Structure, function and antagonism of semen amyloids

Annika Röcker[a], **Nadia R. Roan**[b,c], **Jay Kant Yadav**[d], **Marcus Fändrich**[d], and **Jan Münch**[a,e]

[a]Institute of Molecular Virology, Ulm University Medical Center, 89081 Ulm, Germany, Tel: +49 731 5500 65154

[b]Department of Urology, University of California, San Francisco, San Francisco, CA 94158, USA

[c]Gladstone Institute of Virology and Immunology, San Francisco, CA 94158, USA

[d]Institute of Protein Biochemistry, Ulm University, Helmholtzstr. 8/1, 89081 Ulm, Germany

[e]Core Facility Functional Peptidomics, Ulm University Medical Center, Ulm, Germany

Abstract

Amyloid fibrils are linear polypeptide aggregates with a cross-β structure. These fibrils are best known for their association with neurodegenerative diseases, such as Alzheimer's or Parkinson's, but they may also be used by living organisms as functional units, e.g. in the synthesis of melanin or in the formation of bacterial biofilms. About a decade ago, in a search for semen factors that modulate infection by HIV-1 (a sexually transmitted virus and the causative agent of the acquired immune deficiency syndrome (AIDS)), it was demonstrated that semen harbors amyloid fibrils capable of markedly increasing HIV infection rates. This discovery not only created novel opportunities to prevent sexual HIV-1 transmission but also stimulated research to unravel the natural role of these factors. We discuss here the identification of these intriguing structures, their molecular properties, and their effects on both sexually transmitted diseases and reproductive health. Moreover, we review strategies to antagonize semen amyloid to prevent sexual virus transmission.

1. Seminal plasma: more than just a sperm vehicle

Seminal plasma (SP) is the liquid fraction of semen produced by the male accessory sex organs. SP, far from simply serving as a passive vehicle to transport sperm during conception, harbours many bioactive agents that promote reproductive success. In the absence of the protective effects of SP, oxidative damage to sperm can occur [1]. SP also elicits responses in the female reproductive tract (FRT) that promote conception. Studies in mice have demonstrated that the signalling effects of SP can be long-lasting, spanning from the peri-conception period all the way to post-birth. In particular, the absence of SP during mating decreased the rate of conception, and mice that were conceived despite the absence of SP exhibited metabolic abnormalities relative to mice that were conceived in the presence of SP [2]. In humans, SP can increase the rate of implantation, both when introduced through

capsules and other artificial means and when introduced via sexual intercourse [3]. At the molecular level, SP is a strong inducer of various signal transduction pathways, and elicits a potent and rapid transcriptional response characterized by a gene expression signature associated with inflammation, cellular migration, proliferation, and viability [4-6]. Perhaps not surprisingly, SP also affects infection by sexually transmitted pathogenic microbes, through both direct (e.g., anti-bacterial peptides) and indirect (e.g., by eliciting a pro-inflammatory response in the FRT) means. The complex effects of SP on reproduction and infection by seminal microbes can be mediated through a plethora of different SP constituents, such as the thousands of different proteins, sugars, and lipids present in SP, in addition to the cargo of highly abundant exosomes of which trillions are present in a typical ejaculate [7].

2. Identification of semen amyloids

The initial discovery that semen harbours amyloid-forming peptides that boost HIV infectivity was made rather serendipitously. In order to discover molecules in human semen modulating HIV infection, Münch et al analysed a library of peptides and proteins derived from pooled human semen. In contrast to similar studies conducted using libraries generated from hemofiltrate [8,9], which had identified inhibitors of HIV infection, screening of the semen-derived fractions identified two fractions that potently increased HIV infection rates [10]. Mass spectrometric analysis of the dominant fraction identified a series of peptides derived from the C-terminal region of prostatic acid phosphatase (PAP), with the dominant peptide being comprised of resides 248-286 (Table 1). PAP a homodimeric enzyme that is secreted from the epithelial cells of the prostate gland, and is present at high concentrations (>1 mg/ml) in semen [11]. Freshly synthesized PAP(248-286) did not enhance HIV infection. Intriguingly, however, when PAP(248-286) was agitated, the peptide polymerized into amyloid fibrils (Fig. 1) that potently increased HIV infection rates – by up to several orders of magnitude. These fibrils were termed SEVI, for Semen-derived Enhancer of Viral Infection [10]. SEVI was demonstrated to show key characteristics of amyloid fibrils through a variety of biochemical and biophysical measurements (see below).

Additional studies subsequently revealed that SEVI was not the only amyloid species in human semen. Analysis of the second semen-derived fraction harbouring infection-enhancing activity revealed a second fragment of PAP, PAP(85-120), that, like SEVI, formed fibrils that enhanced HIV infection of cellular targets (Table 1) [12].

Using a completely different approach where antibodies recognizing the general amyloid fold were used to fish out endogenous amyloids from human semen, Roan *et al* identified a second class of semen amyloids, this time derived from the two highly homologous semen proteins semenogelin-1 and semenogelin-2 (Table 1) [13,14]. This second class of amyloids, collectively termed "SEM amyloids", exhibited no sequence homology to either PAP(248-286) or PAP(85-120), but exhibited similar biophysical properties and enhanced HIV infection rates to similar extents.

Fibrils derived from synthetic peptides have a remarkable ability to increase HIV infection rates: only 1–3 virions are sufficient for productive infection in the presence of fibrils, but 1,000–10,000 virions are required in the absence of the fibrils [10,12]. Moreover, physiological

concentrations of fibrils of approximately 30 – 40 μg/ml increase the infectious titer of HIV-1 by more than five orders of magnitude.

3. Structure of semen derived amyloids

The precursor protein of PAP(248-286) and PAP(85-120) is a homodimeric enzyme. In the native state of the protein, the 248-286 segment is located close to the dimer interface and it encompasses two α-helices, which extend between residue K251-R257 and G260-I277. The PAP(85-120) fragment constitutes a part of an α-helix, which extends from V77-F92 and plays a crucial role in dimerization. This helix connects the sixth β-sheet of second subunit through a hydrogen bond between D76 and H112 [15].

The primary structure of PAP(248-286) contains eight basic amino acid residues and two acidic amino acid residues (Table 1). These properties result in a theoretical isoelectric point (pI) of 10.21 (Table 1) and a strongly positive net charge at neural pH. Analysis of freshly dissolved PAP(248-286) peptide at 37°C in 20 mM potassium phosphate buffer, pH 7.5, containing 10% D_2O with ^1H nuclear magnetic resonance (NMR) spectroscopy revealed a poor dispersion of the chemical shifts within the amide proton region. The recorded spectra lacked nuclear Overhauser effects (NOEs) that would have indicated strong intramolecular interactions or a stable secondary structure [16]. Instead, the NMR characteristics suggested that the monomeric peptide was significantly unfolded in aqueous solution but adopted a significantly α-helical conformation in the presence of 20 mM phosphate buffer, pH 7.5, containing 30 or 50% trifluoroethanol (TFE) [17]. ^1H NMR spectroscopy revealed under these conditions strong intramolecular NOEs at 25°C and the recorded spectra were significantly dispersed. The α-helical segment ranged in 50% TFE from residue Q259 to I284 [17]. Evidence for an α-helical conformation of PAP(248-286) was also provided by far-ultraviolet (UV) circular dichroism (CD) spectroscopic analysis of a sample containing lipid vesicles formed from 70% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 30% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol [16]. Under these conditions, the α-helical structure identified by NMR extended between residues H250-Q259 and L263-A274 [16,18]. Hence, binding to these lipid vesicles induced a conformation that is reminiscent of the native conformation of PAP (Jakob et al, 2000).

Overnight incubation of PAP(248-286) peptide in Dulbecco's phosphate buffer saline, pH 7.4, at 37°C led to the aggregation of the peptide and the formation of fibrils that were visible by transmission electron microscopy (TEM) (Fig. 1)[10,19]. Analysis of these *in vitro* formed fibrils with atomic force microscopy (AFM) revealed a height of 5.3 ± 0.01 nm and length up to 5 to 10 μm [12]. Far-UV CD spectroscopy revealed high levels of β-sheet conformation [20]. X-ray diffraction recorded discernible reflections at 4.7 and 10.6 Å, corresponding to the regular inter-strand spacing and inter-sheet distances in aggregated β-sheets [10]. The fibrils interacted with the amyloid-binding dyes thioflavin T (ThT) and Congo red (CR) and displayed, taken together with the above characteristics, key features of amyloid fibril structures [10,20]. Hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS) as well as a protease protection assay suggested that an N-terminal segment of aggregated PAP(248-286) comprising residues G248 to E254 was unprotected from HDX and susceptible to proteolytic cleavage. By contrast, a region from K281 to Y286

was found to be highly protected from exchange suggesting that it was involved in formation of fibril core [21]. Prediction of highly amyloidogenic sequence elements within PAP(248-286) identified a segment extending between residues G260 to K265 (GGVLVN), which formed microcrystals consisting of self-complementary cross-β sheets, termed steric-zippers [22]. This structure allowed the design of a D-amino acid peptide that blocked fibrillation and HIV-1 infectivity enhancement of this peptide (see 10.3.3.).

Similar to PAP(248-286), also PAP(85-120) shows a positive net charge at neutral pH and a theoretical pI of 9.99 (Table 1). The peptide formed amyloid fibrils *in vitro* and displayed ThT and CR binding [12]. PAP(85-120) aggregates showed substantial morphological heterogeneity ranging from small spherical oligomers of a height of 3.58 ± 1.22 nm and protofibril-like structures of a height of 4.01 ± 1.23 nm to 1 to 5 μm-long fibrils as determined by AFM [12].

The precursor protein of SEM amyloids are the two homologous proteins SEM1 and 2 that are mainly expressed in seminal vesicles and form the major component of semen coagulum [13,14]. SEMs are rapidly cleaved after ejaculation by intrinsic proteases and release a number of short peptide fragments, such as SEM1(45–107), SEM2(45–107), SEM1(49–107), SEM2(49–107), SEM1(68–107), SEM2(68–107), and SEM1(86-107)[13,14,23]. Overnight agitation of these peptides in phosphate buffered saline at 37°C led to the formation of fibrils as indicated by an increased ThT fluorescence intensity and detection of fibrillar structures by TEM. Under *in vitro* conditions all the fibrils were capable to enhance HIV infectivity, albeit with different efficiencies [13,14]. Based on HDX-MS aggregated SEM1(86-107) seemed to contain a solvent-protected fibril core, including residues D86-K92, S96-R98 and Q104-L107. The positively charged residues L92, L95, A98 were more solvent exposed [23], suggesting that they might be involved in mediating the electrostatic interactions between SEM amyloids and viral particles or host cells.

While atomic structures of *in vitro* formed fibrils derived from PAP, SEM1 or SEM2 fragments are not yet available, even less is known about the structures of endogenous amyloid fibrils in human semen. Based on AFM and TEM the existence of fibrils in semen has been confirmed (Fig. 2) [19,24]. Some of the fibrils in semen interacted with the amyloid-binding antibodies WO1 and WO2 and could be immunogold-labelled with primary antibodies binding to PAP- or SEM-derived epitopes [19]. These fibrils had diameters of approximately 5-10 nm and showed positive interactions with the amyloid-binding antibodies WO1 and OC [13,19]. Notably, seminal amyloid was detected in all ejaculates derived from healthy individuals or HIV-1 infected men, establishing semen as first human body fluid that naturally contains amyloid fibrils in the absence of disease [19].

4. Endogenous semen amyloids and effect of semen on HIV infection

If fibrils are present in semen and contribute to sexual transmission of HIV, semen itself is expected to exert HIV enhancing activity as well. Until the discovery of SEVI fibrils in 2007, however, only a few studies had examined the effects of semen on HIV infection, likely because this biological fluid is quite toxic to cells *in vitro* [25,26], making it difficult to work with in cell culture-based assays. Since these cytotoxic effects complicate evaluation of

semen *in vitro*, it is critical to study semen under conditions that preserve the viability of target cells [10,27]. Under these conditions, which can be achieved limiting the amount of semen cells are exposed to, semen efficiently promotes viral infection [12–14,19,24,28–34]. Importantly, the infection-enhancing effects of semen are most prominent at low viral inocula, which to some extent mimics the *in vivo* situation since only low amounts of virus are present in semen [10,13]. As a result, the HIV-promoting effects cannot be observed in systems that require high viral inocula, such as most explant systems [35,36]. When low viral doses are used, semen enhances infection of cell types relevant for sexual transmission, including macrophages [27], T cells isolated from cervical and endometrial tissues [10,13], and *ex vivo* infected vaginal explants [37]. This enhancement occurs independently of the HIV-1 strain but still requires the presence of HIV-1 entry receptors CD4 and CXCR4/CCR5 [10,27]. Semen-mediated enhancement of HIV-1 infection is also observed under acidic pH conditions and in the presence of vaginal fluid [27].

That the fibrils are important contributors to the ability of semen to enhance HIV infection is supported by three observations. First, donor-dependent variability in the extent of semen-mediated enhancement of HIV infection correlates with the levels of amyloidogenic SEVI and SEM peptides, as measured by ELISA and quantitative mass spectrometry [13,14,27]. Second, semen lacking these peptides as a consequence of a congenital condition termed ejaculatory duct obstruction cannot enhance HIV infection [13]. Third, extension of the period of semen liquefaction results in progressive loss of the amyloidogenic SEM peptide in a manner that parallels the progressive loss of the infection-enhancing activity of semen [14]. However, because semen retains significant infection-enhancing activity for more than 8 h after emission, it has ample opportunity to enhance viral transmission *in vivo*, which can occur within an hour after exposure in animal models [38]. Furthermore, the detection of amyloidogenic SEM peptides in seminal vesicles [13,39] suggests that amyloid seeds are already present even before ejaculation, consistent with the observation that fresh ejaculates contain amyloid fibrils and have a potent ability to enhance HIV infection [10,19]. Therefore, the "window of opportunity" for semen fibril–mediated enhancement of HIV infection ranges from the time of semen deposition to >8 h later.

5. Mechanism of infection enhancement

Both classes of semen fibrils are derived from peptides with high isoelectric points (Table 1), and only the fibrillar but not the monomeric freshly dissolved peptides enhance viral infection [10,12–14]. Zeta potential measurements confirmed that polymerized PAP and SEM fibrils are all highly cationic at neutral pH (Table 1). These properties enable the fibrils to bind to the negatively charged membranes of both HIV virions and cells, which leads to increased viral attachment and fusion to cellular targets [10,12,13]. Indeed, abrogating the cationic properties of the fibrils through anionic polymers or site-directed mutagenesis largely diminished their ability to enhance infection [12,14,40]. Thus, PAP and SEM derived fibrils enhance viral infection by forming an electrostatic bridge that overcomes the negative repulsions between the viral and cellular membrane, which is normally a rate-limiting step in viral infection [41]. However, this mechanism might be an over-simplification as unpublished data by the Fändrich/Münch group suggest that fibrils with negative net surface

charges may also capture virions and enhance infection, albeit with lower efficiency as the cationic fibrils in semen

One intriguing question is whether amyloid fibrils in semen may also enhance infection of other viruses besides HIV-1. In fact, it was demonstrated that SEVI fibrils increase infection of human immunodeficiency virus type 2 (HIV-2), a mainly sexually transmitted virus prevalent in Western Africa [27]. Similarly, SEVI and SP from humans boosted infection of the closely related simian immunodeficiency virus from macaques (SIVmac) [27,33,42]. Whether macaque SP may also enhance SIVmac infection could not be analysed due to difficulties in collecting this body fluid from monkeys and the rapid coagulation of macaque semen. However, Zhou *et al* demonstrated that synthetic SEVI fibrils derived from the macaque sequence (that differs in one amino acid residue at position 277) enhances infection of HIV-1 and SIV [42]. In addition, SEVI and SEM fibrils as well as SP all enhance infection by herpes simplex virus type 1 and 2 (HSV-1 and -2) and cytomegalovirus (CMV) [43,44]. Addition of the polyanion heparin abolished the HSV/CMV infection-enhancing effects of SEVI, suggesting that the mechanism underlying HSV and CMV infectivity enhancement is similar to that of HIV-1. These findings suggest that seminal amyloids or semen/SP may represent general enhancers of enveloped virus infection.

However, recent findings by Müller *et al* show that SEVI fibrils do not affect the infectivity of Zika and Dengue virus, two members of the Flaviviridae family, which are only sparsely transmitted via sexual contact (Müller, Münch *et al.*, accepted). These findings suggest that seminal amyloids do not interact with the flavivirus particle, which could be explained by the observation that the Zika virion is covered by a dense coat of the viral E protein that renders the viral lipid membrane largely inaccessible to large external factors such as seminal fibrils [45]. In contrast, the membrane of HIV-1 is largely accessible, because only 5-15 viral glycoprotein spikes are embedded [46,47] that may allow efficient interaction of fibrils with HIV-1 particles. Further studies with viruses containing well-defined numbers of viral glycoproteins are needed to clarify whether the accessibility of the viral membrane determines fibril-mediated enhancement of viral infection. In addition, the effect of seminal amyloids on other emerging and re-emerging viruses should be determined as a way to assess the potential of these viruses to be transmitted via sexual intercourse.

The broad and potent ability of seminal amyloid to enhance enveloped virus infection suggests that they might also promote transduction by retro- or lentiviral vectors, which could have practical applications *in vitro* or *in vivo* in the context of gene therapy. Indeed, SEVI fibrils also increased infection by a γ-retrovirus [48] as well as by retro- and lentiviral vectors [49,50]. However, utilization of SEVI as laboratory tool to optimize viral gene transfer is limited because the polypeptide monomers are relatively expensive to produce. These obstacles can be overcome by recently-identified smaller peptides that spontaneously self-assemble into nanofibrils [41,51]. These nanofibrils increase retroviral gene transfer even more efficiently than SEVI, are easy to produce and to handle, and are safe as assessed in an *ex vivo* gene transfer study [51], and have been commercialized as Protransduzin®.

6. Effects on HIV transmission *in vivo*

Experiments in humanized mice and non-human primates (NHPs) have been used to try to dissect the role of both semen and semen amyloids on lentiviral transmission *in vivo*. Humanized mice studies have not found a role for semen [52] or SEVI [53] in affecting HIV transmission rates. However, the difficulty in linking these results to the effects of semen or SEVI on HIV transmission in people is that humanized mouse studies require HIV inocula that are orders of magnitude higher than that naturally present *in vivo*, which becomes problematic because both semen and SEVI enhance HIV infection only under low, physiologically relevant levels of virus [10]. Indeed, in the humanized mouse study characterizing the effects of semen, 5/6 mice were infected in the control group receiving HIV without semen [52]; under these conditions enhancement cannot be readily assessed since the vast majority of mice were already infected in the absence of semen. Similarly, in the study assessing the effects of SEVI, large amounts of HIV were used resulting in high baseline transmission rates of 50% [53]. Although a lower viral dose was used in one experimental group, one could not compare infection rates between mock- and SEVI-treated groups due to the lack of a mock-treated HIV control.

To date, only three studies have examined the effect of SP on lentiviral transmission in NHPs. Two early studies examined the effect of SP on vaginal infection of rhesus macaques by SIV [54,55]. Both reported increased vaginal SIV infection in the presence of SP, but only under conditions of low viral inocula. A more recent rhesus macaque vaginal SIV infection study similarly found that SP may facilitate transmission at low viral doses, and further revealed that animals infected in the presence of SP exhibited 6.9-fold higher peak viral loads [33]. These results are consistent with the notion that SP and SEVI enhance viral infection most potently under conditions of limiting viral inoculum. Unfortunately, in all three of these macaque studies, animals within the experimental group differed strongly in their susceptibility to vaginal SIV infection. For example, in the most recent study the total viral doses required to establish infection in the control group of animals ranged from 5,300 to >128,500 Tissue Culture Infection Doses 50 ($TCID_{50}$) and in the SP group from 100 to >128,500 $TCID_{50}$. As such, the effect of SP on HIV transmission in the NHP system remains unclear. All prior studies have suffered from insufficient statistical power due to a lack of consistency in susceptibility between individual animals within a study group. It should also be noted that SP enhances SIV infection markedly less than HIV-1 infection [33], which blunts the ability of SIV-based studies to assess the infection-promoting effects of SP on HIV *in vivo*. To this end, the effects of SP should be tested using SHIVs which carry HIV-1 T/F envelopes and can be mucosal transmitted to macaques [56]. Such studies should incorporate dose escalation starting with low levels of virus reminiscent of the amount present in the ejaculate of a typical viremic individual. In the absence of such a study, it remains unknown what effect semen is likely to have on HIV transmission in people.

7. Role of semen amyloids in reproduction

As discussed earlier, SP can have protective effects for sperm and can promote reproductive success through inducing responses in the FRT. What role, if any, do semen amyloids play in these processes? That semen amyloids should play a beneficial role in reproductive success

seems likely since the amyloidogenic potential of orthologous peptides is conserved amongst the great apes [14]. Should harbouring these amyloid-forming peptides not confer a species survival advantage, they would have presumably been eliminated over evolutionary time due to their ability to enhance infection by HIV and the other sexually transmitted viruses discussed above. Indeed, a recent study suggests that semen amyloids may play a role in sperm selection and clearance. Single-cell analyses by immunofluorescence and electron microscopy revealed that the membranes of sperm cells interact directly with semen amyloid fibrils [32]. This observation, together with the knowledge that semen elicits a leukocytic response characterized by infiltration of neutrophils and macrophages into the lower FRT [57], led to the hypothesis that entrapment of sperm cells by semen amyloids may promote their engulfment by infiltrating phagocytes. This was shown experimentally by demonstrating the ability of semen amyloids to promote phagocytosis of sperm cells by macrophages [32]. Strikingly, in the presence of amyloids, individual macrophages were seen to engulf up to over a dozen sperm heads (Fig. 3). The rapid removal of sperm cells from the lower FRT may help ensure that no inappropriate immune response is developed against male antigens, and is consistent with the observation that the lower FRT largely returns to its pre-mating state within 24 hours [57]. On the other hand, if semen amyloids promote sperm disposal in the lower FRT, how do spermatozoa make it to the oviduct? Intriguingly, macrophages have the ability to preferentially engulf damaged or dead sperm over live motile ones, and semen amyloids increased the efficiency of this mechanism [32]. Therefore, semen amyloids may promote the efficiency of sperm selection by phagocytes infiltrating the lower FRT, ensuring the rapid removal of damaged sperm and lingering sperm antigens, and allowing for only the most robust spermatozoa (a small fraction of the deposited number [58]) to reach the oviduct isthmus.

8. Role of semen amyloids in innate immunity

Findings by Easterhoff *et al* suggest that semen fibrils may also exert indirect antibacterial activity upon deposition in the FRT [59]. Based on the antimicrobial properties of other amyloidogenic peptides such as amyloid-β (1-42) [60], the antimicrobial activity of SEVI was examined. Neither SEVI fibrils nor the unassembled PAP248-286 peptide displayed any direct antibacterial activity [59]. However, similarly to the interaction with eukaryotic cells, SEVI fibrils bound to and entrapped both Gram-positive and Gram-negative bacteria. Similar to the interactions between SEVI and enveloped viruses, sequestration of bacteria occurred in a charge-dependent fashion. This has been proven by the fact that a non-cationic SEVI variant (Roan et al., 2009) failed to bind bacteria [59]. In turn, SEVI-aggregated bacteria were more efficiently phagocytosed by primary human macrophages, which resulted in an elevated release of bacterially induced pro-inflammatory cytokines. Finally, SEVI fibrils inhibited vaginal colonization with *Neisseria gonorrhoea* in a murine model [59]. Interestingly, it has also been demonstrated that bacterial curli proteins promote conversion of PAP(248-286) into SEVI fibrils [61]. Since curli fibers from bacteria may colocalize with PAP(248-286) at the initial sites of HIV infection, they may induce and accelerate the formation of antibacterial SEVI fibrils trough a cross-seeding mechanism. Together these results demonstrate that SEVI and perhaps other semen fibrils represent a novel class of immune defense molecules with both immunomodulatory and indirect antimicrobial activity.

9. Semen fibrils and microbicide activity against HIV

Anti-HIV microbicides have been sought after as a way to limit worldwide rates of HIV transmission, particularly in resources-limited settings. Microbicides are compounds to be applied inside the vagina or rectum to protect against sexually transmitted viral infections, including HIV. Although many microbicides have demonstrated potent anti-HIV activity in cell culture, they have largely failed to exert protective effects in clinical trials in humans [62]. Zirafi et al showed that in the presence of SEVI amyloid or semen, most candidate microbicides – including neutralizing antibodies and standard antiretroviral drugs – showed greatly reduced antiviral efficacy [31]. This diminished antiviral activity was dependent on the ability of semen fibrils to enhance viral infectivity because semen depleted of amyloid due to prolonged incubation at 37°C [14] or naturally-deficient in amyloid [13] did not impair microbicide efficacy [31]. The molecular basis of how semen and SEVI diminishes the anti-HIV effects of microbicide candidates is not clear. One explanation is that the fibrils bind to HIV virions and efficiently enhance and accelerate their attachment to target cells [10,13,27,40], thereby shortening the time of virion exposure to neutralizing antibodies. In addition, in the presence of fibrils multiple infections of an individual cell may take place, which results in elevated intracellular levels of viral enzymes, requiring increased concentrations of drugs. Regardless of mechanism, the finding that semen impairs the antiviral efficacy of microbicides has important implications. First, before entering clinical trials, promising microbicide candidates should be tested in multiple assays that incorporate the effects of semen [63]. The second implication is that agents that antagonize seminal amyloid may exert dual beneficial effects because they abolish viral infectivity enhancement and restore the antiviral activity of microbicides in the presence of semen.

10. Semen amyloid antagonists

Several strategies are conceivable to antagonize the HIV enhancing activity of semen in order to prevent sexual virus transmission and to increase the antiviral activity of microbicides [10,64–67]. One possibility is to prevent the formation of mature fibrils in semen through blocking the proteolytic generation of PAP and SEM peptides from its precursor proteins. A second approach is to inhibit the assembly of PAP and SEM fragments into the mature infection-enhancing fibrils. However, since mature fibrils are already present in fresh ejaculates, it seems a more effective strategy to remodel or disassemble preformed fibrils in a way that they lose infection-enhancing activity. Finally, shielding or neutralizing the charged surface of the fibrils should disrupt the ability of the fibrils to promote the interactions between viruses and cells, thereby abolishing infection-enhancement. Several agents have been described in the past 10 years that antagonize seminal amyloid through one or more mechanisms (Table 2). These include metal ions, small molecule compounds, peptides, proteins, polymers and nanoparticles, and will be discussed in the following sections.

10.1. Metal ions

10.1.1 Cu^{2+} and Zn^{2+}—Sheftic *et al* found that Cu^{2+} and Zn^{2+} inhibit fibrillization of PAP(248-286) peptide into active SEVI fibrils [68]. Both metals did not affect the morphology

of preformed fibrils. NMR spectroscopy revealed that the metals bind to H3 and 23 in the PAP(248-286) sequence resulting in the formation of oligomeric complexes. Since dissociation constants for Cu^{2+} and Zn^{2+} are comparable to those found in SP, the authors speculated that the metals may modulate the formation of SEVI fibrils under physiological conditions. The effect of both metals on the formation of PAP(85-120) and SEM fibrils have not yet been determined. Moreover, since Zn^{2+} also inhibits seminal proteases that degrade fibrils [14,69], the metal might also have opposite effects by increasing the fibril's half-life in semen.

10.2. Small molecule antagonists

10.2.1. Epigallocatechin-3-gallate—The first published antagonist of SEVI was epigallocatechin-3-gallate (EGCG) [70], the major active constituent of green tea [71]. EGCG not only inhibited the assembly of PAP248-286 into SEVI fibrils, but also remodeled mature fibrils, thereby abrogating their infection-enhancing potential [70]. The mechanism by which EGCG disrupts amyloid formation is mediated by a specific interaction of EGCG with charged lysines in regions K251-R257 and N269-I277 in the PAP(248-286) peptide [72]. EGCG binding was shown to occur in two steps, with the initial formation of a weakly bound complex followed by a pH dependent formation of a tightly bound complex [72]. Castellano *et al* later confirmed that EGCG remodels SEVI and also demonstrated that this activity also occurs with the other seminal fibrils PAP(85-120), SEM1(45-107), and SEM2(49-107) [73]. Interestingly, EGCG also exerted a direct anti-HIV activity suggesting that the combined anti-amyloid and anti-viral properties of EGCG could have utility in preventing HIV transmission [73]. Hartjen et al analysed the activity of EGCG against semen-exposed virus and observed a median inhibition of HIV-1 infection of 70.6% [24]. However, viral inhibition varied substantially and in some cases, no inhibition by EGCG treatment was observed at all, questioning its suitability of EGCG for further clinical development.

10.2.2. Surfen—The aminoquinoline surfen is a small molecule heparan sulfate proteoglycan (HSPG) antagonist that counteracts the interaction between SEVI- and HSPG-expressing target cells, as well as between SEVI and HIV-1 virions [30]. The ability of surfen to directly interact with SEVI was surprising because both are cationic molecules and would be predicted to repel one another. It is possible that hydrophobic ring stacking between the fibrils and the aminoquinoline moieties of surfen mediates this interaction. Of note, not all aminoquinolines inhibit SEVI since chloroquine is inactive [30]. Surfen inhibited both SEVI- and semen-mediated enhancement of HIV infection. Since surfen has been reported to be anti-inflammatory, it may prove useful not only as an inhibitor of semen fibrils but also as a means to limit the inflammatory response that can recruit HIV-susceptible target cells to the genital mucosa. However, manufacturing problems and the low solubility of surfen has hampered clinical development of this compound as a microbicide candidate.

10.2.3. Gallic Acid—LoRicco *et al* used a small molecule screen for antagonists of SEVI and SEM fibrils and identified gallic acid, a polyphenol found in grape seed extracts [74]. This 170 Da compound binds to both SEVI and SEM1 fibrils and renders them less cationic, thereby reducing sequestration of virions. Gallic acid also decreased semen-mediated enhancement of HIV infection but did not decrease the inflammatory response induced by

semen. Together, these observations suggest that gallic acid as a non-polyanionic compound that inhibits semen-mediated enhancement of HIV infection and suggest the potential utility of incorporating gallic acid into a multicomponent microbicide targeting both the virus and host components that promote viral infection.

10.2.4. BTA-EG6—BTA-EG$_6$ is a hexa(ethylene glycol) derivative of benzothiazole aniline that has previously been shown to intercalate into amyloid-β (Aβ) fibrils, which are associated with Alzheimer disease [75]. Olsen and colleagues demonstrated that BTA-EG6 also binds SEVI fibrils and effectively inhibits both SEVI-mediated and semen-mediated enhancement of HIV infection [76]. BTA-EG$_6$ also blocks the interactions of SEVI with HIV-1 virions and target cells but does not cause any inflammation or toxicity to cervical epithelial cells. Interestingly, BTA-EG$_6$ interaction with semen amyloids does not rely on electrostatic properties and is therefore expected to offer a new class of SEVI inhibitors, whose effects are more specifically targeted at the fibrils themselves and are not neutralized by cationic compounds in semen.

10.2.5. ADS-J1—ADS-J1 is an anionic HIV-1 entry inhibitor that exerts its direct antiviral activity by targeting the glycoprotein gp41. Based on its anionic property, Xun et al reasoned that ADS-J1 might also interfere with SEVI function [77]. In fact, ADS-J1 bound to mature SEVI fibrils and antagonized fibril-mediated enhancement of viral infection. Interestingly, in the presence of semen, ADS-J1 exhibited synergistic effects with several antiretroviral drugs against HIV-1 infection. Moreover, ADS-J1 did not facilitate fibril formation in SP, as previously observed for polyanions such as cellulose sulfate [78], that failed in clinical trials to prevent HIV-1 sexual transmission [78]. Thus, ADS-J1 antagonizes semen fibrils and shows synergistic effects against infection by HIV-1 in semen when combined with ARV drugs, and might represent a lead product for the design of combination microbicide candidates.

10.2.6. CLR01—CLR01 is a 'molecular tweezer' that inhibits amyloid fibrillization by engaging specific lysine, arginine, or both residues within a variety of disease-associated amyloidogenic proteins including amyloid-β protein (Aβ) [79–82]. Moreover, CLR01 has been shown to remodel preformed Aβ or α-synuclein fibrils [83]. Lump and colleagues established that CLR01 also selectively interacts with lysine and arginine residues in PAP and SEM derived peptides thereby abrogating fibril formation [29]. Moreover, CLR01 bound mature fibrils, which on a short time frame prevented the interaction with viral particles and on a longer period resulted in fibril disassembly. Strikingly, CLR01 also exhibited a direct antiviral effect by selectively disrupting the membrane of enveloped viruses, and was active in the presence of semen. In contrast to most other antagonists of semen amyloids, CLR01 has already been safely employed in multiple cell and animal models, including systemic applications [83–86]. Conceivably, the application of CLR01 on mucosal surfaces should be even safer relative to systemic administration. The combined antiviral and anti-amyloid activities (Fig. 4) and the encouraging safety data make CLR01 a promising broad-spectrum, topical microbicide against HIV-1 and other sexually transmitted viruses.

10.2.6. Brazilin—Brazilin is a natural compound isolated from the plant *Caesalpinia sappan.* It is a potent inhibitor of amyloid β fibrillogenesis and shows a stronger inhibitory

effect at lower concentrations than EGCG [87]. Li et al found that brazilin inhibited PAP248–286 aggregation mainly through hydrophobic interactions [88]. However, whether this compound is also active against other types of semen amyloids, has activity in semen, and exhibits direct anti-HIV activity remains unknown.

10.3. Peptide-based antagonists

10.3.1. D3—D3 is a D-enantiomeric cationic peptide (RPRTRLHTHRNR) that converts Aβ oligomers and fibrils into non-amyloidogenic, non-fibrillar and non-toxic aggregates, and reduces the cognitive deficits of the central nervous system in transgenic AD model mice [89]. Widera and colleagues found that D3 reduced the HIV enhancing activity of SEVI fibrils [90]. Interestingly they also found that Aβ(1-42) fibrils promote HIV-1 infection (confirming previous data by [91]) and that D3 counteracts this effect. Whether D3 peptide also antagonizes other semen fibrils and whether the peptide is active in the presence of semen needs to be demonstrated. However, since amyloids could play an important role in the progression of AIDS dementia complex, the treatment of HIV-1 infected individuals with D3 may reduce the vulnerability of the central nervous system of HIV patients for HIV associated neurological disorders.

10.3.2. E$_4$(ChaKChaE)$_2$—Easterhoff and coworkers identified amphipathic cationic peptides that efficiently self-assembled into fibril-like structures that were able to enhance HIV-1 infection even more efficiently than SEVI [92]. Using a similar approach, self-assembling anionic peptides were generated and tested for their potential to interfere with SEVI-mediated infectivity enhancement. One self-assembling peptide with the sequence E$_4$(ChaKChaE)$_2$ completely abrogated infection enhancement, most likely by shielding the positive charges of the semen fibrils. However, before these anionic peptide supramolecular assemblies can be further developed as a microbicide, their activity against other types of semen amyloids needs to be evaluated.

10.3.3. WW61 and others—Sievers *et al* applied computer-aided, structure-based design to develop a highly specific peptide inhibitor of PAP(248-286) fibrilization [22]. As template for inhibitor design they used the crystal structure of the hexapeptide GGVLVN (corresponding to residues 260-265 of PAP) that has been predicted to form a steric zipper structure. The peptide Trp-His-Lys-chAla-Trp-hydroxyTic (WW61) blocked PAP(248-286) fibril formation through specifically interacting with the GGVLVN motif at the ends of growing fibrils. The same tools should also allow designing peptide inhibitors of PAP(85-120) and SEM fibrils. However, since mature fibrils are already present in fresh ejaculates, inhibitors of fibril assembly are predicted to not antagonize semen-mediated infection enhancement.

10.4. Proteins

10.4.1. Hsp104—Hsp104 is a broad-spectrum, amyloid-remodeling disaggregase from yeast [93]. Castellano et al repurposed Hsp104 into a three-prong nanomachine that antagonizes seminal amyloids by three different mechanisms [94]. First, Hsp104 and an enhanced engineered variant were shown to remodel preformed SEVI and PAP85-120 fibrils into non-amyloid forms. Second, a catalytically inactive Hsp104 scaffold was shown to

cluster SEVI, PAP85-120, and SEM1(45–107) fibrils into larger assemblies. Third, Hsp104 was modified in way to allow it to interact with the chambered protease ClpP, which enables coupled remodeling and degradation of SEVI and PAP85-120 fibrils. Each of the three strategies diminished the ability of seminal amyloid to promote HIV infection [94], and could have therapeutic utility. However, before entering an advanced preclinical development phase, it needs to be demonstrated these protein-based nanomachines remain active in the presence of semen.

10.5. Polymeric structures

10.5.1. Polyanions—Polyanionic compounds such as heparin or dextran sulfate have been investigated as prophylactic microbicides against HIV for a long time (see 9). They are thought to interfere with HIV infection by binding a basic region of gp120 and inhibiting the attachment of virions to either CD4 or proteoglycans at the surface of target cells [95,96]. Roan and others found that these polymers also directly interact with SEVI and SEM fibrils [12,13,40] and thereby abrogate the ability of these fibrils to increase infection. Unfortunately, anionic polymers such as Naphthalene sulfonate (PRO2000) and the sulphated polysaccharide carrageenan (Carraguard/515) have already failed in clinical trials [97,98]. The failure of these polymers likely stemmed from their induction of an inflammatory environment in the female genital tract, a condition that favours HIV infection. Another explanation is that polyanionic candidate microbicides may accelerate the formation of SEVI fibrils in semen, which may also lead to enhanced HIV-1 infectivity [78]. In any case, due to the unfavourable outcome of polyanion-based microbicide clinical trials, polyanions are likely have limited utility as microbicide against viral infections.

10.5.2. BTA oligomers—As discussed (10.2.4), BTA-E_6 targets SEVI to form a protein resistive coating, thereby preventing interaction with virions and reducing SEVI- and semen-mediated enhancement of HIV infection [76]. Capule *et al* evaluated oligovalent derivatives of BTA for their capability to bind cooperatively to SEVI fibrils and to neutralize its effects on HIV infection [69]. Oligomers of BTA exhibited increased binding to SEVI and an maximum 65-fold improved capability to reduce SEVI-mediated infection enhancement, as compared to the previously reported monomeric BTA-E_6 derivative [76]. However, difficulties in synthesis and isolation of oligomeric amyloid binding materials pose a synthetic challenge for large-scale production [67].

10.5.3. Polymeric BTA-containing nanoparticles—Amyloid-binding polymeric nanoparticles may provide a more practical approach for large-scale production of microbicides that antagonize fibril-mediated HIV infection. Sheik and colleagues synthesized BTA-containing polyacrylate-based polymers and polymeric nanoparticles of comparable size to HIV particles [67]. These polymeric materials reduced SEVI-mediated enhancement of HIV infection with IC_{50} values in the nM range, suggesting that besides charge neutralization also steric interactions may play an important role in counteracting semen fibrils. BTA-carrying polymers were easily prepared through free-radical polymerization and may also be used as vehicles for controlled delivery of antiviral drugs [67].

10.5.4. Hydrophobic Nanoparticles—[99]. A nanoparticle formulation of this hydrophobic polymer reduced SEVI-mediated enhancement of HIV infection in cell culture experiments. These nanoparticles lack specific amyloid-targeting groups and are thought to counteract SEVI through hydrophobic interactions that alter the secondary structure of the fibrils [99]. However, the concentrations required to effectively block fibril-mediated infection enhancement exceed 200 μg/mL, which is too high for microbicide application [99]. Nevertheless, this novel approach to alter the function of amyloids through modulation of its secondary structure could be applied in the design of alternative strategies to combat other amyloid-associated diseases. For a more detailed discussion on the BTA-EG6 monomers and oligomers, BTA-containing polymers and nanoparticles we refer to an excellent review by Sheik, Dewhurst and Yang [67].

10.6. Preclinical and clinical development of semen amyloid antagonists

Although several agents have been shown to counteract the viral-enhancing activity of seminal amyloids in cell culture, none of these have advanced to preclinical development as HIV preventative microbicides. The main reason for why these encouraging *in vitro* findings have not been translated into new prevention strategies is the lack of an appropriate animal model that allows the field to readily assess the HIV-enhancing effects of semen or seminal amyloid on viral transmission (for details see 6). Perhaps even more important is the consideration that most if not all agents that counteract fibril-mediated infection enhancement will likely also interfere with the natural functions of semen amyloids (for details see 7 and 8). Agents that suppress fibril formation, disassemble or remodel fibrils, or form coatings to prevent the interaction with virions and cells, will most likely also abrogate the interaction of fibrils with defective sperm cells [32] or bacterial pathogens [59], and hence disturb sperm clearance and antibacterial immune responses in the FRT. Thus, counteracting semen fibrils to block viral transmission may come at the cost of affecting fertility or general reproductive health.

11. Open questions

This review summarized the current knowledge on seminal amyloids gained in the past decade. Still several questions regarding the structures and functions of these intriguing components in semen remain unanswered. For example, what is the molecular structure of PAP and SEM fibrils, are there additional types of semen fibrils, and what are the concentrations and relative compositions of amyloids in semen? Where and when are PAP and SEM peptides generated and where and with what kinetics do fibrils form? How is fibrillation regulated? What is the exact mechanism underlying the interaction of fibrils with virions and cells, and what is the fate of the fibrils after delivery of the viral cargo to the cell surface? Do the fibrils have signalling effects in the female reproductive tract? Is aberrant fibril formation or degradation associated with infertility or increased susceptibility to sexually transmitted diseases? Are there other amyloids naturally present in the human body that enhance virus infection and may contribute to viral pathogenesis? Clearly, much about the role of human amyloids in health and disease remains to be worked out.

12. Concluding remarks

Semen fibrils are fascinating functional amyloids with unprecedented roles in sperm selection and removal as well as innate immunity. They are present in ejaculates of all men and may be exploited by HIV for effective viral transmission. Since their identification ten years ago, we have learned a lot the structural, functional, and pathogenic effects of these intriguing structures. We suspect the next ten years will yield an additional wealth of information about the role of semen amyloids in reproductive health and viral infections, and whether they can serve as biomarkers for human diseases.

Acknowledgments

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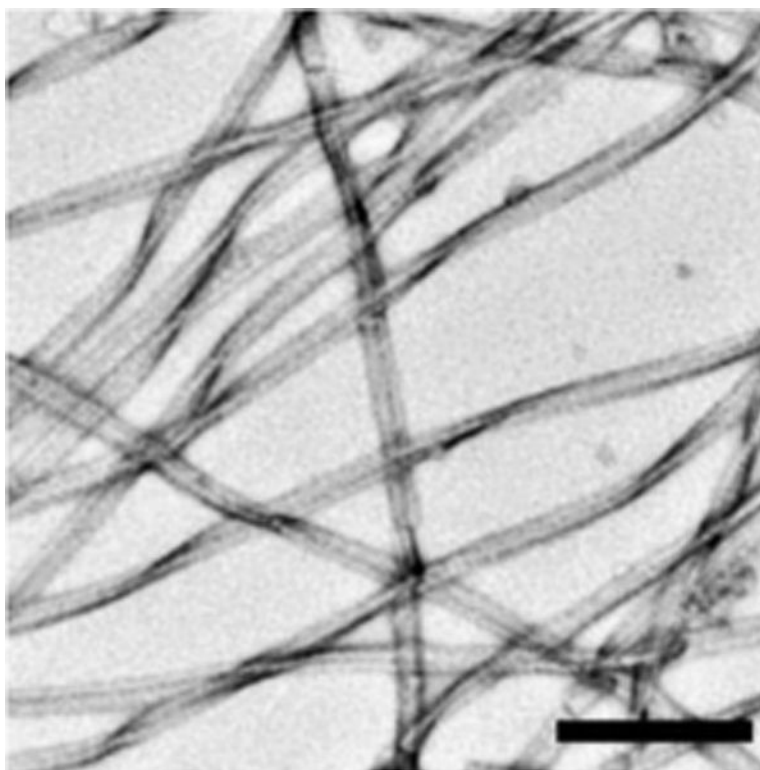
Fig. 1. TEM image of SEVI fibrils
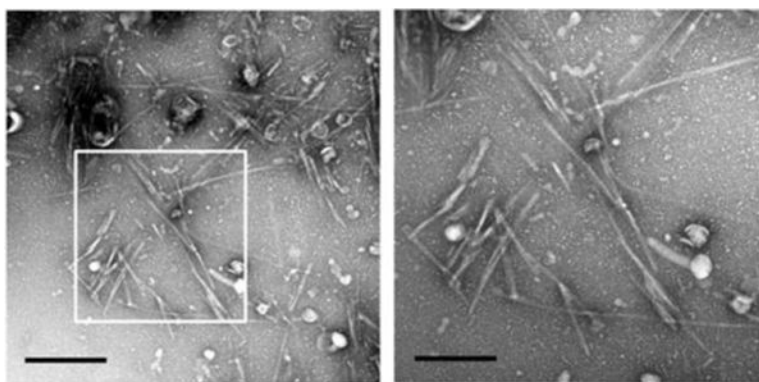The scale bar represents 100nm.

Fig. 2. TEM of a semen sample

Semen from healthy donor contains amyloid fibrils. Scale bar in left panel represents 500 nm, in right panel 200 nm. Reprinted with permission from Springer Nature: Nature communications "Direct visualization of HIV-enhancing endogenous amyloid fibrils in human semen." Usmani, Shariq M., et al. 19, Copyright (2014)..
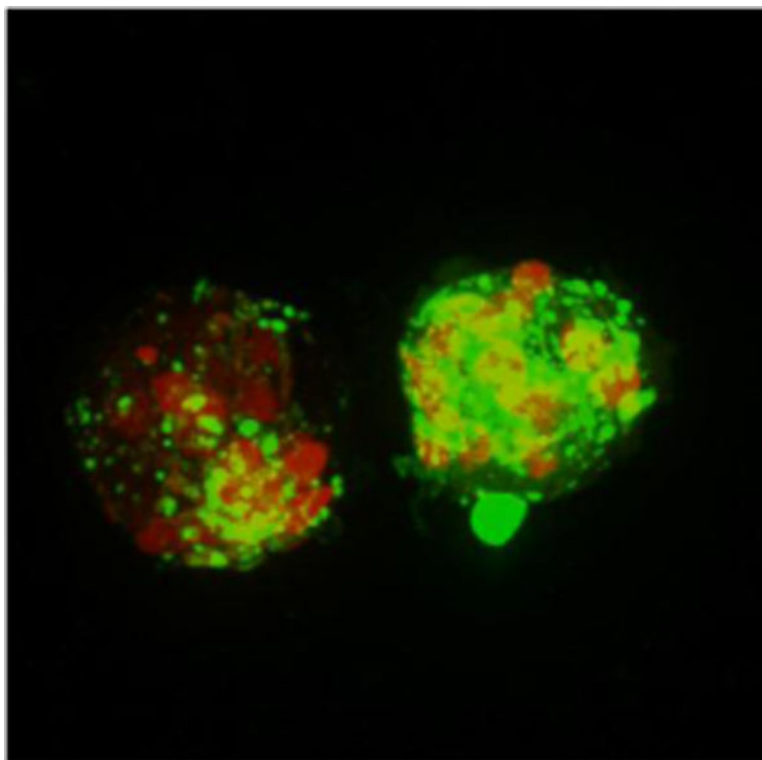
Fig. 3. Image showing two macrophages that in the presence of semen fibrils have phagocytosed over a dozen spermatozoa

Monocyte-derived macrophages were labeled with the membrane dye Vybrant® DiO (green) while human spermatozoa were labeled with eFluor 670 (red). Image was generated with a Nikon Eclipse Ti-E inverted microscope. Image generated by N. Kohgadai and M. Montano.
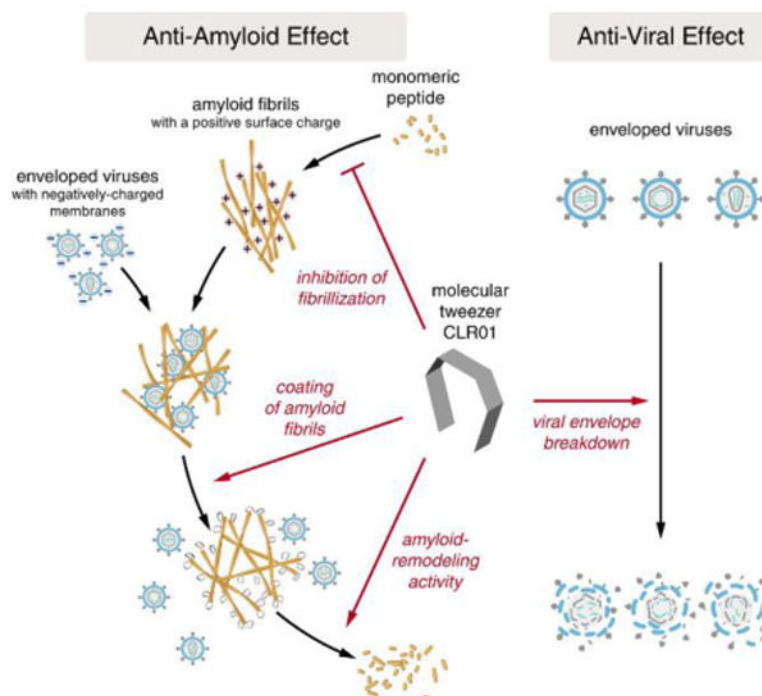
Fig. 4. The molecular tweezer CLR01 acts as a dual-function inhibitor of viral infection featuring both anti-amyloid and antiviral activity
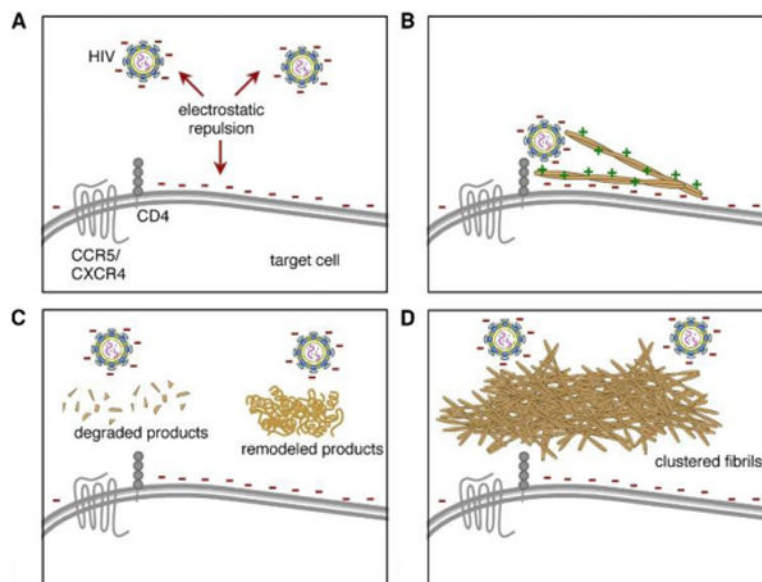Adapted with permissions from [29].

Fig. 5. Hsp104-based treatments that remodel, degrade, or cluster seminal amyloid reduce their ability to stimulate HIV Infection

(A) Electrostatic charge repulsion between the negatively charged surfaces of the viral and target cell membranes. (B) Cationic seminal amyloid fibrils act as "electrostatic bridge" and increase viral attachment and infection. (C). Hsp104 remodels and degrades semen fibrils thereby by antagonizing infection enhancement. (D) Hsp104 promotes the assembly of seminal amyloid into higher-order conglomerates with greatly reduced HIV promoting activity. Reprinted from "Repurposing Hsp104 to antagonize seminal amyloid and counter HIV infection." 22.8, Castellano, Laura M., et al., 1074-1086 94, Copyright (2015), with permission from Elsevier.

Table 1

Overview of amyloidogenic peptides from semen.

Peptide	Precursor	Sequence	#	pI	Charge
PAP248–286	PAP	GIHK K E KS R LQGGVLVN EILN H M KRATQIPSY KKLIMY	39	10.21	6.2
PAP85–120	PAP	I RKRY RKFLN ESY KH EQVYI R ST DV D RTLMSAMTNL	36	9.99	4.1
SEM1(86–107)	SEM1	DLNAL HKTT KSQ RH LGGSQQLL	22	9.99	2.2
SEM1(68–107)	SEM1	TY H V DAN D H DQS RKSQQY DLNAL HKTT KSQ RHLGGSQQLL	40	8.16	1.4
SEM1(45–107)	SEM1	GQ HYSGQ KG KQQT ES KGSFSIQYTY HV DAN D H DQS RKSQQY DLNAL HKTT KSQ RHLGGSQQLL	63	9.4	3.5
SEM1(49–107)	SEM1	SGQ KG KQQT ES KGSFSIQYTY HV DAN D H DQS RKSQQY DLNAL HKTT KSQ RHLGGSQQLL	59	9.46	3.4
SEM2(68–107)	SEM2	TY HV DIN D H DWT RKSQQY DLNAL HKATKS KQ HLGGSQQLL	40	8.12	1.4
SEM2(45–107)	SEM2	GQ HYFGQ K DQQ HT KS KGSFSIQ HTY HV DIN D H DWT RKSQQY DLNAL HKAT KS KQ HLGGSQQLL	63	9.4	3.7
SEM2(49–107)	SEM2	FGQ K DQQ HT KS KGSFSIQ HTY HV DIN D DWT RKSQQY DLNAL HKAT KS KQ HLGGSQQLL	59	9.46	3.6

#: number of amino acids; pI: isoelectric point; positively charged amino acids marked in red; negatively charged residues in blue.

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Table 2

Semen amyloid antagonists

Name	Structure/Class	Mode of action	Amyloid	SP	Refs
Cu^{2+}	Anorganic ion	Assembly blocker	SEVI	n.d	68
Zn^{2+}	Anorganic ion	Assembly blocker	SEVI, SEM1 and SEM1(1-159)	n.d	68,100
Gallic Acid	Small molecule	Neutralization/Coating	SEVI, SEM1(86–107)	yes	74
Brazilin	Small molecule	Assembly blocker	SEVI	n.d	88
Surfen	Small molecule	Neutralization/Coating	SEVI	yes	30
EGCG	Small molecule	Assembly blocker, Remodeling, Direct antiviral activity	SEVI, PAP85-120, SEM1(45-107), and SEM2(49-107)	yes	24,70,72,73
$BTA-EG_6$	Small molecule	Neutralization/Coating	SEVI	yes	76,101
CLR01	Small molecule	Assembly blocker, Remodeling, Neutralization/Coating Direct antiviral activity	SEVI, PAP85-120; SEM1(45-107	yes	29
ADS-J1	Small molecule	Assembly blocker, Neutralization/Coating Direct antiviral activity	SEVI	Yes	77
WW61	Peptide	Assembly blocker	SEVI	n.d	22
D3	Peptide	Remodeling	SEVI	n.d	90
$E_4(ChaKChaE)_2$	Peptide	Neutralization/Coating	SEVI	n.d	92
BTA oligomers	Polymer	Neutralization/Coating	SEVI	n.d	69
Hsp104	Protein	Remodeling	SEVI, PAP85-120, SEM1(45-107)	n.d	94
Polyanions (Heparin, Dextran sulfate)	Polymer	Neutralization/Coating, Direct antiviral activity	SEVI	yes	4012
Polymeric nanoparticles	Nanoparticle	Neutralization/Coating	SEVI	n.d	67
Hydrophobic nanoparticles	Nanoparticle	Remodeling	SEVI	n.d	99

n.d., not determined