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Review Article

TARGETING THE SEMEN DERIVED AMYLOIDS TO CONTROL HIV TRANSMISSION: PERSPECTIVES AND CHALLENGES

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Abstract: Since the discovery of Acquired Immuno Deficiency Syndrome (AIDS) in 1981 in United States, there have been tremendous efforts to reduce the rate of HIV transmission. Although, the epidemic is stabilized in most of the affected regions, its occurrence is reasonably evident in Eastern Europe and Central Asia due to high rate of new HIV infections. It is surprising to know that despite the high rate of infection, the virus is a weak pathogen. This paradox has been answered by a recent discovery stating that human semen contains a proteinaceous factor derived from prostatic acid phosphatase (PAP), which is commonly known as $PAP_{248-286}$ peptide, plays an important role in enhancing the HIV infectivity. It forms well-defined amyloid structure, frequently referred as **S**emen-derived **E**nhancer of **V**iral **I**nfection (SEVI) and enhances HIV infection up to 1,00,000 fold. Serendipitous discovery of this semen derived amyloid has provided an opportunity to design an alternative approach to dismantle the mechanism of HIV infection. It is a need of the hour to search and design novel molecules and compounds that can help in destabilizing SEVI under natural conditions. In this direction, a number of molecules have been identified that have shown promising results under laboratory conditions. However, there are several critical issues that remain untouched and their addressal is highly recommended in order to develop an effective regime to control the HIV transmission via sexual route. This review is an effort to consolidate major challenges in developing a therapeutic strategy against semen derived amyloids to combat HIV transmission.

Key words: Amyloid; SEVI; HIV transmission; $PAP_{248-286}$.

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Introduction

HIV infection is a major health concern all across the world, particularly in the developing countries. According to the UNAIDS fact sheet 2015, the number of people living with HIV infection worldwide reached up to 36.9 million till the end of 2014 and the number is unceasingly growing. Particularly, in India 2.17 million individuals are living with HIV infection and around 86 thousand new HIV infections have been reported in 2015 (India HIV estimation, 2015). The constant rise in the population with HIV infection reflects the high rate of HIV transmission. The severity of the situation can easily be understood by the fact that 1.2 million people globally and around 68 thousand people in India died of AIDS related illness in 2015 (UNAIDS fact sheet, 2015; India HIV estimation, 2015). In spite of the high infection rate of HIV, the virus itself is surprisingly a weak pathogen. Under *in vitro* conditions, only one in every 1,000 to 100,000 viral particles is infectious (Dimitrov *et al.*, 1993; Rusert *et al.*, 2004).

HIV is a lentivirus containing two single stranded positive sense RNA. After entry into the target cells (CD4 + cells), the viral genome is reverse transcribed into double stranded DNA with the help of viral-coded reverse transcriptase and then gets integrated with the host genome

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(Fauci, 2008). It is one of the most polymorphic viruses known so far. The polymorphism is mainly attributed by the error-prone reverse transcription and complexity of cDNA formation (Smith and Daniel, 2006). These viral strains are broadly classified as M-tropic, T-tropic and dual-tropic depending upon their cellular tropism (Feng *et al.,* 1996). The tropism of virus depends upon the type of co-receptor used for its entry into the host cell. The chemokine receptors; chemokine [C-C] motif receptor-5 (CCR5) and CXC chemokin receptor-4 (CXCR4) works as co-receptors for HIV. M-tropic strains primarily infect monocytes using cell surface receptor CCR5 and do not infect T-lymphocytes. The T-tropic strains infect T-cells using CXCR4 (X4). The dual tropic strains are capable of using both the chemokine receptors CCR5 and CXCR4 as co-receptors and can therefore infect both T-Cells and Monocytes (Deeks *et al.*, 2013; Mild *et al.,* 2010). Generally, virus use CCR5 for entry at the early stage of infection. However, at the later stage, CXCR4 is used for the purpose (Bleul *et al.,* 1997). Usually, the HIV infection begins with non-symptomatic illness that may expand up to three months from the time of entry of the virus. During this period, patient may appear to be healthy, but the virus keeps on enhancing its titre in the lymph nodes and blood stream. As a result, the number of CD4+ cells gets significantly decreased and patient starts showing symptomatic illness and susceptibility towards other opportunistic infections; most commonly; infection by *Mycobacterium tuberculosis, Mycobacterium avium, Pneumocystis carnii*, taxoplasmosis and candiasis (El-Atrouni *et al.,* 2005).

Identification of semen derived amyloids

Sexual intercourse is the most common way through which the virus interacts with the target cells and gets entry into the human body. During this process, human semen serves as a medium to facilitate the transportation of the virus from infected male to male (in homosexuals) and male to female (in heterosexuals) (Brennan *et al.,* 2013; Carré *et al.,* 1994; Greene *et al.,* 2013). Different studies of proteomics analysis of human seminal fluid have been carried out to identify different seminal plasma proteins including hormones, growth factors and various bioactive peptides (Pilch and Mann, 2006; Starita-Geribaldi *et al.,* 2001). In a more recent proteomic study, it was identified that the human seminal plasma contains more than 2000 proteins (Batruch *et al.,* 2011). Initially, it was hypothesized that the factor responsible for enhancing HIV infectivity might be present in the human semen that could assist the virus in infection and transmission from one host to another. On the other hand, human semen constitutes a reservoir of components that are necessary for safe journey of the spermatozoa to the egg. Initially, it was hypothesized that these components must have some effect on the HIV transmission and infectivity.

The weak pathogenicity (Dimitrov *et al.,* 1993; Rusert *et al.*, 2004) vs. high rate of new infections of HIV remained a paradox till 2007 when Münch *et al.* discovered a proteinaceous factor in human semen that boosts the HIV infectivity up to 10^5-fold in cell culture when viral inocula are limiting. From a complex proteomic library of human semen, they identified a fraction that consistently enhanced HIV infection. Sequence and mass spectroscopic measurements revealed that the peptide fragments are derived from prostatic acid phosphatase (PAP), which is produced and secreted by prostate gland. The major peptides were derived from the same region of PAP but differed in length from 34 to 40 residues. The predominant form of peptide corresponds to the sequence of amino acids from G248 to Y286 (G I H K Q K E K S R L Q G G V L V N E I LNHMKRATQIPSYKKLIMY) of PAP. The synthetic analogue of this peptide is shown to form well-defined amyloid structure that exhibit HIV infection enhancement activity. They named the aggregated $PAP_{248-286}$ as Semen-derived Enhancer of Viral Infection (SEVI). The involvement of amyloid structure in enhancing the HIV infectivity came into light by an observation that after incubation/agitation and centrifugation of the peptide solution, the HIV enhancing potential lies in the pellet fraction and not in the supernatant. The systematic biochemical analysis of the pellet led to the identification that this peptide forms well-defined amyloid structure (Münch *et al.,* 2007). which are usually present in the patients with neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and others (Selkoe, 1993).

Along with SEVI, some other semen derived amyloids were identified. It was found that coagulum component semenogelin protein gets cleaved by PSA proteases (in the process of liquefaction). These cleaved peptide fragments aggregate together and form SEM amyloids that also can enhance the HIV infectivity like SEVI amyloid (Roan *et al.,* 2011). Another study identified that the N-terminal sequence of PAP (PAP_{85-120}) also forms fibrils similar to SEVI (Arnold *et al.,* 2012).

Structure of SEVI

$PAP_{248-286}$ is a 39 amino acid residues peptide fragment, abundantly present in human semen. It has a theoretical isoelectric point (*pI*) of 10.21 and consists of 8 positively charged amino acids (2 Arginine and 6 Lysine), constituting more than 20% of the total amino acid composition, and 2 negatively charged (glutamic acid) residues (Easterhoff *et al.,* 2011; Rönnberg *et al.,* 1981). $PAP_{248-286}$ peptide has high aggregation propensity in aqueous buffer. After incubation and agitation, the solution produces amyloid characteristics. For example, the peptide forms fibrillar structure which can be seen under electron microscope, exhibits Thioflavin T (ThT) binding and green birefringence upon staining with Congo red (CR). The fibrillar structure also produced specific X-ray diffraction reflections at 4.7 and 10.6 Å (Münch *et al.,* 2007). Although, these data indicated the presence of high level of β-sheet structures, which are commonly observed in many amyloid structures. The aggregated $PAP_{248-286}$ are significantly different from many other amyloidogenic peptides (Nanga *et al.,* 2009). The monomeric $PAP_{248-286}$ is predominantly unstructured in solution. The N-terminus of $PAP_{248-286}$ is rich in positively charged residues having high affinity for cell surface. $PAP_{248-286}$ peptide preferentially binds to the surface of the micelle in contrast to the membrane disrupting amyloidogenic peptides. The structure of $PAP_{248-286}$ is mostly disordered when bound to the surface of the micelle as opposed to the α helical structures typically found in the case of many amyloidogenic peptides. It was observed that two regions of helical structure may serve as nucleation sites thus may be essential for amyloid formation. The first region extends from V262 to H270 having a regular R-helix and the second region (A274-Q276 and Y280-I284) having two transient R-helices, disconnected by a distortion at residues I277-S279 (Nanga *et al.,* 2009). It was also found that these results were in comparison with the prediction results. The prediction algorithm 'AGGRESCAN' identified the sequences from G260 to M271 and Y280 to Y286 as 'hot spot areas' having higher tendency to form amyloid aggregates. Both the hot spot areas lie in those two important helical regions that were identified by NMR studies (Nanga *et al.,* 2009).

Mechanism of SEVI mediated enhancement of HIV infection

It is usually believed that the cationic property of SEVI underlies its ability to enhance the viral infection ability (Roan *et al.,* 2009; Tan *et al.,* 2013). A mutant form of SEVI in which the positive charge is replaced with alanine retains its potential to form amyloid fibrils but is defective in binding with virus and enhancing the infection.

Furthermore, it was also shown that addition of polyanionic component could completely abolish the binding of SEVI with virion and its attachment to the target cells (Tan *et al.,* 2013). As mentioned earlier, SEVI precursor has net 6 positive charges that facilitate the viral attachment and fusion with the target cells. These findings suggest that SEVI serves as a polycationic bridge that neutralizes the negative charges on the viral surface as well as on the target cells to facilitate the easy attachment.

Strategies to inhibit semen derived amyloids

Several inhibitors have been either chemically synthesized or screened from natural resources which either partially or fully block the amyloid formation. It was shown that epigallocatechin-3-gallate (EGCG), a major catechin present in green tea has been attributed to exert antitumorogenic, antioxidative, antibacterial and antiviral effects that can block the formation of many amyloids (Babu *et al.,* 2013; Gharib *et al.,* 2013; Kim *et al.,* 2009; Kim *et al.,* 2013; Mitrica *et al.,* 2012; Singh and Katiyar, 2013). Recently, Hauber *et al,* (2009) demonstrated that EGCG can inhibit the SEVI amyloid formation as well as can degrade

preformed SEVI amyloid fibrils in a dose-dependent manner and subsequently abrogates its HIV infection enhancing properties (Hauber *et al.,* 2009). EGCG poses its effect by specifically interacting to the side chains (K_{251}-R_{257}, N_{269}-I_{277}) of monomeric $PAP_{248-286}$ (Popovych et al., 2012). More recently, it is identified that EGCG is the first small molecule that can remodel all four classes of semen derived amyloids including $PAP_{248-286}$, PAP_{85-120}, SEM 1_{45-107} and SEM 2_{49-107} (Castellano *et al.,* 2015b).

Another molecule, bis-2-methyl-4-amino-quinolyl-6-carbamide commonly called as surfen which is an antagonist of cell-surface heparan sulphate proteoglycans. This molecule interferes with the binding of SEVI with both HIV type 1 virion and to the target cells (Roan et al., 2010). In a different approach, polyanionic candidates have been used for SEVI inhibition. It is identified that an anionic HIV-1 entry inhibitor ADS-J1 can inhibit the formation of SEVI amyloid fibrils and can also block SEVI-mediated HIV infection enhancement (Xun *et al.,* 2015).

Most recently, Sheik *et al.,* (2015) developed a BTA consisting amyloid binding polymer that works on the principle of steric hindrance. This polymer has reasonably good affinity for SEVI fibrils and effectively inhibits SEVI-mediated HIV infectivity (Sheik *et al.,* 2015). Another finding in the field of inhibition is the development of a molecular tweezer CLR01 that inhibits the formation of SEVI amyloid and also remodels preformed fibrils. The tweezer specifically binds and targets Lys and Arg residues in the peptide (Lump *et al.,* 2015). Currently, a new approach is identified to inhibit the SEVI-mediated HIV infectivity enhancement. In the same direction, Castellano *et al.* used Hsp104 to remodel seminal amyloids into non-amyloid forms. It also generates a scaffold and thus gathers the seminal amyloids into larger assemblies that do not have the capability to enhance HIV infectivity (Castellano *et al.,* 2015a). Some other molecules that have been shown to have anti-SEVI activity under laboratory conditions are summarized in table 1.

It is assumed that the above inhibitory molecules in combination with HIV virucidal agents may represent an attractive microbicidal solution.

Major challenges in targeting Semen derived amyloids

Discovery of SEVI in human semen is undoubtedly a landmark discovery in the course of prevention of HIV infection. *In vitro* studies confirmed the role of semen derived amyloids in enhancing HIV infectivity. Therefore, targeting these amyloids may possibly lead to development of an attractive strategy to combat HIV transmission via sexual route. However, the situation is not so straight forward as it appears that there are certain key issues that need to be addressed while concentrating on anti-SEVI approach. Today, most of the studies available on anti-SEVI are predominantly based on the formation of SEVI fibrils derived from synthetic $PAP_{248-286}$ peptide under *in vitro* conditions using significantly higher peptide concentration (1-10

Table 1
The molecules with anti-SEVI activity

Name of inhibitors	*Mechanism*	*References*
1. BTA-EG6	It intercalates into the amyloid fibril structure and block the interaction of SEVI with HIV-1 virus and that with the target cells	(Olsen *et al.,* 2010)
2. Surfen	It antagonizes the ionic interaction of SEVI with HSPG (heparan sulphate proteoglycan) expressing target cells and HIV-1 virus	(Roan *et al.,* 2010)
3. WW1 (Trp-His-Lys-chAla-Trp-hydroxyTic)	It is a hexa-peptide and inhibits the amyloid formation by capping the growing ends of the fibrils	(Sievers *et al.,* 2011)
4. EGCG	It disassembles preformed SEVI fibrils and completely abrogates the formation of new fibrils in a dose dependent manner.	(Hauber *et al.,* 2009)

mg/ mL). Such conditions may not be apparent in human semen. On the other hand, the existence of well-defined amyloid structures in every human semen sample is doubtful. However, in a recent study, direct imaging of amyloid fibrils in human ejaculates has been carried out showing the presence of semen derived amyloids in semen samples (Usmani *et al.,* 2014). It is obvious that SEVI fibrils that are formed under *in vitro* conditions may vary significantly in their physicochemical and functional properties compared to the ones which are likely to be present in human semen. The seminal fluid (semen without spermatozoa) is a protein-rich fluid containing various enzymes (such as, proteases, esterases and phosphatases), cytokines, immunoglobulins, prostaglandins, polyamines and Zinc ions (Alghamdi and Foster, 2005; Rodríguez Martínez *et al.,* 2011; Rolland *et al.,* 2013) that are likely to alter the overall dynamics of amyloid formation. Such biomolecules provide unique viscosity to human semen and confer macromolecular crowding effect. It is possible that $PAP_{248-286}$ and other related peptides may acquire different aggregation pathways and aggregated species may assume variety of morphologies that lead to the formation of various conformational strains. In one of our study, we have found that in the presence of macromolecular crowding agents, the morphology of SEVI amyloid fibrils gets significantly altered resulting in the formation of shorter amyloid fibrils (Gaharwar *et al.,* 2015). It is hypothesized that these shorter fibrils or intermediates states may be more efficient in binding with HIV virion and thus enhancing the HIV infectivity (Figure 2). While the compact mesh like structure of mature fibrils around host cell may decrease the flexibility of virion to bind with the cell, it is also possible that the nature and composition of human semen vary significantly from one individual to another depending upon the health conditions, fitness and nutrition. It was also evident that out of 47 semen samples examined, EGCG exerted its anti-SEVI effect against only 41 samples (Hauber *et al.,* 2009). This observation confirms the existence of heterogeneity in structure and function of amyloids derived from different human semen samples. Under high macromolecular conditions, it is also possible that the amyloids may exist as conformations that resemble the intermediates states, such as oligomers and protofibrils. Therefore, the phenomenon of polymorphism in semen derived amyloids should be given due consideration to identify biologically active conformers. Hence, in order to develop a successful strategy to combat HIV transmission the above issues must be considered and addressed judiciously.

Missing biological context of semen derived amyloids in HIV transmission

Another important aspect of semen derived amyloids is the consideration of biological context of HIV transmission, which might play significant role in altering the functions of semen derived amyloids. For example, the occurrence of physical trauma after consensual unprotected intercourse results in the release of neutrophils into female reproductive tract. Immune response is also triggered by the deposition of the seminal plasma in female reproductive tract (Coombs *et al.,* 2003). After ejaculation, the pH of semen gets decreased because of the acidic environment of female genital tract; the pH of female reproductive tract is maintained by commensal *Lactobacilli* that range from pH 2.0-4.0. Hence, there is a possibility that after encountering such a high pH transition, there might be some alteration in the amyloid structure and function. Such a modification might affect the susceptibility of epithelial lining that may help in establishing infection in susceptible target cells (Cohen *et al.,* 2011; Haase, 2010). The role of spermatozoa on amyloids and HIV transmission should also be investigated in order to obtain the appropriate biological context and its importance in determining the transmissibility of HIV virus.

The identification of the missing link between *in vitro* and *in vivo* SEVI amyloid is essential to understand the mechanism associated with the SEVI-mediated HIV infectivity enhancement. Various studies have provided some interesting facts that empower the construction of the biological perspective of SEVI amyloid. It is identified in a study that addition of seminal plasma reduces the effect of HIV infectivity enhancement by SEVI fibrils (Martellini *et al.,*

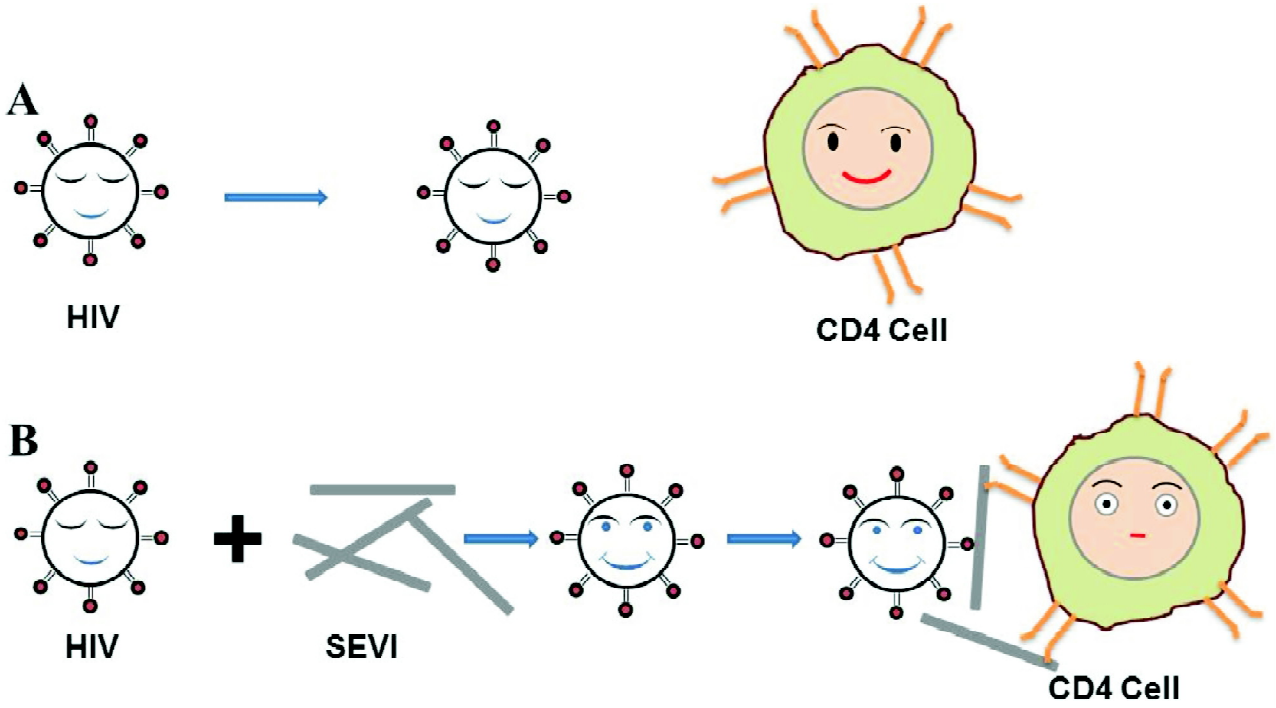

Figure 1: **The HIV virion in the absence of SEV1 remains silent and unable to reach in close proximity to the target cell (A) that results in poor infectivity. The presence of SEV1 facilitates the virus to reach closer to the target cell and enables it to infect CD4+ cell (B). In this process, the amyloid fibrils serve as sandwich between virus and the target cell**

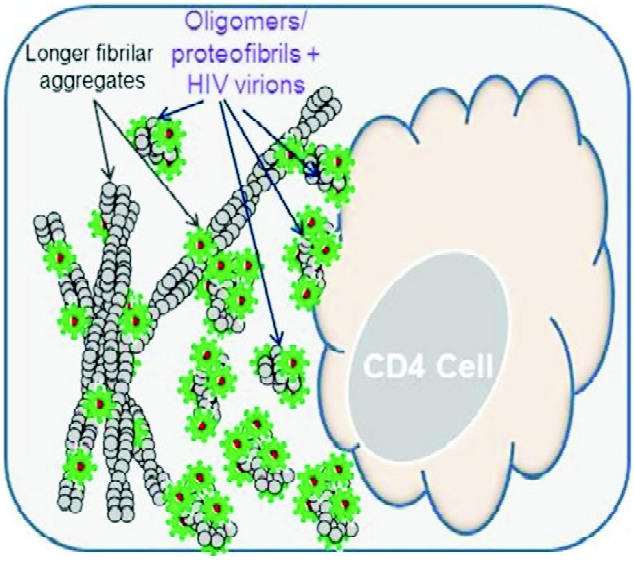

Figure 2: **Mechanism of SEVI mediated enhancement in HIV infection. The SEVI fibrils act as a cationic bridge between HIV virion and host cell. Shorter fibrils and intermediate states more efficiently interact with both HIV virion and CD4+ target cells. Thus, the shorter fibrils or protofibrils might possess greater infectivity enhancement effect.**

2011). Another study shows that Cu II and Zn II ions inhibit the fibrilization of SEVI at concentration comparable to their actual concentration in human semen (Sheftic *et al.*, 2012). Most surprisingly, a study in recent times has shown that SEVI fibrils are unable to increase the infectivity in rectum of a humanized mouse model (Dis *et al.*, 2015). All these studies together indicate that there exist some untouched aspects of SEVI that need to be addressed.

Undoubtedly, the discovery of semen derived amyloids has provided a new window of opportunity to prevent HIV transmission through sexual route, however, the above points should be considered. Identification of functional and active conformers will help in establishing strain phenomenon that will provide a comprehensive strategy to target the SEVI-mediated transmission of HIV. Nevertheless, the data presented in the review is mainly extracted from *in vitro* experiments and the stability of inhibitors has not been discussed well. In order to achieve successful crafting strategies, safety, stability, toxicity and distribution and excretion of the inhibitors are important aspects that are required to be considered.

Abbreviations

AIDS, Acquired Immuno Deficiency Syndrome; cDNA, Complementary Deoxyribonucleic Acid; CR, Congo Red; EGCG, Epigallocatechin-3-Gallate; HIV, Human Immunodeficiency Virus; PAP, Prostatic Acid Phosphatase; *pI*, isoelectric point; RNA, Ribonucleic Acid; SEVI, Semen-derived Enhancer of Viral Infection; ThT, Thioflavin T; UNAIDS, United Nations Programme on HIV and AIDS.

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