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Review

Multifaceted action of Fuzeon as virus–cell membrane fusion inhibitor

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ABSTRACT

The viral peptide fusion inhibitor Fuzeon (T-20/DP178/enfuvirtide) is an essential part of the drug combination that has significantly increased the quality of life and life span of many acquired immunodeficiency syndrome (AIDS) patients. Its development as a drug preceded the elucidation of its precise inhibitory mechanism, as well as its molecular targets. The initial model was that Fuzeon inhibits human immunodeficiency virus (HIV) entry by targeting one site within the viral transmembrane envelope protein. Herein, we describe the emerging discoveries that extend this model towards a multifaceted mechanism for the drug in targeting HIV. This significantly advances the understanding of how viruses enter host cells and opens a new window of opportunity for designing future viral fusion inhibitors.

Contents

1. Introduction

Acquired immunodeficiency syndrome (AIDS) was discovered in 1981 [1,2], and is a major threat to human health since. The cause of AIDS was identified in 1983 [3–5] and later was named the human immunodeficiency virus (HIV). HIV is the most known membrane enveloped retrovirus [6] which mainly infects cells from the immune system; T-cells and macrophages [7,8]. In 2010, the joint United Nations program on HIV/AIDS (UNAIDS) published a report on the global AIDS pandemics. This report indicated that around 33.3 million people live with HIV. Each year 2.6 million people are infected by the virus and 1.8 million die from AIDS. There is no cure or a vaccine for this disease, and currently the treatment involves combinatorial anti retroviral therapy targeting several steps in the virus life cycle [9]. This treatment strategy has dramatically elongated the life span of patients and results in a change from a progressive lethal disease to a chronic condition with slow disease progression [10]. The magnitude of this epidemic and the challenge to develop an effective treatment has led to an extensive research on the molecular mechanisms of compounds which inhibit different stages in the life cycle of the virus [11,12].

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2. HIV entry into target cells

The first step in human immunodeficiency virus (HIV) infection is fusion between the membranes of the virus and the host cell, leading to the insertion of the viral genetic material into the cytoplasm. Following reverse transcription, the viral cDNA is integrated into the chromosomes of the cell [11]. The membrane fusion step is mediated by the viral envelope glycoprotein (ENV) [13–16] (Fig. 1a). Like other type I viral fusion proteins, the HIV ENV adopts trimeric organization on the viral surface [17,18]. It is composed of two non-covalently associated subunits: The gp120 subunit enables cell receptors and co-receptors binding, while the gp41 transmembrane subunit endows the physical membrane fusion [19–21]. The extracellular part (ectodomain) of gp41 includes different regions that are involved in membrane fusion (Fig. 1b): (i) the fusion peptide (FP), a hydrophobic stretch at the N-terminus of the gp41 [22,23], (ii) the fusion peptide proximal region (FPPR) [24], (iii) the N-terminal heptad repeat (NHR) which creates a coiled-coil structure [25–27], (iv) the loop which reverses the polypeptide chain [28,29], (v) the C-terminal heptad repeat (CHR), a region which also creates a coiled-coil structure [30,31], (vi) the membrane proximal external region (MPER) [32,33], and another hydrophobic region at the C-terminus of the protein, the transmembrane domain (TMD) [34]. The intracellular part of gp41 is also involved in membrane fusion and comprised of a cytoplasmic tail (Cyt) [35].

The model of HIV membrane fusion asserts the conversion between at least three major ENV conformations. Firstly, the ENV is in its metastable native state on the virion surface [36,37]. In this state gp41 is considered to be sheltered by gp120. Secondly, binding of gp120 to CD4 and co-receptor (such as CXCR4 and CCR5) involves conformational changes in both gp120 and gp41 resulting in the pre-hairpin conformation [36,38,39]. At this point, gp41 is exposed and extended leading to the insertion of the FP into the host cell membrane [23,40,41]. Thirdly, additional conformational changes generate the hairpin conformation [17]. This conformation comprises a trimeric central coiled-coil that is created by three NHR regions, into which three CHR regions pack in an anti-parallel manner. This structure is usually referred to as the “six helix bundle” or “core” structure and it is suggested to be essential for complete membrane merger as well as pore formation [33,42–44] (see structure in detail in Fig. 2 a, b).

3. The discovery of Fuzeon

The first indications for functional regions in gp41 originated from studies predicting a coiled-coil structure in its N- and C-termini [45]. Synthetic peptides are used to model coiled-coils, therefore, a peptide, DP107, corresponding to the N-terminal region of gp41 was synthesized. It exhibited a strong α-helical structure in solution validating its coiled-coil nature [46]. The authors also tested the ability of the peptide to inhibit HIV replication and demonstrated inhibitory capabilities. Following the prediction of a high α-helical secondary structure for another region, in the C-terminus of gp41, another peptide, Fuzeon (T-20/DP178/enfuvirtide), was synthesized and analyzed [47]. In contrary to DP107 it was unstructured in solution but surprisingly, much more potent in inhibiting the virus. Fuzeon did not affect the function of HIV reverse transcriptase, or protease, and it was established that the peptide functions at an early stage of viral replication, consistent with inhibiting the fusion ability of the virus [48,49]. From that stage the work with Fuzeon diverged into the following directions; the first, involved research towards understanding the inhibition mechanism of the peptide, while the second focused on developing the compound as a therapeutic drug [50].

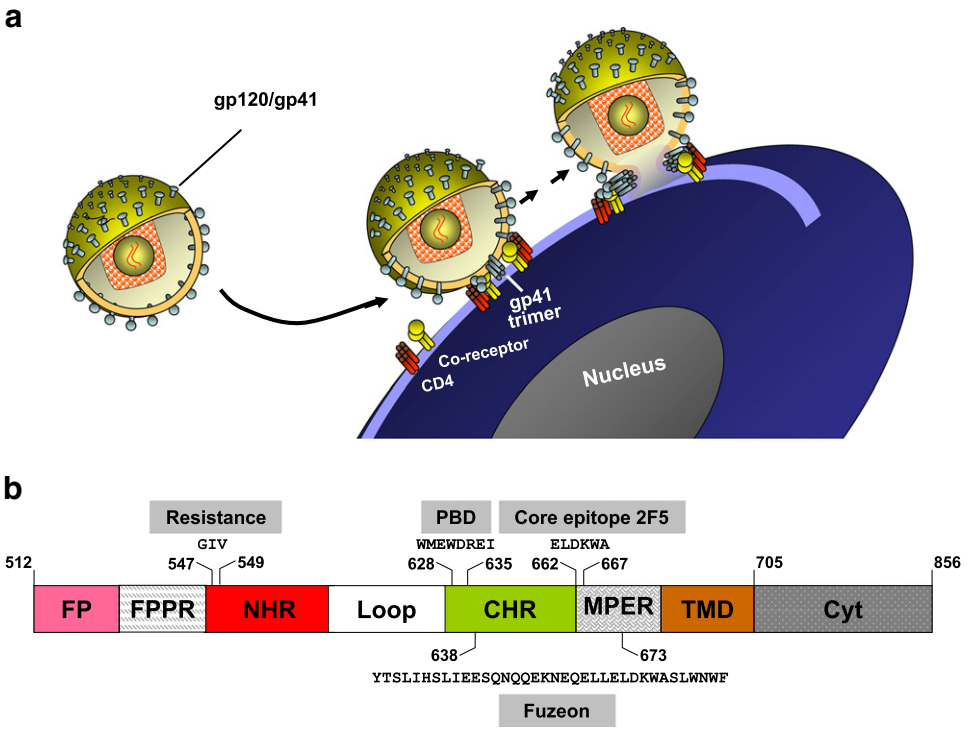

Fig. 1. Entry of HIV into its host cells. (a) The first step in retrovirus infection is fusion between the membranes of the virus and the host cell, leading to the insertion of the viral genetic material. The trimeric ENV of HIV mediates the fusion event [15,16,88,89]. It is composed of two subunits; gp120 subunit endows CD4 and co-receptors binding, while gp41 transmembrane subunit endows the physical membrane fusion [21,90]. (b) Organization of the ENV transmembrane subunit, gp41. The domain abbreviations are: fusion peptide (FP); fusion peptide proximal region (FPPR); N-terminal heptad repeat (NHR); loop; C-terminal heptad repeat (CHR); membrane proximal external region (MPER); transmembrane domain (TMD) and cytoplasmic tail (Cyt). Regions within gp41 that are discussed in the review are indicated. Residues are numbered according to the HXB2 gp160 variant.

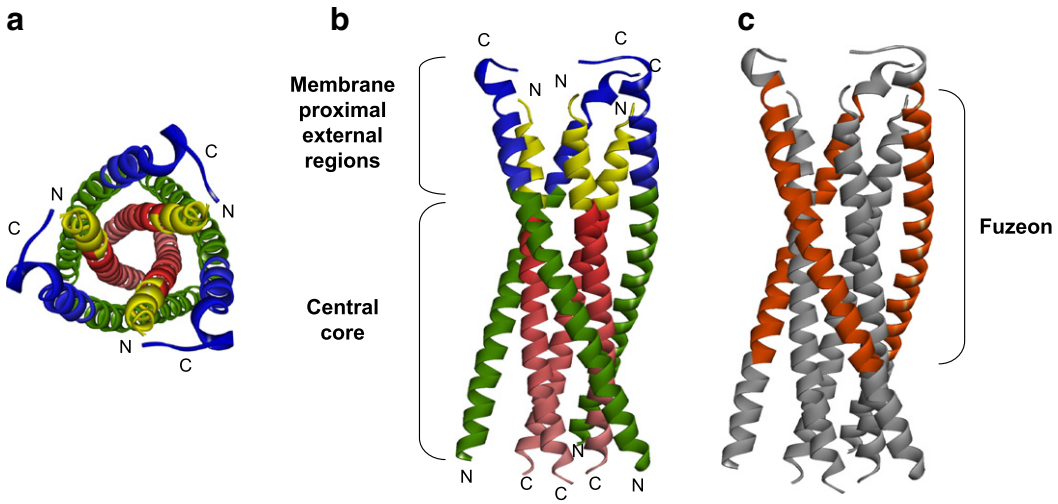

Fig. 2. The hairpin structure of gp41. The very recent high resolution structure (PDB ID: 2x7r) [33] provides an insight into (i) the central core which is created by a trimeric inner coiled-coil of three NHR regions, into which three CHR regions pack in an antiparallel manner, and (ii) the fusion peptide and the membrane proximal external regions (FPPR and MPER, respectively). A top (a) and side (b) view of the structure is presented. C, carboxyl terminus; N amino terminus. In section b, the trimeric inner coiled-coil of the NHR is highlighted in red and the three packing CHR regions are highlighted in green. The FPPR is highlighted in yellow and the MPER is highlighted in blue. (c) The same structure as in section b with the location of Fuzeon which is highlighted in orange. Fuzeon overlaps two functionally different regions from the ectodomain of gp41. The N-terminus of the peptide overlaps the CHR region while its C-terminus overlaps the hydrophobic MPER.

4. Fuzeon comprises a unique sequence location

The peptide Fuzeon is unique amongst other fusion inhibitors in its sequence location. It partially overlaps two functionally different regions from the ectodomain of gp41, the CHR and the MPER (Figs. 1b and 2c).

4.1. The CHR region

The CHR region contains a pocket-binding domain (PBD) that includes three conserved hydrophobic residues. These residues bind to a deep cavity termed “the pocket” on the outer surface of the NHR coiled-coil structure. This is believed to be crucial for the six helix bundle stability [51]. Also, the CHR contains a NHR-binding domain consisting of hydrophobic amino acid heptad repeats [43]. Fuzeon includes the NHR-binding domain and lacks the PBD [30,31] (Fig. 1b).

4.2. The MPER

The MPER of gp41 is rich in specifically tryptophan amongst other hydrophobic residues. It is also the target of broadly neutralizing anti-HIV-1 antibodies suggesting an important role for this region in gp41-mediated membrane fusion [52–54]. It has been demonstrated already in 1999 that this region is important for virus fusion and infectivity [55]. Deletion of 17 amino acids or mutations of tryptophan residues to alanines abrogated the ENV ability to induce cell–cell fusion and viral entry. In contrast, the ENV maturation, transport and CD4 binding properties were unaltered. The importance of the conserved tryptophan residues was indicated to be at the level of glycoprotein incorporation into virions. It was speculated that the tryptophan residues interact with components of the membrane, such as cholesterol, that result in glycoprotein enrichment in the membrane subdomains through which the virus buds [55]. Later, it was established that the MPER serves as a lipid domain targeting sequence that causes membrane destabilization, and gp41 clustering at the fusion site [56]. Utilizing cryoelectron microscopy tomography, the structures of ENV spikes on the membranes of SIV and HIV-1 virions were discovered; the first [18] supports binding of the MPER region to the membrane of the virus, whereas the second [57] supports inter-subunit interactions of this region. A recent crystal structure of the HIV-1 gp41 core including the MPER (Fig. 2) provides the evidence that part of MPER will be membrane inserted within trimeric gp41 [33].

5. The molecular targets and inhibition mechanism of Fuzeon

5.1. Targeting the NHR region of gp41 and acquiring viral resistance

It was demonstrated already in 1994 [49] that Fuzeon blocks an early step in the virus replication cycle prior to reverse transcription. It was suggested that the peptide interferes with virus entry or uncoating. Later on, a research showed that the helical structure of the N-peptide, DP107, was disrupted in a dose dependent manner following the addition of Fuzeon, indicating a possible interaction between these two regions. Fuzeon peptides with alterations or mutations demonstrated reduced interactive ability with DP107 and exhibited reduced anti-viral activity [58]. Using Fuzeon, researchers capture an early conformation of gp41 [38]. They showed that the peptide binds gp41 and inhibits HIV-cell fusion after gp120 interacts with cellular receptors. These studies provide insights into early events leading to ENV-mediated membrane fusion.

Additional evidence for this mode of action lies in studies examining viral strains that are resistant to Fuzeon. Resistant strains were derived by serial virus passage in the presence of increasing doses of the peptide [59]. A contiguous three amino acid sequence within the NHR region, GIV (aa 547–549), was associated with the resistance phenotype. This phenomenon of resistant strains with alterations in that specific region was also observed during phase I of the clinical trial with Fuzeon [60]. However their analysis, as well as others, suggests that mutations in adjacent residues also contribute to the resistance [61,62]. There are several proposed escape mechanisms for the peptide resistant strains depending on the type of mutation in the NHR region: reduced contact, steric obstruction, electrostatic repulsion, and electrostatic attraction [63].

Using a six helix bundle specific monoclonal antibody with isolate-restricted ENV reactivity, it was directly shown that Fuzeon binds to the gp41 NHR coiled-coil to form a peptide/protein hybrid structure [64]. These observations led to a broad belief that this peptide inhibits the virus replication cycle by blocking six helix bundle formation, and preventing progression from the pre-hairpin conformation to the hairpin conformation resulting in inhibiting virus–cell membrane fusion (Fig. 3).

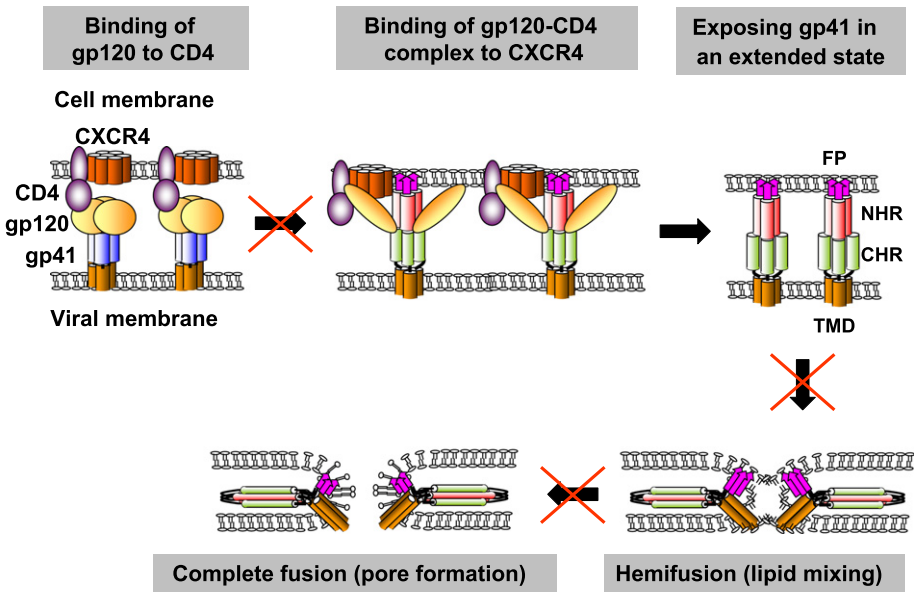

Fig. 3. Fuzeon inhibits multiple intermediate conformations within the viral ENV. Early on, it targets the gp120 co-receptor binding sequence, blocking the interaction of gp120–CD4 complexes with the CXCR4 co-receptor [65,68,77]. This prevents the conversion into the pre-hairpin conformation. Later, Fuzeon targets the NHR in gp41 pre-hairpin conformation thus preventing the conversion into the hairpin conformation [61,64,72]. Lastly, it inhibits late fusion events which subsequently prevent fusion pore expansion [44,66,67].

5.2. Targeting other regions in gp41

Several discoveries expand the initial inhibitory model of Fuzeon that relies on prevention of the six helix bundle formation by targeting the NHR region. First, the peptide does not contain the PBD (Fig. 1b) that was previously demonstrated to be crucial for fusion ability and six helix bundle stability [51]. Second, virus strains resistant to Fuzeon are still sensitive to other CHR derived peptides such as T649 [65] raising the possibility of an additional inhibitory mode of action. Indications for additional inhibitory modes of action began to surface, and in 1998, the first study that presented strong evidence for it was published [44]. The authors monitored the fusion between effector cells (containing HIV-1 ENV) and target cells (containing the receptors and co-receptors) labeled with lypophilic and cytosolic fluorescent probes. Upon addition of Fuzeon they monitored inhibition of the cytosolic probe redistribution while the lypophilic probes were redistributed freely. In a second study [66] it was also demonstrated that Fuzeon binds and oligomerizes on the surface of membranes. The authors concluded that the peptide has two binding sites with different binding affinities; the lower affinity site is the NHR, while the higher affinity site is yet unknown. It was speculated that the higher affinity site is the parallel endogenous region, and that by binding to it Fuzeon prevents aggregation of several ENV trimers needed to enlarge the fusion pore. Therefore, binding to the high affinity site is associated with transition from the lipid mixing state to the content mixing state. This conclusion was further supported by a study demonstrating that the peptide inhibits the late steps of membrane fusion after the lipid mixing stage [67]. The claim that Fuzeon has more than one binding site on gp41 was also demonstrated by blocking its inhibitory activity by peptides derived from the TMD of gp41. The TMD is thought to be a possible second site in gp41 for Fuzeon binding [68].

5.3. Targeting the cell membrane

It has been demonstrated that the forces governing protein–protein interactions in a membrane environment are different from those in a solution environment. Specifically, the constraints involving specific recognition based on chirality are not necessarily applicable [22,69]. Based on this knowledge, Fuzeon variants with substitutions to D-amino acids in either their N- or C-termini were designed [70]. It was shown that substitution in the N-terminal region of the peptide severely decreased the ability of the variant to inhibit fusion, while such substitution in the C-terminus did not. This demonstrated the involvement of the C-terminal region in membrane binding. Additionally, monitoring the changes in membrane dipole potential demonstrated an interaction of Fuzeon with erythrocyte and lymphocyte membranes [71].

Strong evidence for the membrane binding ability of the peptide was provided in a study published in 2007 [72]. Therein, the entire C-terminal region of the peptide was truncated leading to loss of activity. Strikingly however, replacement of the truncated amino acids with a fatty acid resulted in the recovery of the activity. The activity of the conjugated peptide correlated to the fatty acid carbon chain length, to its concentration on cells, and to the directionality of fatty acid conjugation (N- or C-terminus). The studies involving fatty acid conjugation to Fuzeon variants [72,73] demonstrated that the nC-terminal region of the peptide functions also as an anchor to the cell membrane enabling its N-terminus to inhibit fusion by interacting with the endogenous NHR coiled-coil.

5.4. Targeting the gp120 co-receptor binding sequence

The involvement of gp120 in the susceptibility to Fuzeon is supported by several lines of evidence. A reduced susceptibility to the peptide was demonstrated by chimeric viruses containing a CCR5 V3 loop compared with the CXCR4 parent strains [74,75]. The authors speculated that a reasonable explanation to these results would be that the affinity of gp120 to the co-receptor governs the length of time in which the C-inhibitory peptides can bind their NHR targets. Indeed, later on, it was revealed that the sensitivity to Fuzeon and a co-receptor antagonist is modulated by two determinants: the affinity of the gp120 co-receptor interaction, and the availability or expression level of the co-receptor [65]. These in turn correlated to the fusion kinetics. These results confirmed previous speculation that co-receptor affinity and expression level modulate the kinetic window in which the ENV is sensitive to Fuzeon. Additionally, in 2004 it was observed that in some HIV-1 patients prior to peptide treatment, resistant strains emerged. These strains did not contain any mutations in the NHR in contrast to a number of substitutions in the CHR and

MPER [76]. It was established that the inhibitory capability of the peptide does not merely depend on the CHR affinity to the NHR but rather involves other thermodynamic determinants. The authors hypothesized that the resistance mechanism of these virus strains depends on reducing the pre-hairpin conformation time window. They suggested a model in which sequences from both gp120 (co-receptor binding regions) and gp41 modulate the kinetics of six helix bundle formation thereby shortening the time in which the CHR-peptides can interact with their NHR targets. Fuzeon was able to block the interaction of gp120–CD4 complexes with the CXCR4 co-receptor [77] and peptides derived from the co-receptor binding site in gp120 competitively inhibited the activity of Fuzeon [68,78]. These results help to explain the increased sensitivity of CXCR4-specific HIV-1 isolates to Fuzeon and propose the co-receptor binding site in gp120 as a target for the peptide during viral entry.

5.5. Targeting the N-terminus of gp41

The C-terminus of Fuzeon overlaps with the hydrophobic MPER which contains the epitope for the 2F5 monoclonal antibody. A crystal structure of the monoclonal antibody 2F5 bound to its corresponding epitope demonstrated that this binding is based on electrostatic interactions creating an elongated structure [52]. This type of interaction produces a highly unbound hydrophobic face of the epitope. The authors suggested that this hydrophobic face is occluded by other hydrophobic gp41 regions. Later, several studies provided evidence that the interaction between FP or its proximal region and the MPER domains of gp41 increased the efficacy of 2F5 binding to its MPER epitope [24,32]. These observations suggest the N-terminus of gp41 as a potential target for Fuzeon during viral entry. Fuzeon may prevent the self-interaction of the N-terminus of gp41 in the early fusion steps while gp41 is extended; alternatively, it may block the interaction of the MPER with the N-terminus of gp41 in the late fusion steps.

6. HIV-1 fusion inhibitors are important candidates in AIDS therapy

Fuzeon was approved as a drug by the food and drug administration (FDA) in March 2003 [79]. It created a new therapeutic category of anti HIV-1 drugs: entry inhibitors [80]. It shows good antiviral potency and long-term safety in clinical use. However, it must be subcutaneously injected twice a day, which is highly inconvenient. The virus exhibited wide ranging susceptibilities to Fuzeon, and the compound has a relatively low genetic barrier for resistance. Therefore, it should only be applied as part of a combinatorial drug application with others antiretroviral agents [81].

Recent progress in the field of HIV therapy has led to alternative entry inhibitor compounds that are generally divided into three classes: (i) attachment inhibitors, (ii) co-receptor binding inhibitors, and (iii) fusion inhibitors. Many of these inhibitors are at different stages in clinical trials [82]. Until now, fusion inhibitors have been the most successful and promising class of HIV entry inhibitors. For several compounds, the identified targets are regions within the ectodomain of gp41. In addition to the first generation fusion inhibitor, Fuzeon, a rational peptide design approach has led to other attractive peptides. The second generation fusion inhibitor T-1249 was designed from the CHR region. It is more potent than Fuzeon and preserves its potency to Fuzeon resistant strains [83]. Recently, a series of more potent third generation fusion inhibitors were designed. These include T-2635, which has an improved helical structure that increases stability and activity against both wild type HIV-1 and fusion inhibitor resistant variants [84,85]. A natural HIV-1 inhibitor, designated as VIRIP, was recently discovered. This is a 20-residue peptide which targets gp41 fusion peptide. The peptide is potent against a wide variety of HIV-1 strains including those resistant to current antiretroviral drugs [86]. Clinical trials with VIRIP provides a proof of concept that fusion peptide inhibitors suppress viral infection in human patients, and offer prospects for the development of a new class of drugs that prevent the virus from anchoring to and infecting host cells [87].

7. Conclusions and prospects

Three decades have passed since the discovery of AIDS and still it is a major concern for public health. Its causing agent, the HIV retrovirus, was detected and was extensively studied throughout the years to seek how it spreads the disease. In order to start an infection cycle, the virus delivers its genetic material into the host cell by merging both viral and host cell membranes into one. This step is orchestrated by the trimeric HIV ENV similarly to many other viruses. Its peripheral subunit, gp120, mediates host tropism, while its transmembrane subunit, gp41, mediates the actual membrane fusion. Compounds that interfere with membrane fusion induced by viruses are important to reveal the mechanism of this process, and to develop drugs that would inhibit it. The discovery of gp41 derived peptide, Fuzeon, led to a successful achievement of these two goals. Fuzeon was synthesized from the C-terminal part of the ectodomain gp41. It was the first peptide that showed very potent inhibition of HIV infection and created a new therapeutic category of anti HIV-1 drugs: entry inhibitors. Initially, it was believed that the sole inhibition mechanism of the peptide is targeting the NHR region of gp41 during the formation of the pre-hairpin conformation. This would subsequently prevent the formation of the hairpin conformation leading to inhibition of membrane fusion. Only recently, accumulating evidence suggest a multifaceted nature for Fuzeon in inhibiting HIV infection.

In this review we provided a comprehensive summary of the different modes of action of Fuzeon, suggesting multiple targets on the viral ENV because of its unique sequence (Fig. 3). The peptide's sequence is comprised of two regions in gp41 (CHR and MPER) that are important to its fusogenicity. Early on, it targets the gp120 co-receptor binding sequence, blocking the interaction of gp120–CD4 complexes with the CXCR4 co-receptor. Later, it targets the NHR in gp41 pre-hairpin conformation thus preventing the conversion into the hairpin conformation. Lastly, it has an additional inhibitory mode of action involving its C-terminal region and membrane binding. This alternative mode of action might involve interfering with the assembly of gp41 trimers (by binding the MPER region), thus preventing fusion pore expansion to arrest the fusion process.

Fuzeon was approved by the FDA in 2003 for AIDS patients and was considered a promising drug, but resistant strains quickly emerged. Today there are alternative compounds that are at different stages in clinical trials. Besides its important clinical contribution, the discovery of this peptide heralded a new era of thinking within the field of membrane proteins. The use of peptidic fragments from a membrane protein that interfere with its function is now an emerging and promising approach for understanding protein structure and function.

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