Resistance to enfuvirtide, the first HIV fusion inhibitor

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Fusion inhibitors are a new class of antiretroviral drugs (ARVs) for the treatment of human immunodeficiency virus infection. Enfuvirtide is the first in this class to reach market approval. Fusion inhibitors block the last step in the three-step viral entry process consisting of attachment, co-receptor binding and fusion, thereby preventing viral capsid entry into the host cell. Enfuvirtide has a unique mechanism of action and high viral target specificity, and in clinical trials has been shown to exhibit both high efficacy and low toxicity. Enfuvirtide is a peptide mimetic of an essential region within viral envelope glycoprotein gp41 that functions by blocking gp41 structural rearrangements at a transitional pre-fusion conformation. Although different clinical isolates show variation in susceptibility to enfuvirtide, primary resistance has not been observed, and thus enfuvirtide-naive isolates remain clinically sensitive. Acquired resistance centres round a 10 amino acid motif between residues 36 and 45 in gp41 that forms part of the binding site of enfuvirtide. The 10 amino acid motif is critical for viral fusion, and enfuvirtide-resistant mutants show poor replicative capacity compared with wild type. Reversion to a wild-type, drug-sensitive state has been reported following enfuvirtide withdrawal.

Keywords: fusion inhibitors, resistance, gp41

Introduction

Within the last decade combinatorial drug regimens, known as highly active antiretroviral therapy (HAART), have significantly improved the prognosis for individuals infected with human immunodeficiency virus (HIV).1 These typically comprise at least three drugs chosen from three well-established drug classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs).2–6 Despite these therapeutic advances, almost a quarter of first-time HAART patients discontinue treatment within 8 months often due to virological failure, poor adherence to treatment schedules or excessive toxicity.7,8 Indeed, all of the drugs conventionally incorporated into HAART regimens have significant toxicity profiles. For example, nucleoside/nucleotide analogues can inhibit cellular mitochondrial polymerase3,9–13 leading to lactic acidosis, while PIs can prevent the function of essential cellular proteins resulting in insulin resistance and hypercholesterolaemia.12–16 These severe adverse events and other tolerability issues with these drugs can lead to low patient adherence resulting in sub-optimal drug usage, the development of resistance and virological failure. However, even with good patient adherence, the high replicative rate of the virus and low fidelity of its reverse transcriptase enzyme make HIV mutants common and drug resistance inevitable.17 The range of possible therapeutic options available to HIV-infected individuals is narrowed further by the fact that resistance to one particular drug often causes resistance to other drugs in the same inhibitor class.

The recent approval of enfuvirtide (FUZEON, formerly known as T-20 or DP178) for the commercial market therefore provides a much-needed fourth class of drugs for the treatment of HIV, the fusion inhibitors. Operating early in the viral life cycle, fusion inhibitors prevent viral entry, and have a novel, highly specific mechanism of action with a low toxicity profile.

Viral entry and fusion

From a therapeutic perspective, viral entry is one of the most attractive points for intervention in the viral life cycle, since drug activity is independent of intracellular access. The HIV entry process has three discrete steps: attachment, co-receptor binding and fusion, each representing a unique drug target.

The initial step in the entry process involves attachment of the viral envelope glycoprotein (gp120) to the CD4 cell surface receptor on helper T-cells, and on other susceptible cell types.18–21 The host-derived viral lipid membrane is studded with virus-encoded trimeric envelope structures consisting of each of two glycoproteins, gp120 and gp41. The structure is formed via intramolecular disulphide bridges and by non-covalent
intermolecular bonds. In each envelope structure, a trimer of gp120 molecules makes up the cap and the stalk is formed from a gp41 trimer that is anchored in the viral lipid bilayer. Upon binding of gp120 to CD4 and chemokine receptors, a conformational change in the structure of gp41 occurs so that the membranes of virus and cell are brought into close proximity, resulting in membrane fusion and subsequent infection.

Many inhibitors, known as attachment inhibitors, have been designed to block the binding of gp120 to the CD4 receptor and a number are in pre-clinical or clinical development including BMS-806 and BMS-043, PRO 2000,\textsuperscript{24} TNX-355\textsuperscript{25} and PRO 542.\textsuperscript{24,26,27} However, while these act extracellularly prior to viral cell invasion and have the advantage that they do not require entry into the cell in order to function, some (e.g. TNX-355) target the essential host CD4 receptor rather than a specific viral target, which may result in unwanted side effects.

Following its attachment to the host CD4 receptor, the gp120 protein undergoes a conformational change that facilitates its binding to a second (chemokine) co-receptor.\textsuperscript{28,29} CCR5 and CXCR4 are the most important HIV-1 co-receptors. All strains of the virus can use either CCR5 or CXCR4 (monotropic viruses) or both (dual-tropic viruses) to enter CD4 cells. Viruses that use CCR5 are largely transmitted and endure throughout infection. Those that exploit CXCR4 often emerge later on in the course of infection and have been associated with more rapid disease progression and CD4 cell decline.\textsuperscript{30,31} The binding of both CD4 and chemokine co-receptor by gp120 are the first two steps of viral entry, and the prevention of co-receptor binding, like the prevention of attachment, is an important therapeutic target. Many co-receptor binding inhibitors are in various stages of development, including TAK-779,\textsuperscript{32} SCH-C and -D,\textsuperscript{33–35} PRO-140,\textsuperscript{36} UK-427,857,\textsuperscript{37,38} GW873140 and AMD887, which target the CCR5 receptor, and AMD3100\textsuperscript{39} and AMD070, which block the CXCR4 receptor. However, since these drugs target essential host molecules, rather than specific viral targets, they will not only prevent HIV entry but also may affect normal functions of the co-receptors that they block, leading to the possibility of undesirable side effects. In addition, since each co-receptor inhibitor is directed at only one type of co-receptor, HIV could enter via the other co-receptor, particularly where individuals are infected with dual-tropic viruses or dual-tropic viral populations.

The third and final step in the viral entry process, fusion, holds the best promise for the development of highly specific, low toxicity therapies by combining a virus-specific target with an extracellular mode of action. Fusion of the viral and cellular membranes is a highly complex process that results in the release of the viral capsid into the cytoplasm of the host cell. The amino acid sequence of gp41 predicts four regions of essential functional importance (Figure 1):\textsuperscript{18–21,30,40–45} a transmembrane-spanning region anchors the protein into the viral membrane; two regions known as heptad repeats (HR1 and HR2) show periodic hydrophobicity predictive of α-helical structures that can unite to form a trimer of antiparallel heptad repeat dimers also referred to as a six-helix bundle or hairpin structure; and a fusion peptide region that is capable of piercing the host cell membrane. The binding of gp120 to CD4 and either CCR5 or CXCR4 is believed to favour the formation of the six-helix bundle hairpin conformational structure in gp41. Once formed, the hairpin structure is stabilized by intramolecular disulphide bridges. The transition into this altered conformation pulls the viral and cellular membranes together and promotes the insertion of the N-terminal fusion peptide into the cellular membrane.\textsuperscript{41} Evidence suggests that the aggregation of several envelope trimers of these complexes inserted into the cellular membrane creates a pore through which the viral capsid can then pass.\textsuperscript{46,47}

**Mechanism of action of fusion inhibitors**

Fusion inhibitors exploit the gp41 conformational transition that follows gp120–CD4 binding and co-receptor binding, and

![Figure 1](https://academic.oup.com/jac/article-abstract/54/2/333/767439/6667678148)
precedes pore formation. A fundamental step in the fusion process involves the interaction between the two different peptide motifs (HR1 and HR2) that make up the six-helix bundle, and fusion inhibitor development has tended to concentrate on molecules that act as peptide mimics, blocking the interactions that are necessary for entry.46,48,49 Prior to the establishment of the hairpin structure that promotes membrane fusion, a gp41 pre-hairpin intermediate structure forms and temporarily exposes the two separate portions of the six-helix bundle. Peptide mimetic fusion inhibitors operate at this stage by binding along the hydrophobic grooves of the trimeric coiled coil HR1 region, locking the gp41 structure into this transitional form, preventing the formation of the six-helix bundle hairpin structure.

Enfuvirtide is homologous to a segment of the HR2 region of gp41 corresponding to amino acids 643–678.48–50 As a linear 36 amino acid peptide, enfuvirtide binds to the HR1 region of gp4148–53 blocking the formation of the six-helix bundle necessary for fusion. Enfuvirtide exhibits potent and selective inhibition of HIV-1 both in vitro and in vivo.48,54–59 The clinical efficacy of enfuvirtide was demonstrated in the pivotal Phase III TORO trials, in which almost 1000 triple-class-experienced patients were randomized to receive an optimized background regimen with or without enfuvirtide over 48 weeks. These trials showed significantly greater CD4 count increases (a change from baseline of 91, versus 45 cells/mm3) and HIV viral load decreases (a change from baseline of −1.48, versus −0.63 log10 HIV-1 RNA copies/mL) were observed for the enfuvirtide arm, compared with the control arm at 48 weeks.60,61

A slightly longer, 39 amino acid peptide, T-1249 (not currently in clinical development), binds to a region of HR1 that overlaps with the binding site of enfuvirtide and is active against HIV-1 and HIV-2 as well as simian immunodeficiency virus. Several other fusion inhibitor therapeutics have been studied solely at the research level, and many of these are peptide mimetics, including T-649, which has homology with the HR2 region of gp41 and blocks hairpin structure formation in a similar way to enfuvirtide; C34 peptide, which has homology to a region that makes up the six-helix bundle and blocks its formation;62 D-peptide, a cyclic molecule designed to bind to a pocket region within the six-helix structure;63 and DP-107, a peptide with homology to HR1 that binds HR2.64,49 Other types of inhibitors that have been researched include 5-helix, which prevents the formation of the six-helix bundle by sequestering one of the helices necessary for its formation, and RPR103611, a non-peptide triterpene compound that targets the loop region linking the two halves of the gp41 leucine zipper and disrupts the association of gp120–gp41 in CXCR4-tropic viruses.

The benefits of fusion inhibitors

Fusion inhibitors act extracellularly prior to invasion of the host cell. They are therefore not susceptible to cellular efflux transporters that lower the effective intracellular concentrations of other classes of antiretrovirals.64,65 Furthermore, enfuvirtide shows little or no drug–drug interaction with drugs metabolized by the CYP 450 or N-acetyltransferase route.66,67 Their lack of intracellular processing compared with other antiretroviral drug classes may contribute to their low toxicity profile. Furthermore, their site of action mimizes cross-resistance with established intracellular agents.68 The fusion inhibitor class of entry inhibitors, unlike the co-receptor inhibitor class and some members of the attachment inhibitor class, are specific to virus components rather than targeting elements of the host immune system and they show activity against both CCR5- and CXCR4-tropic virus. However, while enfuvirtide is highly efficacious both in vitro and in vivo,8,34–39 it is uncertain whether other fusion inhibitors will be as effective.

A further strength of enfuvirtide and other entry inhibitors lies in their potential inter-class synergy.27,34,36,39 Enfuvirtide is the fourth drug in treatment options available to antiretroviral-experienced patients, including HAART regimens using NNRTIs, NNRTIs and PI. The development of drugs with different extra-cellular targets in the future may even lead to the possibility of potent extracellular regimens (E-HAART).53 Synergy has already been seen with enfuvirtide and the other entry inhibitors (i.e. AMD3100 and SCH-C)14,38 and in the future there may be the potential for E-HAART regimens.

Several of the entry inhibitors are large peptidomimetic molecules (e.g. antibodies or peptides) that cannot be administered orally. Many, such as PRO 542, TNX-355 and enfuvirtide require regular injection that may affect long-term patient adherence. Peptides have also been shown to induce an antibody response,58 potentially blocking antiviral activity, affecting drug clearance and causing an inflammatory response.70 However, Walmsley et al.71 report that gp41 antibodies that cross-react with enfuvirtide do not affect clinical responses to it.

Factors affecting baseline susceptibility to enfuvirtide

Enfuvirtide and T-1249 are active against both B and non-B viral subtypes of HIV-1.50,52 Primary genotypic resistance to enfuvirtide has not been detected among enfuvirtide-naive patients.72 However, there is a wide range of susceptibility to enfuvirtide among primary isolates derived from these patients but this does not appear to have any clinical relevance.73–81

There was initial controversy over the basis for differences in the in vitro susceptibility to inhibition of fusion inhibitor-naive viruses. Several studies suggested that HIV-1 envelope co-receptor tropism4,59 or affinity72 may contribute to this observed range of in vitro susceptibility. However, further investigations were not able to demonstrate the link between co-receptor tropism and susceptibility to enfuvirtide.68,61,83,84

A small-scale study on 14 viral isolates initially reported that CCR5-tropic isolates were less susceptible to inhibition by enfuvirtide than CXCR4-tropic isolates with a mean IC50 that was 0.8 log10 higher for CCR5-tropic viruses.74 However, a larger study of 55 isolates by these same investigators found much smaller differences in the enfuvirtide susceptibility of CCR5- and CXCR4-tropic viruses.75 When over 100 baseline isolates from Phase II studies were examined, however, such differences in enfuvirtide susceptibility as a function of virus co-receptor tropism were not found.81 Furthermore, an examination of genetically linked longitudinal samples from fusion inhibitor-naive subjects who underwent a switch in co-receptor tropism from CCR5-tropic to CXCR4-tropic failed to find alterations in enfuvirtide susceptibility associated with these changes in virus co-receptor tropism.81

A different approach to examining this issue was taken by Stanfield-Oakley and colleagues.55 These investigators constructed gp120–gp41 chimeric envelopes derived from...
C Coreceptor type is an important determinant of susceptibility to enfuvirtide. Enfuvirtide differs in its ability to inhibit CCR5- and CXCR4-tropic fusion inhibitor-naive isolates with widely differing sensitivities to enfuvirtide in order to map determinants of enfuvirtide susceptibility. The results indicated that the major determinants of enfuvirtide susceptibility mapped to the gp41 subunit and were independent of the co-receptor phenotype of the starting isolates. Results with additional chimeric constructs and site-directed mutants from this group and another suggested that both HR1 and the HR2 regions of gp41 contribute to the enfuvirtide susceptibility of fusion inhibitor-naive viruses.

The basis for the initial discordant results regarding the role of co-receptor phenotype in susceptibility to inhibition by enfuvirtide is unclear, though they may have resulted from either selection bias due to an insufficient number of isolates examined or to artefactual influences associated with the different phenotypic assay systems employed. Nevertheless, analysis of virological responses in the pivotal Phase III clinical trials of enfuvirtide have demonstrated that patients harbouring either CCR5-tropic, CXCR4-tropic or dual-tropic virus populations respond to enfuvirtide therapy with equivalent decreases in viral load. Thus, any differences in the in vitro susceptibility of CCR5- and CXCR4-tropic viruses do not manifest in a clinical setting, and enfuvirtide shows potent activity in vivo against viruses that use either or both co-receptors.

**Acquired resistance to enfuvirtide**

Under selective drug pressure, the high replication rate of HIV and the low fidelity of the HIV reverse transcriptase enzyme can lead to the development of resistance to ARVs which includes the occurrence of resistance to enfuvirtide. Early in vitro studies using enfuvirtide showed that differences in susceptibility in enfuvirtide-naive virus and the development of resistance are associated with changes in a conserved amino acid triad (GIV) at positions 36–38 in the HR1 region of gp41. The mutation of this region under selective pressure supports the predicted notion that HR2–HR1 binding is a prerequisite for fusion and that homologues of HR2, such as enfuvirtide, function by blocking this interaction. These findings were confirmed by site-directed mutagenesis experiments and in vivo studies which expanded the core region of functional importance to amino acids 36–45. Interestingly, regions associated with co-receptor specificity and susceptibility to enfuvirtide, such as the V3 loop of gp120 were not selected under drug pressure, indicating that variations in these regions do not play a primary role in the development of clinical resistance.

A variety of different mutations has been noted in the amino acid region 36–45 (Table 1), each conferring a distinct level of resistance or susceptibility to enfuvirtide in a defined molecular background. Single amino acid substitutions in this region are the most common and cause variable degrees of susceptibility loss. Serial mutations, where the reversion of the primary mutation coincides with the generation of the second, are known. Single substitutions exhibit a 5- to 10-fold reduction in susceptibility to enfuvirtide. Double amino acid substitutions have also been observed and these are associated with the highest levels of resistance, some combinations (G36S/V38M) exhibiting an 100-fold reduction in enfuvirtide susceptibility. However, there is a significant overlap in the ranges observed for single and double mutations. In addition, considerable differences in enfuvirtide IC₅₀ have been observed between primary isolates bearing the same pattern of mutations in gp41 amino acids 36–45 as well as differences between viruses isolated from patients enrolled in Phase II clinical trials and site-directed mutants bearing the same mutations (e.g., G36S/L44M), suggesting that other viral factors (e.g., the V3 loop, or the HR2 region) may modulate the sensitivity of the gp41 36–45 amino acid core region. Mutations in the HR2 region, although rare, have been detected. The incidence of genotypic changes at amino acids 36–45 in enfuvirtide-naive populations is low (Los Alamos database; http://hiv-web.lanl.gov), indicating a natural conservation of the motif and underscoring its importance as a region that is critical for viral fusion.

Although double substitutions usually require higher inhibitory concentrations of enfuvirtide, in general, there is a lack of association between genotype and phenotype. Population sequencing of samples from over 300 patients experiencing virological failure in Phase II and Phase III enfuvirtide clinical trials (T20-205, T20-206, T20-208, TORO 1 and TORO 2) confirmed the role of the 36–45 amino acid region in gp41 resistance. Differences in baseline susceptibility to enfuvirtide do not correlate with clinical outcome and isolates with a low susceptibility to enfuvirtide are no more likely to develop resistance or exhibit virological failure than are those with a high susceptibility.

**Fitness of enfuvirtide-resistant mutants and revertants**

The conservation of amino acids 36–45 in the HR1 region of gp41 illustrates the essential function of this domain. Predictably therefore, the fitness (as measured by replicative
capacity) of viruses bearing mutations in this region was found to be lower than wild type. 58,90 In the absence of drug, wild-type virus was able to replicate with faster kinetics than viruses bearing mutations. Within amino acids 36–38 of HR1 a relative order of GIV > DIV > DTV > DIM > SIM was found for the replication kinetics of mutant clones in the absence of drug. This relative order of fitness was reversed in the presence of enfuvirtide. 99,100 Viruses with double amino acid substitutions were less fit than those with single substitutions. 78

One consequence of the development of enfuvirtide-resistant mutants with attendant reduced fitness is that following discontinuation of enfuvirtide, wild-type virus has been found to outgrow resistant virus, restoring replicative capacity associated with normal drug susceptibility. 101 The clinical relevance of these observations requires further study.

Cross-resistance with enfuvirtide

Cross-resistance between enfuvirtide and drugs belonging to the well-established classes used in HAART has not been observed, and since enfuvirtide has a different drug target this would not be expected. However, the durability of an antiviral agent is influenced by how effective the entire treatment regimen is. Resistance to enfuvirtide was found to be minimized where patients maintained maximum susceptibility to other classes of inhibitors in their HAART regimen. 91

Summary and conclusions

The fusion inhibitors mark the beginning of a new era in HIV disease management. With a unique mechanism of action they represent a new fourth class of ARVs. Enfuvirtide has been shown to exhibit potent antiretroviral activity, and is approved for treatment in combination with other antiretrovirals in treatment-experienced patients with evidence of HIV-1 replication despite ongoing antiretroviral therapy. The development of resistance to enfuvirtide centres round a 10 amino acid motif in the envelope glycoprotein to which the drug binds. However, by virtue of the fact that the 10 amino acid motif is critical for viral function, enfuvirtide-resistant mutants show poor replicative capacity and revertnce to a drug-susceptible state following drug withdrawal has been reported. The development of enfuvirtide and other agents that target viral fusion or entry is a welcome respite for the growing population of HIV-infected patients with virus that is resistant to some or all of the other available drug classes and could herald the advent of an entirely new therapeutic approach.

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