To experimentally confirm the ability of

these peptides to inhibit formation of SEVI fibrils, freshly dissolved monomeric PAP248-286 was incubated in the presence or absence of the respective peptides. Fibril formation was monitored by the amyloid-specific dye Thioflavin T (ThT). Under control conditions, PAP248-286 readily formed amyloid fibrils after 1 hour of incubation (Fig. 7), as evidenced by increasing ThT fluorescence intensity. In the presence of a 2-fold molar excess of the GGVLN-targeting peptides HM6, WI6 and WI62 only low amounts of SEVI fibrils were formed (Fig. 7a). Similarly, the KLIMY-targeting peptides HW5 and YW5 also exhibited low amounts of fibril formation (Fig. 7b). This data indicates that the five analysed peptides prevent the formation of SEVI fibrils.

Fig. 7. Designer peptides targeting GGVLN and KLIMY delay PAP248-286 fibril formation. 2-fold molar excess of peptides were added to monomeric PAP248-286 and agitated at 340 g and 37 °C. The fibril formation was monitored by fluorescence intensity of Thioflavin T as measured by spectrofluorometry (excite at 444 nm and read emission at 482 nm). **(a)** The peptides HM6 (red), WI6 (brown) and WI62 (blue) were designed to target the GGVLN structure (PAP260-265). **(b)** The peptides HW5 (red) and YW5 (blue) were designed based on the KLIMY structure (PAP282-286). Shown are the fluorescence intensities as raw data (mean relative fluorescence units $\pm$ standard deviation from 4 independent experiments). The assays were performed by Anni Zhao (UCLA, USA). RFU, relative fluorescence units.

To prove these observations in a functional manner, HIV-1 particles were treated with PAP248-286 solutions that were agitated for 0 or 5 hours in the presence or absence of the respective peptides. Thereafter, TZM-bl indicator cells were infected with these mixtures and infection rates were determined 2 days later by quantifying β -galactosidase activities in cellular lysates. Freshly dissolved PAP248-286 (0 h) had no effect on HIV infection (Fig. 8), but agitated for 5 hours increased HIV infection rates by 23-fold. In the most dramatic instance, I observed an increase from 6.2×10^4 RLU/s to 1.4×10^6 RLU/s in the presence of 150 μ g/ml PAP248-286. This demonstrates that effective SEVI fibril formation occurs after

incubation and agitation. Infection rates obtained after inoculation with virions exposed to PAP248-286 and the GGVLVN-targeting or the KLIMY-targeting peptides were similar to those measured in the absence of SEVI (6.2×10^4 RLU/s). Thus, the three GGVLVN and the two KLIMY targeting peptides prevent the formation of HIV-enhancing SEVI fibrils.

Fig. 8. The designed peptides prevent the formation of infection promoting SEVI fibrils. PAP248-286 solutions that had been agitated in the presence or absence of 2-fold molar excess of peptides (a) HM6, WI6 and WI62 (directed against GGVLVN), and (b) HW5 and YW5 (directed against KLIMY) for 0 or 5 hours were mixed with HIV-1 particles (0.1 ng p24) and added to TZM-bl cells. PAP248-286 concentrations during virion treatment are indicated above. Shown are the infection rates as raw data (mean β -galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second.

3.1.1 Development of optimised inhibitors of SEVI fibrillation

Peptides that block the formation of SEVI in semen must be resistant against proteolytic degradation in semen. Since WI6 was most effective in delaying fibril formation (Fig. 7 and data not shown) peptide derivatives thereof were generated. The optimised peptides, specifically WW61, WW62, and WW63 contain non-natural amino acids, which should aid in protease resistance (Table 2, see page 32). These peptides were tested as described above. SEVI fibrils were detectable after ~5 hours of incubation and fluorescence intensities increased constantly thereafter (Fig. 9). In the presence of WW61, WW62, and WW63 fibril formation was blocked. Even after 20 hours of incubation, no significant amounts of ThT-reactive material was detected. Thus, I conclude that WW61, WW62, and WW63 prolong the lag time of PAP248-286 fibrillation compared to the first set of inhibitory peptides (Fig. 7) from 2 to more than 20 hours (Fig. 9).

Fig. 9. Optimised inhibitors with non-natural amino acids delay PAP248-286 fibril formation for more than 20 hours. 2-fold molar excess of inhibitory peptides were added to monomeric PAP248-286 and agitated at 340 g and 37 °C. The fibril formation was monitored by Thioflavin T fluorescence. The peptides WW61 (red), WW62 (blue) and WW63 (grey) are derivatives of the previous peptide WI6 (against GGVLVN). Shown are the fluorescence intensities as raw data (mean relative fluorescence units $\pm$ standard deviation from 4 independent experiments). The assays were performed by Anni Zhao (UCLA, USA). RFU, relative fluorescence units.

For further experiments, these optimised inhibitors were analysed for their ability to prevent the formation of HIV infection promoting factors. PAP248-286 was agitated for 0 or 20 hours in the presence or absence of the optimised inhibitors. As described above, the samples were mixed with HIV-1 and TZM-bl cells were infected. After 20 hours of incubation, fibrils formed and enhanced HIV-1 infection from 4.7×10^3 RLU/s measured in the absence of PAP248-286 to 4.9×10^5 RLU/s if virions were exposed to 150 $\mu\text{g/ml}$ SEVI (Fig. 10a). WW61 and WW63 completely abolished the formation of infectivity-enhancing factors. Infection levels with inhibitors were similar to those obtained in the absence of SEVI (4.7×10^3 RLU/s) (Fig. 10a). Peptide WW62 was slightly less active than WW61 and WW63, since some enhancing activity could be detected (Fig. 10a). Control experiments performed with the inhibitory peptides alone revealed that none of them affected HIV-1 infection directly (Fig. 10b). Thus, the lack of infectivity enhancement observed in the infectivity assays is due to the prevention of PAP248-286 fibril formation.

Fig. 10. Optimised peptides containing non-natural amino acids prevent the formation of infection promoting SEVI fibrils. (a) PAP248-286 solutions that had been agitated in the presence or absence of 2-fold molar excess of respective inhibitors for 0 or 20 hours were mixed with HIV-1 (0.1 ng p24). After 5 minutes, TZM-bl cells were infected. PAP248-286 and inhibitory peptide concentrations during virion treatment are indicated above. (b) Optimised inhibitory peptides alone were mixed with HIV-1 (0.1 ng p24) and used for infection. Shown are the infection rates as raw data (mean β -galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second.

3.1.2 The effect of WW61 on PAP248-286 fibrillation is specific

WW61 was designed to specifically inhibit PAP248-286 fibril formation by interacting with the segment GGVLVN. Next, I sought to exclude the possibility that the GGVLVN-inhibiting peptide WW61 interferes with fibrillation in a sequence-independent manner. For this, two control peptides, G6K as a positively charged control, and PN6 with the same charge as WW61, were synthesised and analysed as described above (Table 2, see page 32). The control peptides slightly affected fibril formation (Fig. 11).

Fig. 11. Sequence-independent control peptides slightly delay PAP248-286 fibril formation. 2-fold molar excess of control peptides were added to monomeric PAP248-286 and agitated at 340 g and 37 °C. The fibril formation was monitored by Thioflavin T fluorescence. The peptide G6K (red) was designed based on the amino terminal part of PAP248-286, which is not necessary for fibrillation. While, the peptide PN6 (blue) was synthesised with the same charge as the optimised inhibitor WW61. Shown are fluorescence intensities as raw data (mean relative fluorescence units $\pm$ standard deviation from 4 independent experiments). The assays were performed by Anni Zhao (UCLA, USA). RFU, relative fluorescence units.

To confirm this finding, PAP248-286 solutions were agitated in the absence or presence of G6K and PN6 for 23 hours, and analysed for their infection-enhancing activity. In the absence of control peptides, SEVI fibrils formed, increasing viral infection 30-fold. Similarly, infection-promoting fibrils also formed in the presence of G6K and PN6 after 23 hours of incubation, indicating enhanced HIV-1 infection (Fig. 12a). Notably, the control peptides itself did not show an effect on HIV-1 enhancement (Fig. 12b). Thus, the designed inhibitors specifically affect the formation of SEVI.

Fig. 12. G6K and PN6 do not prevent the formation of SEVI fibrils. (a) PAP248-286 solutions that had been agitated in the presence or absence of 2-fold molar excess of respective control peptides for 0 or 23 hours were mixed with HIV-1 (1 ng p24). (b) G6K and PN6 alone were mixed with HIV-1. After 5 minutes, TZM-bl cells were infected. PAP248-286 (a) and peptide (b) concentrations during virion treatment are indicated above. Shown are the infection rates as raw data (mean β -galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second.

Like seminal amyloids, Poly-L lysine (PL) is known to enhance HIV infection by its positive charge thereby facilitating attachment of HIV to the cellular membrane [20, 157]. To exclude that WW61 inhibits PAP248-286 assembly by just electrostatic interactions, various concentrations of PL and WW61 were mixed with virus and used to infect TZM-bl cells. As expected, poly-L lysine dose-dependently enhanced HIV-1 infection from 5.4×10^4 RLU/s (in the absence of PL) to 1.7×10^6 RLU/s (32-fold enhancement) (Fig. 13). Similarly, when mixed with WW61, poly-L lysine enhanced HIV-1 infection by 36-fold. As already shown in Fig. 10, WW61 did not affect HIV-1 infection directly. Thus, WW61 did not have an effect on the poly-L lysine-mediated enhancement of HIV-1 infection further confirming that the designed peptide inhibits the PAP248-286 assembly by binding to the GGVLVN motif.

Fig. 13. WW61 does not affect poly-L lysine-mediated enhancement of HIV-1 infection. HIV-1 particles containing 1 ng p24 antigen were incubated with indicated concentrations of WW61 and poly-L lysine (PL) alone or in combination, and used to infect TZM-bl cells. Shown are the infection rates as raw data (mean β -galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second.

Table 2. Sequence of analysed inhibitors and control peptides.

	Peptide	Segment	Sequence
First round inhibitors	HM6	GGVLVN	HYRLYM
	WI6		WHKLWI
	WI62		WHRLYI
	HW5	KLIMY	HYKWW
	YW5		YTWKW
Optimised inhibitors	WW61	GGVLVN	WHK-Cha-W-hydroxylTic
	WW62		WHK-MeNle-W-Melle
	WW63		WHK-MePhg-W-Q
Control peptide	G6K	Positive charge	GIHKQK
Control peptide	PN6	Same charge as WW61	PYKLWN

3.1.3 SEVI inhibitors do not prevent semen-mediated enhancement of HIV-1 infection

Finally, I investigated whether the designed peptides, including WW61, are capable of reducing semen-mediated enhancement of HIV-1 infection. To this end, a low dose of HIV-1 particles (0.1 ng p24) was mixed with 10% semen that had been incubated for 0 or 2 hours in the presence or absence of 300 μ M of the respective peptides similar to previous experiments. Thereafter, TZM-bl cells were infected, after 2 hours, the inoculum containing cytotoxic semen was removed, and fresh medium was added. Infection rates were determined 2 days later by quantifying β -galactosidase activities in cellular lysates. As previously observed [69, 90], pretreatment of virions with 10% semen enhanced HIV-1

infection 47-fold (from 1.5×10^3 RLU/s to 7.2×10^5 RLU/s) (Fig. 14). However, none of the peptides reduced semen-treated virus infection at the tested concentrations. In the presence of WI6 and semen, HIV-1 infection increased from 1.4×10^3 RLU/s up to 5.4×10^5 RLU/s, a 35-fold increase (Fig. 14). This slight reduction, however, was not statistically significant. Thus, the peptides that prevent fibril formation of synthetic peptides do not affect semen-mediated enhancement of HIV-1 infection, suggesting that the peptides are either inactive in semen or that SEVI fibrils may already be present in semen.

Fig. 14. The designed inhibitors do not abrogate semen-mediated enhancement of HIV-1 infection. HIV-1 particles containing 0.1 ng p24 antigen were mixed with 10% semen (SE)/inhibitor or PBS/inhibitor solutions that had been incubated for 2 hours at RT and TZM-bl cells were infected. After 2 hours, the inocula were removed and replaced with fresh medium. Shown are the infection rates as raw data (mean β galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 2 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second. Student's t-test; *ns*, no statistically significant difference.

3.2 Semen diminishes the antiviral activity of microbicides

In the second part of my thesis, I evaluated the efficacy of microbicides in the presence of semen. Microbicides are currently being tested for their potential to limit the spread of HIV/AIDS. Although many microbicides effectively inhibit HIV infection *in vitro*, they almost invariably fail to prevent viral transmission *in vivo* [3, 38, 55, 73, 84, 145, 146]. One possible reason for this discrepancy in the efficacy of antiretroviral microbicides is that the preclinical testing of these agents does not reflect the conditions of sexual transmission of HIV-1 in a realistic way. This is because studies have not considered the well-documented enhancement in infectivity of semen [69, 90]. To test whether semen interferes with the antiviral efficacy of microbicides, I used the following experimental design: semen- or mock-treated CCR5 tropic (R5) HIV-1 was added to TZM-bl reporter cells containing serial dilutions of microbicides or antivirals of interest (Fig. 15). In these experiments, a low viral inoculum was used to quantify semen-mediated HIV-infection enhancing effects. In addition, I inoculated the cells with a control 10-fold higher virus dose to determine inhibition of untreated virus infection and to have a similar background infection rate (“infectivity-matched” control) as in the presence of semen. After 2 hours, the inoculum containing cytotoxic semen was removed, and fresh medium supplemented with inhibitors was added. Infection rates were determined 3 days later by quantifying β -galactosidase activities in cellular lysates.

Fig. 15. Schematic overview of the assay. TZM-bl cells containing increasing concentrations of microbicides are inoculated with 0.05 ng p24 antigen of mock-treated (upper half of the microtiter plate) or semen-treated R5 HIV 1 (bottom half). 2 hours later, the inocula are removed to prevent semen-mediated cytotoxic effects, and replaced with fresh medium containing microbicides. Infection rates are determined by measuring β -galactosidase activities in cellular lysates 3 days post-infection. A protease inhibitor was analysed the same way, except that CEMx-M7 cells were used and infection rates were quantified 4 days post infection by measuring luciferase activities (see chapter 2.2.4.10).

3.2.1 Semen reduces the antiviral efficacy of polyanions

I first analysed the effect of semen on the antiviral activity of three anionic polymers: Polystyrene acid (PSA) [131], Polynaphthalene sulfonate (PNS, PRO 2000) [119] and Cellulose sulfate (CS) [131], as well as the dendrimer SPL7013, a branched molecule capped with 32 naphthalene disulfonic acid groups that impart an anionic charge to the surface [64]. These antiviral agents represent “first-generation” microbicides that efficiently block virion attachment to cells *in vitro* [22, 141] but were inactive in clinical trials [55, 83, 84, 145]. As previously observed [69, 90], pretreatment of virions with 10% semen enhanced low-dose HIV-1 infection up to 2.1×10^5 RLU/s compared to mock-treated virions (2.2×10^4 RLU/s) (Fig. 16a, 0.05 ng p24), a 10-fold increase in infection rate. These infection rates were similar to those obtained after infection with a 10-fold increased amount of mock-treated HIV-1 (0.5 ng p24 antigen) (Fig. 16a), which served as “infectivity-matched” control. Treatment of cells with PSA reduced infection at both doses of untreated virus by more than 99% and blocked infection with a mean half minimal (50%) inhibitory concentration (IC_{50}) of 1.0 ± 0.1 μ g/ml (Fig. 16b and c). Remarkably, PSA was substantially less active against semen-exposed virus. Even at concentrations of 100 μ g/ml of PSA, semen-treated virus readily infected cells, and the absolute infection rates were similar to those obtained for untreated virus in the absence of drug (red and black lines in Fig. 16b). To determine the antiviral efficacy of PSA and all other analysed drugs, infection rates were normalised and used to calculate the IC_{50} values (Table 3, see page 41). This analysis revealed that PSA inhibited infection of low dose mock-treated HIV-1 with an IC_{50} of 1.0 ± 0.3 μ g/ml (Fig. 16c). This IC_{50} value was similar to that obtained after infection with a high-dose mock-treated HIV-1 (1.2 ± 0.4 μ g/ml) (Fig. 16c). In contrast, the IC_{50} obtained after infection with semen-exposed virus was increased by 20-fold. Thus, pretreatment of virions with as little as 10% semen results in a 20-fold reduced antiviral efficacy of PSA.

Fig. 16. The effect of semen on the antiviral activity of the polyanion PSA. (a) Infection rates of TZM-bl cells containing increasing concentrations of PSA that were inoculated with 0.05 ng mock- and semen-exposed R5 HIV-1, and 0.5 ng R5 HIV-1 as ‘infectivity-matched’ control. Shown are the infection rates as raw data (mean β -galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second. PSA, Polystyrene acid. (b) Normalised infection rates as % infection (β -galactosidase activities obtained from infected cells in the absence of inhibitor were set to 100%). (c) Calculated IC₅₀ values derived from normalised infection rates shown in b. The number above the bar represents the fold change in the IC₅₀ values derived from 0.05 ng semen-treated relative to 0.05 ng, and 0.5 ng untreated virus infection.

Similar to the results described for PSA, the polyanions PNS and CS also efficiently blocked 0.5 and 0.05 ng p24 HIV-1 infection in the absence of semen with an IC₅₀ of 1.0 ± 0.2 or 1.2 ± 0.3 μ g/ml (Fig. 17a), and 1.1 ± 0.1 or 1.2 ± 0.4 μ g/ml, respectively (Fig. 17b). Likewise, PNS and CS were less active against 0.05 ng semen-treated virus (IC₅₀ values = 21.0 ± 1.2 and 20.3 ± 1.3 μ g/ml) (Table 3, see page 41). The dendrimer SPL7013 inhibited 0.5 and 0.05 ng p24 untreated HIV-1 infection with an IC₅₀ of 1.2 ± 0.1 and 1.1 ± 0.2 μ g/ml, respectively (Fig. 17c). Again, pretreatment of HIV-1 with semen increased the IC₅₀ to 23.0 ± 1.9 μ g/ml (Table 3). Thus, PNS, CS and SPL7013 were 17 to 21-fold less active against semen-treated virus, and did not inhibit semen-exposed virus infection entirely.

Fig. 17. Polyanions and the dendrimer do not efficiently block semen-exposed virus. TZM-bl cells containing increasing concentrations of (a) PNS, (b) CS, and (c) SPL7013 were inoculated with 0.05 ng mock- and SE-exposed R5 HIV-1, and 0.5 ng R5 HIV-1. The raw data were analysed and evaluated as described above. Shown panels depict normalised infection rates (% infection). PNS, Polynaphthalene sulfonate. CS, Cellulose sulfate.

Polyanion concentrations exceeding 100 μg/ml could not be analysed in a meaningful manner as they caused cytotoxic effects (Fig. 18). Thus, polyanion-based microbicides markedly lose antiviral efficacy in the presence of semen, the main vector for the sexual transmission of HIV-1.

Fig. 18. Elevated concentrations of polyanions and the dendrimer are cytotoxic. TZM-bl cells were incubated with indicated concentrations of (a) the polyanions PSA, PNS, and CS, or (b) the dendrimer SPL7013 for 3 days and cell viability was determined by MTT assay. Data shown represent mean normalised viability values as % viability obtained from triplicate measurements $\pm$ standard deviation (untreated cells were set to 100%).

3.2.2 Semen diminishes the antiviral efficacy of neutralising antibodies

In a second set of experiments, I determined whether semen affects the efficacy of antiviral agents targeting different steps in the viral life cycle. Similar to anionic polymers, neutralising antibodies (nAb) such as the gp41-specific 2F5 and the gp120-specific 2G12 antibodies inhibit HIV attachment to, and fusion with, target cells [15, 45]. In contrast to polyanions, nAbs exhibit more specificity, since they target the HIV envelope glycoprotein [25, 65, 102, 134, 135, 158]. In the absence of semen, nAb 2F5 (Fig. 19a) blocked mock-treated HIV-1 (0.05 and 0.5 ng p24 antigen) with an IC_{50} of 2.6 ± 0.3 and 2.7 ± 0.1 $\mu\text{g/ml}$, respectively (Table 3, see page 41). Virion treatment with semen resulted in an 8-fold increased IC_{50} of 22.2 ± 1.2 $\mu\text{g/ml}$ (Fig. 19a). Similar results were obtained for the gp120 targeting nAb 2G12 (Fig. 19b). Thus, 10% semen reduces the antiviral efficacy of the tested neutralising antibodies by 8 to 9-fold.

Fig. 19. Neutralising antibodies lose efficacy against semen-exposed HIV-1. TZM-bl cells containing increasing concentrations of **(a)** the nAb 2F5, and **(b)** 2G12 were inoculated with 0.05 ng mock- and SE-exposed R5 HIV-1, and 0.5 ng R5 HIV-1. The raw data were analysed and evaluated as described above. Shown panels depict normalised infection rates (% infection). Please note that higher concentrations of antibodies could not be tested due to the limited amounts of available material.

3.2.3 Intracellular drugs are substantially less active against semen-exposed HIV

To continue my investigation of whether semen affects the efficacy of antiviral agents, I turned to compounds targeting the intracellular machinery of the HIV replication cycle [9]. This included the NRTI Tenofovir disoproxil fumarate (Tenofovir df, TDF), the INI Elvitegravir, and the PI Indinavir. TDF, a prodrug of Tenofovir, exerts a higher antiviral activity than Tenofovir [47, 62, 114]. TDF has been developed as a vaginal gel, but failed in a clinical phase III trial [80, 118]. TDF efficiently blocked both doses of HIV-1 with an IC_{50} of 1.7 ± 0.3 nM and 1.4 ± 0.2 nM (Fig. 20a) but was markedly less active against semen-exposed virus (IC_{50} to 17.9 ± 0.6 nM) (Table 3, see page 41). Like TDF, the INI Elvitegravir targets an intracellular step of the HIV-1 replication cycle [128, 129]. Elvitegravir efficiently inhibited HIV-1 infection (0.05 and 0.5 ng p24 antigen) with an IC_{50} of 0.60 ± 0.07 and 0.72 ± 0.05 nM, respectively (Fig. 20b), whereas 5.6 ± 0.5 nM was required for half-maximal inhibition in the presence of semen (Table 3). As a final step, I addressed the PI Indinavir. The antiviral activity of PIs cannot be determined in the TZM-bl infection system, since spreading infection has to be evaluated. The infection of cells from exclusively newly produced virions and not of the viral inoculum can be ensured by performing a multi-cycle replication assay in a suspension cell line. Thus, the T-cell line CEMx-M7 was pretreated with Indinavir, and infected with mock- and semen-exposed virus. After 2 days of infection, supernatants were replaced by fresh medium containing the PI. After 4 days, when spreading infection occurred, infection rates were determined by quantifying luciferase activities (see chapter 2.2.4.10). Indinavir inhibited infection of 0.05 and 0.5 ng mock-treated HIV-1 with IC_{50} values of 2.3 ± 0.2 and 1.9 ± 0.2 nM. After infection with semen-treated virus, however, the IC_{50} was increased by 10-fold ($IC_{50} = 23.2 \pm 3.0$ nM) (Table 3). Notably, the indicated concentrations had no cytotoxic effects (Fig. 21). These results demonstrate that inhibitors targeting HIV-1 glycoproteins or post-fusion steps of the viral life cycle lose antiviral activity if the viral inoculum was exposed to as little as 10% semen.

Fig. 20. Antivirals targeting various steps in the viral life cycle lose efficacy against semen-exposed HIV. TZM-bl cells containing increasing concentrations of (a) the NRTI Tenofovir df and (b) the INI Elvitegravir were inoculated with 0.05 ng mock- and SE-exposed R5 HIV-1, and 0.5 ng HIV-1. (c) The PI Indinavir was analysed the same way, except that CEMx-M7 cells were used, and 2 days post infection the cells were pelleted and fresh medium containing Indinavir was added. Infection rates were quantified 2 days later by quantifying luciferase activities in cellular supernatants. The raw data were analysed and evaluated as described above. Shown panels depict normalised infection rates (% infection). NRTI, Nucleotide reverse transcriptase inhibitor. INI, Integrase inhibitor. PI, Protease inhibitor.

Fig. 21. Non-polyanionic compounds are not cytotoxic. TZM-bl cells were incubated with (a) the nAbs 2F5 and 2G12, as well as (b) the NRTI Tenofovir df, (c) the INI Elvitegravir, and (d) the PI Indinavir for 3 days and cell viability was determined by MTT assay. Data shown represent mean normalised viability values as % viability obtained from triplicate measurements $\pm$ standard deviation (untreated cells were set to 100%). NRTI, Nucleotide reverse transcriptase inhibitor. INI, Integrase inhibitor. PI, Protease inhibitor.

Table 3. Antiviral activity of microbicides against mock- and semen-exposed R5 HIV-1.

Compound	Class	Inhibitory concentration 50% (IC ₅₀)			Fold increase in IC ₅₀ of SE-exposed relative to non-exposed HIV	
		0.5 ng HIV	0.05 ng HIV	0.05 ng HIV + 10% SE	0.5 ng HIV	0.05 ng HIV
PSA	Polyanion	1.0±0.1 µg/ml	1.0±0.1 µg/ml	20.1±0.5 µg/ml	20	20
PNS	Polyanion	1.0±0.2 µg/ml	1.2±0.3 µg/ml	21.0±1.2 µg/ml	21	18
CS	Polyanion	1.1±0.1 µg/ml	1.2±0.4 µg/ml	20.3±1.3 µg/ml	19	17
SPL7013	Dendrimer	1.2±0.1 µg/ml	1.1±0.2 µg/ml	23.0±1.9 µg/ml	19	21
2F5	Neutralising antibody	2.6±0.3 µg/ml	2.7±0.1 µg/ml	22.2±1.2 µg/ml	9	8
2G12	Neutralising antibody	0.7±0.1 µg/ml	0.7±0.1 µg/ml	6.0±0.5 µg/ml	9	8
TDF	NRTI	1.7±0.3 nM	1.4±0.2 nM	17.9±0.6 nM	10	13
ELV	Integrase inhibitor	0.60±0.07 nM	0.72±0.05 nM	5.6±0.5 nM	9	8
IDV (CEMx-M7 cells)	Protease inhibitor	1.9±0.2 nM	2.3±0.2 nM	23.2±3.0 nM	12	10
MVC	CCR5 antagonist	3.7±0.7 nM	3.9±0.2 nM	4.3±0.3 nM	1.2	1.1

3.2.4 Semen reduces the antiviral efficacy of microbicides in primary target cells of HIV

The findings described above were obtained from experiments using immortalised HeLa-derived TZM-bl and CEMx-M7 T-cells (Table 3, see page 41). Therefore, I tested whether semen also overcomes the antiviral activity of microbicides in primary target cells of HIV-1, in particular Peripheral blood mononuclear cell (PBMCs). Therefore, PHA/IL2-activated PBMCs were supplemented with PSA, 2F5, Tenofovir df, Elvitegravir and Indinavir, and inoculated with either 5 ng semen- or mock-exposed viruses encoding luciferase, as well as 50 ng mock-treated virus as “infectivity-matched” control. A higher viral dose was used since PBMCs are less permissive and are infected with lower efficiencies than, for example, TZM-bl cells [153, 156]. After 2 hours, the cells were pelleted and the inoculum containing cytotoxic semen was removed, and fresh medium supplemented with inhibitors was added. Infection rates were determined 3 days later by quantifying luciferase activities in cellular supernatants (see chapter 2.2.4.8).

As previously described [69, 90], pretreatment of low viral-doses of HIV-1 with 10% semen enhanced infection of PBMCs by approximately 10-fold from 4.3×10^5 to 4.5×10^6 RLU/s (Fig. 22a, 5 ng p24). These infection rates were similar to those obtained after infection with 10-times more mock-treated HIV (50 ng p24 antigen) (Fig. 22a). The polyanion PSA inhibited mock-treated HIV infection and exhibited an IC_{50} of $1.0 \pm 0.3 \mu\text{g/ml}$ (Fig. 22b and c). As observed with TZM-bl cells, PSA resulted in a 20-fold decrease compared to the same amount of semen-treated virus ($IC_{50} = 20.4 \pm 1.7 \mu\text{g/ml}$) (Table 4, see page 44).

Fig. 22. Semen reduces the antiviral efficacy of PSA to block HIV-1 infection of PBMCs. (a) PBMCs containing increasing concentrations of PSA were inoculated with 5 ng mock- and SE-exposed R5 HIV-1-luc, and 50 ng R5 HIV-1-luc. Shown are the infection rates as raw data (mean luciferase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying luciferase activities in cellular supernatants). RLU/s, relative light units per second. (b) Normalised infection rates as % infection (luciferase activities obtained from infected cells in the absence of inhibitor were set to 100%). (c) Calculated IC₅₀ values derived from normalised infection rates shown in b. The number above the bar represents the fold change in the IC₅₀ values derived from 5 ng SE-treated relative to 5 ng, and 50 ng untreated virus infection.

Semen also reduced the efficacy of antivirals targeting different steps in the viral life cycle. For example, the nAb 2F5 (Fig. 23a) blocked 50 and 5 ng p24 HIV-1 infection in the absence of semen with an IC₅₀ of 3.0 ± 0.4 and 3.2 ± 0.5 µg/ml, respectively (Table 4, see page 44). However, pretreatment of 5 ng HIV-1 with semen increased the IC₅₀ to 22.2 ± 1.6 µg/ml (Fig. 23a). Similar results were observed in cell lines (Table 3, see page 41), IC₅₀ values in the presence of 2F5 (Fig. 23a), Tenofovir df (Fig. 23b), Elvitegravir (Fig. 23c) and Indinavir (Fig. 23d) increased by 7 to 12-fold if cells were inoculated with semen-exposed virus (Table 4). Thus, semen reduces the antiviral efficacy of antiretroviral drugs, not only in cell lines but also in primary HIV-1 target cells.

Fig. 23. Semen reduces the antiviral efficacy of candidate microbicides to block HIV-1 infection of PBMCs. PBMCs containing indicated concentrations of (a) the nAb 2F5, (b) the NRTI Tenofovir df, (c) the INI Elvitegravir, or (d) the PI Indinavir were inoculated with 5 ng mock- and SE-exposed R5 HIV-1-luc, and 50 ng R5 HIV-1-luc. Infection rates were determined 3 (a-c) or 4 days (d) post infection by quantifying luciferase activities in cellular supernatants. The raw data were analysed and evaluated as described above. Shown panels depict normalised infection rates (% infection).

Table 4. Antiviral activity of microbicides against mock- and semen-exposed HIV-1 in PBMCs.

Compound	Class	Inhibitory concentration 50% (IC ₅₀)			Fold increase in IC ₅₀ of SE-exposed relative to non-exposed HIV	
		50 ng HIV	5 ng HIV	5 ng HIV + 10% SE	50 ng HIV	5 ng HIV
PSA	Polyanion	1.2±0.4 µg/ml	1.0±0.3 µg/ml	20.4±1.7 µg/ml	17	20
2F5	Neutralising antibody	3.0±0.4 µg/ml	3.2±0.5 µg/ml	22.2±1.6 µg/ml	7	7
TDF	NRTI	2.7±0.1 nM	3.1±0.3 nM	21.0±1.5 nM	8	7
ELV	Integrase inhibitor	0.67±0.08 nM	0.64±0.28 nM	5.3±0.7 nM	8	8
IDV	Protease inhibitor	1.6±0.2 nM	1.4±0.4 nM	16.5±0.3 nM	11	12
MVC	CCR5 antagonist	3.5±0.1 nM	3.6±0.4 nM	4.3±0.1 nM	1.2	1.2

3.2.5 SEVI antagonises the anti-HIV-1 activity of microbicides and antiviral drugs

The previous results demonstrated that the antiviral activity of the surveyed compounds is not greatly affected by the viral inocula (Table 3, see page 41). However, the IC_{50} values obtained after infection with 0.05 ng p24 of semen-exposed virus were 7 to 21-fold higher than those obtained in the absence of semen. This was also the case for the “infectivity-matched” control (0.5 ng p24) (Table 3). These findings indicate that it is not just an increase in the infectious dose, but also a specific property of semen that is responsible for the diminished antiviral efficacy [90]. To examine whether the infectivity-enhancing activity of amyloid in semen is responsible for this effect, I evaluated the effect of synthetic SEVI amyloid under identical experimental conditions.

Pretreatment of virions with 35 μ g/ml SEVI enhanced low dose HIV-1 infection (0.05 ng p24 antigen) of TZM-bl cells by 10-fold from 2.3×10^4 to 2.4×10^5 RLU/s (Fig. 24a), which results in a similar background infection rate as in the presence of semen. Treatment of cells with PSA reduced infection of both doses of untreated virus by more than 99% and blocked infection with a IC_{50} of 1.1 ± 0.2 and 1.2 ± 0.1 μ g/ml, respectively (Table 5, see page 48). However, PSA was markedly less active against SEVI-treated virus ($IC_{50} = 21.4 \pm 2.5$ μ g/ml) (Fig. 24b and c). 35 μ g/ml SEVI affected PSA activity and increased the IC_{50} 20-fold compared to low dose untreated HIV-1 (Fig. 24c).

Fig. 24. The infectivity-enhancing activity of SEVI antagonises the anti-HIV-1 activity of PSA. (a) Infection rates of TZM-bl cells containing increasing concentrations of PSA that were inoculated with 0.05 ng mock- and 35 μg/ml SEVI-exposed R5 HIV-1, and 0.5 ng R5 HIV-1. Shown are the infection rates as raw data (mean β-galactosidase activities ± standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β-galactosidase activities in cellular lysates). RLU/s, relative light units per second. (b) Normalised infection rates as % infection (β-galactosidase activities obtained from infected cells in the absence of inhibitor were set to 100%). (c) Calculated IC₅₀ values derived from normalised infection rates shown in b. The number above the bar represents the fold change in the IC₅₀ values derived from 0.05 ng SE-treated relative to 0.05 ng, and 0.5 ng untreated virus infection.

Interestingly, SEVI also reduced the efficacy of antiviral agents targeting various steps in the viral life-cycle. For example, the dendrimer SPL7013 (Fig. 25a) blocked 0.5 and 0.05 ng p24 HIV-1 infection in the absence of SEVI with IC₅₀ values of 1.1±0.1 and 0.9±0.1 μg/ml, respectively (Table 5, see page 48). However, pretreatment of 0.05 ng HIV 1 with SEVI increased the IC₅₀ to 20.1±1.5 μg/ml (Fig. 25a and Table 5). The presence of SEVI amyloid also increased the IC₅₀ values of other tested microbicides and antiretroviral drugs, such as SPL7013, 2F5, Tenofovir df, Elvitegravir, and Indinavir (Fig. 25b-e and Table 5). Thus, exposure of HIV-1 to just 35 μg/ml SEVI results in 7 to 22-fold reduced antiviral efficacy of polyanions, nAbs, and antiretroviral drugs targeting intracellular steps of the viral life cycle.

Fig. 25. The infectivity-enhancing activity of SEVI fibrils antagonises the anti-HIV-1 activity of antivirals. TZM-bl cells containing increasing concentrations of (a) the dendrimer SPL7013, (b) the nAb 2F5, (c) the NRTI Tenofovir df, and (d) the INI Elvitegravir were inoculated with 0.05 ng mock- and SEVI-exposed R5 HIV-1, and 0.5 ng R5 HIV-1. (e) The PI Indinavir was analysed the same way, except that CEMx-M7 cells were used. The raw data were analysed and evaluated as described above. Shown panels depict normalised infection rates (% infection). NRTI, Nucleotide reverse transcriptase inhibitor. INI, Integrase inhibitor. PI, Protease inhibitor.

Table 5. Antiviral activity of microbicides against mock- and SEVI-exposed HIV-1.

Compound	Class	Inhibitory concentration 50% (IC ₅₀)			Fold increase in IC ₅₀ of SEVI-exposed relative to non-exposed HIV	
		0.5 ng HIV	0.05 ng HIV	0.05 ng HIV + SEVI	0.5 ng HIV	0.05 ng HIV
PSA	Polyanion	1.1±0.2 µg/ml	1.2±0.1 µg/ml	21.4±2.5 µg/ml	20	19
SPL7013	Dendrimer	1.1±0.1 µg/ml	0.9±0.1 µg/ml	20.1±1.5 µg/ml	19	22
2F5	Neutralising antibody	3.4±0.2 µg/ml	3.5±0.4 µg/ml	23.1±1.0 µg/ml	7	7
TDF	NRTI	1.7±0.1 nM	2.0±0.3 nM	16.1±1.4 nM	10	8
ELV	Integrase inhibitor	0.63±0.08 nM	0.66±0.11 nM	4.3±0.6 nM	7	6
IDV (CEMx-M7 cells)	Protease inhibitor	2.0±0.3 nM	3.1±1.7 nM	28.4±3.1 nM	14	17
MVC	CCR5 antagonist	3.1±0.4 nM	3.4±0.5 nM	4.4±0.5 nM	1.4	1.3

3.2.6 Semen lacking amyloid does not impair polyanion activity

The differences in the IC₅₀ values observed in the presence of synthetic SEVI amyloid (19 to 22-fold decrease for polyanions; 7 to 17-fold decrease for neutralising antibodies, reverse transcriptase inhibitor, integrase and protease inhibitor) largely reflect decreases obtained with semen-treated virus (Table 1-3, see page 41, 44, 48), suggesting a similar underlying mechanism. If seminal amyloid accounts for the reduced antiviral efficacies of the tested compounds, then semen deficient of amyloid should not have an effect. To test this hypothesis, I took advantage of seminal plasma (SP) derived from patients with ejaculatory duct obstruction (EDO) which does not enhance HIV-1 infection due to a lack of amyloids [107, 111]. To this end, virions were treated with 10% SP from healthy individuals or EDO patients and used to infect cells containing Cellulose sulfate. SP from EDO patients did not enhance HIV infection, as evidenced by similar reporter gene activities (3.1×10^4 RLU/s) as those obtained after mock-treated infection (2.9×10^4 RLU/s) (Fig. 26). Strikingly, the polyanion CS reduced HIV-1 infection of mock and EDO-semen-treated virus, and CS was only marginally active in the presence of semen from healthy individuals (Fig. 26). Unfortunately, the effect of SP from EDO patients on other classes of microbicides could not be determined, since the volumes of this body fluid were limited. However, these findings indicate that amyloid enhancers of HIV-1 infection appear to be both necessary and sufficient for the ability of semen to diminish the antiretroviral efficacy of the analysed

compounds that all target viral components either extra- (polyanions, nAbs) or intracellular (RT, IN and PR inhibitors).

Fig. 26. Seminal plasma (SP) from EDO patients that lacks amyloid and viral-enhancing activity does not antagonise the antiviral activity of a polyanionic microbicide. Infection rates of TBM-bl cells containing increasing concentrations of the polyanion Cellulose sulfate that were inoculated with 0.1 ng p24 R5 HIV-1 mock-treated or exposed to pooled SP derived from healthy individuals or EDO patients. Shown are the infection rates as raw data (mean β -galactosidase activities $\pm$ standard deviation derived from triplicate infections obtained 3 days post infection by quantifying β -galactosidase activities in cellular lysates). RLU/s, relative light units per second.

3.2.7 The CCR5 antagonist Maraviroc retains antiviral activity against semen and SEVI

Maraviroc (MVC) prevents the binding of HIV-1 gp120 to CCR5, thus preventing viral entry [35]. I analysed the ability of MVC to block HIV-1 infection in TBM-bl cells using the same experimental design outlined above. Remarkably, MVC, at concentrations ranging from 0.01 to 1,000 nM, inhibited TBM-bl infection with untreated HIV-1 in a dose-dependent manner, with IC_{50} values of 3.7 ± 0.7 nM (0.5 ng of p24) and 3.9 ± 0.2 nM (0.05 ng of p24) (Fig. 27a). In contrast to antivirals targeting viral components, elevated concentrations of MVC (between 200–1,000 nM) also efficiently blocked semen-exposed HIV-1 (Fig. 27a). The IC_{50} value increased only marginally to 4.3 ± 0.3 nM in the presence of semen (Table 3, see page 41). Importantly, the indicated concentrations had no cytotoxic effects (Fig. 27b).

Fig. 27. Maraviroc retains antiviral activity against semen-exposed HIV-1 and lacks cytotoxicity. (a) TZM-bl cells containing increasing concentrations of MVC were inoculated with 0.05 ng mock- and SE-exposed R5 HIV-1, and 0.5 ng R5 HIV-1. The raw data were analysed and evaluated as described above. Shown panels depict normalised infection rates (% infection). (b) TZM-bl cells were incubated with indicated concentrations of MVC for 3 days and cell viability was determined by MTT assay. Data shown represent mean normalised viability values as % viability obtained from triplicate measurements $\pm$ standard deviation (untreated cells were set to 100%).

To verify the TZM-bl results, I analysed whether MVC retains its antiviral activity in primary target cells of HIV-1 (Fig. 28a). In this case the IC_{50} increased 1.2-fold, from 3.6 ± 0.4 nM to 4.3 ± 0.1 nM in the presence of semen (Table 4, see page 44). This result suggests that MVC retains its antiretroviral activity, not only in cell lines, but also in primary HIV-1 target cells.

Similar results were obtained when virions were exposed to SEVI amyloid in the presence of MVC (Fig. 28b). The IC_{50} increased 1.3-fold (Table 5, see page 48) demonstrating that seminal amyloid does not inhibit the antiviral activity of MVC. Notably, this CCR5-antagonist entirely inhibited semen-treated HIV-1 infection of TZM-bl cells (Fig. 27a) and PBMCs (Fig. 28a) at concentrations between 200 and 1,000 nM, which are lower than those achievable in the human genital tract (~ 2.4 μ M in cervicovaginal fluid and ~ 1.7 μ M in vaginal tissue following oral administration) [24, 36, 147]. Potent inhibition of HIV-1 by MVC in the presence of semen was confirmed in a transmission assay from cervical epithelial cells (CaSki) to CEMx-M7 T cells (Fig. 28c). Attachment of HIV to epithelial cells followed by their transfer to susceptible target cells may play an important role in sexual transmission of HIV-1 [24]. To determine whether MVC could block cell-to-cell transmission of semen-exposed HIV-1, I inoculated cervical epithelial cells (CaSki) with a Transmitter/Founder virus and then co-cultivated the cells with CEMx-M7 cells. I found that semen increased HIV-1 transmission rates, but that 4 nM MVC fully blocked transmission

in the absence of semen (Fig. 28c). Although semen partially counteracted the antiviral effects of MVC, concentrations of the drug exceeding 20 nM potently inhibited *trans*-infection (Fig. 28c). Thus, unlike the antiretroviral drugs targeting viral components that lose efficacy when presented with semen-exposed virus, MVC remains potent in the presence of semen, suggesting that this CCR5-antagonist holds great promise as an HIV preventative therapy.

Fig. 28. Maraviroc retains antiviral activity against semen-exposed virus in PBMCs and SEVI-exposed virus in TZM-bl cells, and inhibits *trans*-infection of semen-treated virus from cervical epithelial cells to target cells. (a) PBMCs containing indicated concentrations of MVC were inoculated with 5 and 50 ng mock-, and 5 ng SE-exposed R5 HIV-1-luc. (b) TZM-bl cells containing increasing concentrations of MVC were inoculated with 0.05 and 0.5 mock-, and 0.05 ng SEVI-exposed R5 HIV-1. (c) CaSki cells supplemented with MVC were inoculated with mock- or SE-treated HIV (1 ng p24). After 2 hours, inocula were removed, and CEMx-M7 reporter cells were added onto CaSki cells in the presence of MVC. 3 days post infection, luciferase and β-galactosidase activities were determined in cellular supernatants (PBMCs) and lysates (TZM-bl), respectively. Shown are normalised infection rates as % infection (luciferase and β galactosidase activities obtained from infected cells in the absence of inhibitor were set to 100%).

4 DISCUSSION

4.1 Development of PAP248-286 fibrillation inhibitors

The identification of infectivity-enhancing seminal amyloid fibrils pose interesting novel opportunities to prevent sexual HIV-1 transmission [69, 90]. In particular, a promising strategy to counteract the infectivity-enhancing activity of semen is to prevent the formation of these fibrils by preventing peptide assembly. Prior to this study, PAP248-286 was the only seminal peptide described to form amyloid. In a collaborative approach with David Eisenberg's laboratory, peptide inhibitors were developed to block fibrillation of PAP248-286 by a novel, structure-based mechanism. Since the native structures of many peptides are not known, an algorithm was developed by the Eisenberg lab [139]. This algorithm identifies segments that form fibrils, based on the peptide sequence. In a first round, peptides consisting of D-amino acids were generated against segment PAP260-265 and PAP282-286. These peptides delayed fibrillation and thus the SEVI-mediated enhancement of HIV-1 infection. The inhibitors were improved by inserting L- or commercially available non-natural amino acids to exert resistance against proteolytic degradation in semen [18]. For the first time, we could demonstrate that designed inhibitors specifically delay PAP248-286 fibril formation *in vitro* and thus SEVI-mediated enhancement of HIV-1 infection is diminished [130]. At the time these experiments were performed it was unclear whether fibrils formed after ejaculation or during liquefaction of semen. We now know that fibrils are already present in fresh ejaculates [143] and that semen loses enhancing activity over time, suggesting that fibrils get degraded [110]. The presence of amyloid in fresh ejaculates explains the lack of activity of developed peptide inhibitors in the presence of semen. Although these results were disappointing with respect to microbicide development, it was the first time that the prevention of fibril formation from proteins with still unknown native structures, such as PAP248-286 and tau protein [124, 130], was shown. Some other approaches have been described that block the HIV infectivity-enhancing activity of seminal amyloid. The aminoquinolene surfen and the Thioflavin T derivative BTA-EG(6) prevent the binding of SEVI to virus particles and target cells [97]. The green tea constituent Epigallocatechin-3-gallate (EGCG) not only blocks SEVI-mediated enhancement, but also disaggregates pre-formed fibrils [59]. Nonetheless, these approaches are generally unspecific. In addition to focusing on specific processes, our novel approach is of high interest to a wider research community, since amyloid fibrils in different tissues have similar β -sheet structures. Thus, the possibility exists to generate specific inhibitors that block the

generation not only of PAP248-286, but also of other amyloid fibrils, such as PAP85-120 and SEMs. Indeed, no specific amyloid inhibitors are known that prevent the formation of amyloid, such as A β or α -synuclein, which are involved in amyloid-related diseases such as Alzheimer's and Parkinson's disease. This thesis outlines the generation of specific fibrillation inhibitors using a novel structure-based approach, which holds the potential as a promising new tool for the design of fibril-inhibitors in amyloid-related diseases.

4.2 Semen diminishes the antiviral activity of microbicides and antiretroviral drugs

As semen is the main vector for HIV transmission, candidate microbicides must be active in its presence. Research from both our own and other laboratories has revealed that semen and seminal plasma enhance viral infection [8, 21, 57, 59, 69, 90, 97, 111–113]. Therefore, in this study we focused on whether this HIV-1-enhancing effect might interfere with the antiviral activity of microbicides. I found that all tested microbicides targeting viral components (anionic polymers, neutralising antibodies, as well as reverse transcriptase, protease and integrase inhibitors) exhibited strongly reduced antiviral activity against HIV-1 when in the presence of semen. Similar results were obtained when untreated- and semen-treated virus were normalised for the same infectivity. Thus, the reduced antiviral efficacies were not due to higher infection rates. The work presented herein suggests that the antiviral activities of most microbicides determined in the absence of semen in standard infection assays are grossly misleading and do not reflect the antiviral potency of the compounds against HIV-1 in semen during sexual transmission in real life. Anionic polymers were among the first compounds to be considered for HIV microbicide development because of their potent antiviral activity *in vitro* [131]. Although several anionic polymers advanced into clinical phase III trials, none of them showed significant protection against sexual HIV transmission [3, 55, 83, 145, 146]. A possible reason for this is that the preclinical testing of microbicides does not faithfully reflect the conditions of sexual transmission of HIV. In the present study, I found that anionic polymers exhibit greatly reduced antiviral activity against HIV-1 when in the presence of semen, which may explain these clinical results. Of note, a prior study also found that the antiviral activity of anionic polymers was reduced in the presence of seminal plasma [94]. Neurath *et al.* (2006) reported that a variety of polyanionic compounds have diminished anti-HIV-1 activity when mixed at a 1:1 (v/v) ratio with seminal plasma. They postulated that positively charged factors in seminal plasma neutralise the

negative charges of these compounds, thereby rendering them less effective. Charge neutralisation might explain why polyanion-based microbicides lose antiviral activity. However, my experimental protocol was designed to more closely mimic the native conditions in which HIV-1-contaminated semen becomes diluted and exposed to microbicides during transmission. To accomplish this, a small volume (20 μ l) of HIV-1 treated with 10% semen was added to a relatively large volume (280 μ l) of cells containing various inhibitors. This results in a final semen concentration in cell culture of less than 0.7%, which is too low to directly neutralise the anionic polymers [94]. Since I still observed reduced antiviral activity of polyanion-based microbicides in the presence of semen, charge neutralisation alone is unlikely to be the sole factor responsible for the loss of antiviral activity. Instead, I showed that the ability of semen to enhance HIV-1 infection is responsible for the decreased antiviral activity of the anionic polymers observed in our assays. VivaGel® is a microbicide containing the active ingredient SPL7013. Like anionic polymers, SPL7013 exhibits a negatively charged outer surface and potently inhibits HIV infection *in vitro* and *in vivo* [141]. Similar to the findings with anionic polymers, SPL7013 was substantially less active against semen-exposed HIV-1. We tried to experimentally determine the SPL7013 concentration required to fully protect cells from semen-exposed HIV-1 infection, but were unable to do so, since elevated doses of the inhibitor caused cytotoxic effects in TZM-bl cells. Therefore, we estimated the 99% effective concentration by graphical extrapolation and found that approximately 1 mg/ml of SPL7013 would result in complete protection against semen-treated virus (data not shown). Although this concentration might be achievable *in vivo* [106], it is apparent that the antiviral activities determined in standard infection assays in the absence of semen greatly underestimate the dose needed to block infection by semen-exposed virus.

Like anionic polymers, neutralising antibodies inhibit HIV attachment to and fusion with target cells [15], but exhibit more specificity as they target the HIV envelope glycoproteins [25, 65, 102, 134, 135, 158]. Several animal studies demonstrated that neutralising antibodies have been successfully used to protect monkeys and mice from intravenous infection by SIV or HIV, and thus have potential to be developed as microbicides [13, 14, 16, 49, 60, 103, 147–149]. As observed for the polyanions and SPL7013, neutralising antibodies lose antiviral efficacy in the presence of semen as well. Since “first-generation” microbicides that were based on surfactants, polyanions, acid buffer and dendrimers failed in the clinics, the development of “next-generation” microbicides focused on antiretroviral drugs that have been proven successful in treatment of HIV-infected individuals [125]. In a

phase II study, a vaginal gel containing the NRTI Tenofovir, showed protection rates of up to 54% [2]. Despite this high protection rate, the key issue in this study was the willingness of the participants to use the vaginal gel as prescribed. A subsequent larger phase III trial (MTN-003) testing the same formula failed to confirm these protective effects [80]. Since the gel has to be applied before and after sexual intercourse, the main reason for the failure was attributed to poor adherence, similar to the previous clinical trial. In my study, I found that the NRTI Tenofovir df, as well as the INI Elvitegravir and the PI Indinavir have a reduced antiviral activity against HIV-1 in the presence of semen. The observation that antiretroviral agents are less active in the presence of semen may explain the ineffectiveness of TDF in the MTN-003 study [31, 80]. Conclusively, I could show that semen affects the antiviral activity of inhibitors targeting viral components.

Remarkably, one exception of a drug that remained active against semen-exposed virus was observed, namely Maraviroc (MVC). In contrast to antiretroviral drugs targeting viral components that act intracellularly, MVC blocks viral entry via binding to the cellular CCR5 receptor [35, 79]. MVC, an allosteric inhibitor, renders CCR5 more rigid, and thus prevents conformational changes that allow HIV to enter target cells [7, 138]. The CCR5-antagonist is in clinical use and protects animals against vaginal virus challenge [92, 147]. Notably, oral administration of MVC in humans leads to relatively high average concentrations of the drug in cervico-vaginal fluid ($\sim 2.4 \mu\text{M}$) and vaginal tissue ($\sim 1.7 \mu\text{M}$) [36]. The presence of high concentrations in the female genital tract implies that MVC is a promising candidate for further microbicide development, since R5-tropic viruses predominate in the sexual transmission of HIV-1. It is plausible that a drug concentration that blocks all CCR5 receptors at the cell surface prevents HIV entry in a manner that is largely independent of the number of viral particles. Therefore, compounds that target cellular factors hold great promise for the further development of effective microbicides [77]. However, as with other antiretroviral drugs, treatment with a CCR5-antagonist can lead to drug resistance, and thus to a viral rebound of resistant HIV-1 strains [115, 116, 152]. This suggests that MVC-resistant strains have to be isolated and tested in the presence of a MVC-based microbicide. Several not-yet-approved CCR5 inhibitors that exhibit anti-HIV-1 activity *in vitro* are still undergoing clinical trials. These include variants of the natural ligand CCL5 [28], e.g. Vicriviroc (SCH-D) [98] or Cenicriviroc [12]. Other cell-targeting antivirals are monoclonal antibodies against CCR5 and CD4 that inhibit HIV-1 infection *in vivo*, but have not yet been approved for use outside of clinical trials, such as PRO140 [108] and Ibalizumab

[23]. Thus, antiviral agents that target cellular components are still promising candidates for further microbicide development.

What is the mechanism underlying the diminished activity of microbicides against semen-treated HIV-1? As noted above, seminal amyloid, in the form of SEVI fibrils, drastically enhances HIV-1 infectivity [69, 90, 110, 111]. In this study, I have demonstrated that synthetic SEVI fibrils reduce the antiviral efficacy of microbicides as efficiently as semen, whereas semen samples from patients suffering from ejaculatory duct obstruction (EDO), lacking amyloid, do not reduce antiviral efficacy. Since amyloid present in semen clusters and concentrates virions towards the site of infection [8, 90, 110, 111, 113, 143, 155], I postulated that the increased amount of virus, at the cell surface or inside the cell, might overcome the activity of microbicides targeting viral components, including NRTI, INI and PI. However, this does not completely account for the reduced activity of antiviral drugs that act intracellularly. A reasonable explanation might be differential infection kinetics. The rate-limiting step is the attachment of virions to the cell membrane of target cells [66, 142]. Indeed, amyloids carry many viral particles, and thereby not only facilitate the attachment of the virions to the cell membrane but concentrate them to the cell surface. As a result, simultaneous infections of individual cells may occur by semen-exposed virus, which in turn results in higher numbers of viral enzymes within the infected cell. Our findings are in agreement with the recent observation that local increases of the number of virions at the entry site are responsible for the decreased efficacy of microbicides and CD4-binding antibodies on cell-to-cell transmission [4]. In contrast to viral proteins, the HIV-targeted CCR5 co-receptor is a host-derived drug target with relatively constant expression levels at the cell surface. Saturating MVC concentrations, which occupy all receptor molecules, should prevent infection independently of the number of viral particles. Thus, it is plausible that MVC remains active against semen-treated HIV-1. To reinforce the results obtained in cell lines in the presence of semen, experiments were performed in PBMCs, the primary HIV target cells. As expected, similar effects were observed, since previous studies demonstrated that semen enhances HIV-1 infection in different cell lines and in PBMCs [8, 69, 90].

My results in cell lines and primary target cells of HIV-1 provide insight into general HIV 1 infection and transmission mechanisms *in vivo*. These models are still important to further understand the mechanisms of HIV-1 transmission but they neither mimic the functions nor reproduce the morphology of living tissues. As such, they do not reflect tissue responses of real organisms [85]. Therefore, explants or *ex vivo* tissue models have been established to

more closely mimic the *in vivo* situation. I performed some preliminary studies in cervico-vaginal explants and found that, at low viral titres, none of the tissues became productively infected in the absence of semen, although occasional infections were observed after exposure of virions to semen. At high viral titres, reproducible infection was achieved [30, 41, 43, 53] although semen exhibited no enhancing effects (data not shown). From this, I conclude that explants are suitable for studying neither the viral enhancing effects of semen nor the inhibitory effects of antiretroviral drugs, since semen-mediated enhancement is more pronounced at a low viral titre [8, 69, 90, 112, 113]. Despite the unsuitability of explants for analysing semen-mediated enhancement of HIV infection, there might be other variables that influence results derived from cervico-vaginal explants, such as the hormonal state, topical microbicides that have been applied prior to a surgery, or a related pre-surgical hormone therapy [6].

Different animal models have been developed to elucidate the sexual transmission of HIV-1 *in vivo*: e.g. SIV infection of macaques or HIV infection in humanised mice [33, 58, 86]. Despite this, current models used for studying sexual transmission of HIV-1 might not really imitate human HIV-1 transmission since animals are directly challenged with a concentrated virus stock to vaginal or rectal surfaces in the absence of semen [32, 33, 75]. More recently, sexual transmission of HIV-1 by coitus has been described in a new mouse model [54]. An animal model reflecting the *in vivo* situation of sexual transmission of HIV-1 might be the best approach to prove the reduced antiviral activity of microbicides obtained using cell lines and primary cells in the presence of semen. Notably, our laboratory has recently demonstrated that in macaque, semen and SEVI fibrils do not efficiently enhance vaginal SIV transmission at high viral titres but seemed to promote infection at low doses, although the results did not yield statistical significance because of the high variability in the susceptibility toward infection in the control group [91]. Nonetheless, the work offers early evidence that semen and seminal amyloids may facilitate SIV transmission after exposure to a low viral titre, which accurately reflects the *in vivo* situation. Further *in vivo* studies on the effects of semen on HIV-1 transmission and the efficacy of microbicides are warranted.

This thesis demonstrates that our assay allows the identification of compounds that are active against semen-exposed virus. Such compounds are promising for development as microbicides, since semen is the main vector for HIV-1 transmission. The reduced antiviral activity of microbicides targeting viral components against semen-treated virions may explain why many compounds that showed high efficacy *in vitro* failed in subsequent studies

in vivo. Meanwhile, further development of polyanions and SPL7013 as microbicides was halted as a result of higher incidences of HIV-1 infection. The higher susceptibility was due to toxic effects that led to local inflammation in the female genital tract [29, 83, 84, 145]. Notably, I found that toxic effects in the presence of polyanions and SPL7013 occur in TZM-bl cells at concentrations $> 100 \mu\text{g/ml}$. However, MVC did not exert toxic effects and retained its antiviral activity in the presence of semen. This suggests that microbicides targeting cellular components may be superior to those that target virions (anionic polymers) or viral components (anti-HIV antibodies, NRTI, INI and PI). Furthermore, this thesis suggests that an effective microbicide should contain agents targeting CCR5 (such as MVC) and counteracting semen-mediated infectivity enhancement. Indeed, different agents that prevent fibril formation or antagonise the HIV-1 infectivity-enhancing activity have been described [59, 97, 113, 127]. Therefore, a combination of compounds that antagonise seminal amyloids and those that exert antiviral activity should be more effective than the use of a single formulation. This represents a promising means for prevention of sexual transmission of HIV-1.

5 SUMMARY

The identification of SEVI fibrils in semen represents an interesting novel opportunity to prevent sexual HIV transmission. For the first time, this study demonstrated the development of peptides that block the fibrillation of PAP248-286 by a novel, structure-based mechanism. We generated peptide inhibitors that specifically delay PAP248-286 fibril formation *in vitro* and thus SEVI mediated enhancement of HIV-1 infection is abrogated. These inhibitors were inactive to prevent semen-mediated enhancement of HIV-1 infection, since fibrils already form before ejaculation. However, this approach might help to develop inhibitors that block assembly of A β or α synuclein, which are involved in amyloid-related diseases such as Alzheimer's and Parkinson's disease.

Many topical microbicides effectively inhibit HIV infection *in vitro*. Nonetheless, they almost invariably failed to prevent viral transmission *in vivo*. One possible reason for this discrepancy is that the preclinical testing of microbicides does not realistically reflect the conditions of sexual transmission of HIV-1. Here, I have demonstrated that candidate microbicides targeting viral components exert reduced efficacies in the presence of semen, the main vector for HIV transmission. This diminished antiviral activity results from the ability of semen to enhance the infectiousness of HIV particles. Our data support this idea by showing that microbicides efficiently suppress infection with virus that has been treated with seminal plasma from patients suffering from ejaculatory duct obstruction lacking amyloid fibrils. Thus, antiviral efficacies determined in standard infection assays in the absence of semen greatly underestimate the drug concentrations needed to block semen-exposed virus during sexual HIV transmission. Interestingly, MVC, a HIV entry inhibitor that binds to the CCR5 co-receptor, displayed strong antiviral activity against both untreated and semen-exposed virus. Thus, antiviral compounds targeting host factors hold greater promise for microbicide development than those targeting viral structures. My results further imply that the *in vitro* efficacy of microbicides should be determined in the presence of semen to identify the best candidates for the prevention of sexual HIV-1 transmission. If we extend our *in vitro* findings to the *in vivo* setting, this would suggest that "next-generation" microbicides should include components targeting cellular components the virus needs to replicate, but also factors in semen that enhance HIV infectivity, such as semen amyloid. This currently seems to be the most promising way to reduce sexual HIV-1 transmission.

6 References

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