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Invited review

HIV entry inhibitors: a new generation of antiretroviral drugs

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Abstract

AIDS is presently treatable, and patients can have a good prognosis due to the success of highly active antiretroviral therapy (HAART), but it is still not curable or preventable. High toxicity of HAART, and the emergence of drug resistance add to the imperative to continue research into new strategies and interventions. Considerable progress in the understanding of HIV attachment and entry into host cells has suggested new possibilities for rationally designing agents that interfere with this process. The approval and introduction of the fusion inhibitor enfuvirtide (Fuzeon) for clinical use signals a new era in AIDS therapeutics. Here we review the crucial steps the virus uses to achieve cell entry, which merit attention as potential targets, and the compounds at pre-clinical and clinical development stages, reported to effectively inhibit cell entry.

Introduction

Acquired immunodeficiency syndrome (AIDS) was recognized in 1981, and the first human immunodeficiency virus (HIV) was isolated 2 years later, heralding a new era in the fight against pathogenic viruses[1,2]. Since then, HIV infection has become a major public health problem worldwide, with an estimated 39.4 million infected people as at the end of 2004 (Table 1)[3]. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS) epidemic update, in 2004 there were more than 3.1 million AIDS deaths, including 500 000 children under 15 years of age[4]. The prevalence of HIV-1 is greater in developing countries, and especially in Sub-Saharan Africa, where the infrastructure to prevent and treat the infection is limited[5]. These "hotspots" absorb most of the attention of international committees and organizations, and a significant part of the funding for AIDS prevention and treatment goes towards attempting to scale up antiretroviral (ARV) therapy in developing and transitional countries[6].

HIV is a lentinovirus that is predominantly transmitted by sexual contact, as virus particles can cross the mucosal epithelium and infect specific cells[7,8] expressing the CD4 receptor. Cells bearing CD4 receptors on their membrane belong to the macrophage/monocyte lineage and to a subset of T-cells[9,10]. Initial indications were that HIV-1 used only CD4 to identify and enter the target cells. Soon, however, it became apparent that additional co-receptors were probably required in order for the virus to complete cell entry. Subsequently, several such potential co-receptors were proposed[11,12], but the CCR5 and CXCR4 chemokine receptors are today considered to be the major co-receptors for HIV-1 entry[13–15]. T-cell tropic HIV strains use mainly CXCR4 as a co-receptor and are called X4 strains, whereas macrophage-

Table 1. Worldwide distribution of estimated number of people living with HIV[4].

Region	Estimated number[1)]
North America	1.0 million (540 000–1.6 million)
Caribbean	440 00 (270 000–780 000)
Latin America	1.7 million (1.3–2.2 million)
Western and Central Europe	610 000 (480 000–760 000)
Eastern Europe and Central Asia	1.4 million (920 000–2.1 million)
North Africa and Middle East	540 000 (230 000–1.5 million)
Sub-Saharan Africa	25.4 million (23.4–28.4 million)
East Asia	1.1 million (560 000–1.8 million)
South and South-East Asia	7.1 million (4.4–10.6 million)
Oceania	35 000 (25 000–48 000)

[1)]The ranges around the estimates define the boundaries within which the actual numbers lie based on the best available information[4].

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tropic strains, responsible for host-to-host transmission, use CCR5 as a co-receptor, and are referred to as R5 strains. Thus, macrophages are the principal targets for the establishment of the infection in new individuals[16]. Although it is not always the case[17,18], the transition from viral isolates that use the CCR5 receptor to isolates that use the CXCR4 receptor has been linked with the transition from the latent asymptomatic phase to the clinical manifestations associated with AIDS[19,20].

The most striking feature of HIV-1 infection is the gradual depletion of circulating $CD4^+$ T cells, which leads to increased sensitivity of the patient to opportunistic and chronic infections and to oncogenesis. The cause of the $CD4^+$ T cell depletion is still under debate[21–23]. It is generally accepted, however, that during the asymptomatic phase the daily replenishment rate of $CD4^+$ T cells is much higher than the turnover of infective virus particles for the cytopathicity model to explain the progressive depletion of $CD4^+$ T cells from circulation[24]. An alternative hypothesis proposes that certain viral components contribute to dysfunction of a vital immune mechanism[25].

Over the past 23 years, the main objective in the field of HIV research has been the discovery of drugs that will combat the disease. Satisfactory progress has already been made and there are now more than 20 anti-HIV drugs approved by the American Food and Drug Administration (FDA)[26]. ARV drugs are categorized according to their mode of action into three main groups: 1) the nucleoside reverse transcriptase inhibitors (NRTI); 2) the non-nucleoside reverse transcriptase inhibitors (NNRTI)[27,28]; and 3) the protease inhibitors (PI)[29]. ARV drugs from these categories are now administered in combination (as cocktails) to produce more efficient treatment[30]. This type of therapy, termed "highly active antiretroviral therapy" or HAART, has markedly decreased mortality and morbidity in the developed world. Efforts have been made by the World Health Organization (WHO) and UNAIDS to substantially increase the number of people on HAART in developing and transitional countries[6].

Despite the fact that current antiviral treatments have improved prognosis, drug resistance and high toxicity are serious limitations to current treatments that justify the continuation of research efforts for new strategies and interventions[31,32]. Today, AIDS is treatable, and patients can have a good prognosis, but it is still not curable. A new generation of drugs was recently introduced that inhibit viral cell entry (to be discussed later). HIV entry inhibitors appear to be a rational step forward in ARV therapy, because they prevent the virus from infecting new host cells, and may potentially stop or significantly limit HIV transmission[33–35]. In order to rationally design effective drugs, the pathophysiology of HIV must be better understood for ARV therapy research to target specific events in the biology of the virus within the host cell[36,37].

HIV entry

HIV-1 predominately infects cells that have the CD4 receptor on their surface membrane, although this is not always the case[38,39]. Achievement of infection of these cells involves three discrete steps: viral attachment, then co-receptor binding, and finally fusion (see Figure 1). Recognition of the "correct" target cell and attachment to it is primarily achieved through envelope glycoprotein gp120, which binds to CD4 molecules. Gp120 is generated within the infected host cell after cleavage of gp160 by cellular proteases into two functional proteins: gp120 and gp41. It consists of 5 variable (V1-V5) and 5 conserved (C1-C5) regions[40]. Gp120 and gp41 are glycosylated in the Golgi apparatus, and then transported to the membrane that is later incorporated in the viral envelope during the budding of the viral particles to form mature viruses[41]. The envelope membrane is studded with trimers of gp120-gp41 heterodimers, where gp41 forms

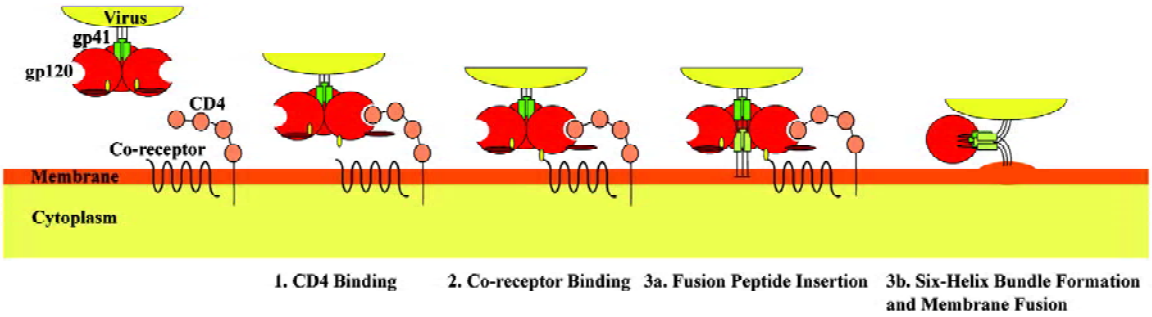

Figure 1. Schematic representation of the mechanism for HIV-1 cell entry.

the cytosolic part and gp120 the extracellular part[42].

Binding of viral gp120 to host cell CD4 is achieved through interactions of several conserved gp120 residues with the second complementarity-determining region (CDR2) of CD4[43,44]. This interaction alone is not sufficient to achieve cell entry, but it is necessary in order to identify the target cell and also to increase the affinity of other viral components for the co-receptor molecules. Indeed, binding of gp120 to CD4 causes conformational changes to the variable loop regions V1/V2 and V3 of gp120, causing the V3 loop to evaginate, thus becoming exposed to the co-receptors[45] (Figure 1). The major co-receptors that HIV-1 uses are the CCR5 and CXCR4 chemokine receptors. The exact mechanism of interaction between the variable loop regions V1/V2 and V3 and the chemokine receptors is not well understood and it merits a more detailed investigation. It has been suggested, however, that the interaction between V3 and CCR5 is ionic in nature, and results in enhancement of the process of activation-induced cell death of responding effector $CD4^+$ T cells during antigen presentation[22,46,47].

The final step for viral entry requires fusion of the viral envelope components with the target surface membrane; this is achieved with the use of gp41, which is a glycoprotein consisting of 3 main domains: an intracellular domain (endodomain), a transmembrane anchor and an extracellular domain (ectodomain). The ectodomain is the key structure responsible for fusion and consists of a hydrophobic fusion peptide sequence at the N-terminal, two hydrophobic heptad repeats (HR1 and HR2) at the C-terminal, and a hinge region, where a disulfide-bond loop is formed between the two heptad repeats during fusion[48,49]. On binding of gp120 to CD4 and subsequently to the co-receptor, further conformational changes occur that lead to gp41 dissociation from gp120. The gp41 unfolds and the hydrophobic fusion peptide sequence extends out of the viral membrane towards the host cell membrane. Insertion of the fusion peptide into the host cell membrane leads gp41 to fold into a hairpin-like structure where the two hydrophobic heptad repeats (HR1 and HR2) lie antiparallel, forming a 6-helix bundle[50,51]. This hairpin structure is believed to be responsible for the fusion of the HIV envelope to the host cell membrane.

Enfuvirtide: the first FDA-approved fusion inhibitor

Enfuvirtide (formerly known as T-20) is the first fusion inhibitor approved by the FDA and the European Commission for the Treatment of AIDS, and is available under the trade name Fuzeon (Trimeris and Roche). It is a 36 amino acid synthetic peptide homologous to the HR2 region of gp41 (residues 127-162) [52,53], that has the ability to interfere with the fusion pathway by mimicking the HR2 domain[54]. The accepted mode of action proposes that enfuvirtide targets conformational changes during fusion by binding to the HR1 domain. Recent evidence indicates that enfuvirtide interacts with multiple sites in gp41 and gp120[55]. This binding prevents the formation of the 6-helix bundle by preventing HR2 from refolding antiparallel to HR1[56,57]. Thus, inhibition of fusion of the viral envelope to cell membranes is achieved by blocking a critical step in the fusion pathway (Figure 2).

In the initial stages of discovery, enfuvirtide appeared to inhibit HIV-1 replication very effectively in various cell types and clinical trials proved to be very promising. Phase I/II trials provided proof that HIV entry was inhibited after treating patients with 100 mg enfuvirtide twice daily for 14 d. The levels of plasma HIV RNA after 14 d of treatment demonstrated a 1.96 lg median decline[58]. Phase II clinical trials were performed on 71 HIV-infected individuals who were treated with 50 mg enfuvirtide together with other ARV drugs for 48 weeks. There was a 1.0 $\log_{10}$ decline from baseline in HIV RNA and a median gain of CD4 cell counts of 84.9 cells/μL, with no significant toxicity[59].

Furthermore, two TORO (T-20 *vs* Optimized Regimens Only) Phase III clinical trials were performed in America (TORO 1) and in Europe and Australia (TORO 2). The trials had similar protocols: they compared the efficacy and safety of enfuvirtide plus an optimized antiretroviral regimen with the efficacy and safety of an optimized antiretroviral regimen alone[60,61]. In both studies the least-squares mean change from baseline in the plasma viral load indicated a significant difference in the decrease in the enfuvirtide group compared with the control ($P<0.01$). In the same way, the mean count of CD4 cells/mL was significantly greater in the enfuvirtide group compared with the controls ($P<0.01$).

Further studies are currently being performed on the exact metabolic pathway of enfuvirtide, potential drug resistance problems, and identification of synergistic interactions with other drugs. Several reports concluded that enfuvirtide does not appear to interfere with the activities of cytochrome P450, probably because it is a peptide and is easily hydrolyzed in the body[62,63]. Enfuvirtide was found to act synergistically with other potential entry inhibitors *in vitro*, such as AMD3100 and PRO542, producing results that encouraged the use of combinations of entry inhibitors as part of a new generation of ARV strategies[64,65]. However, HIV resistance has been reported in patients treated with enfuvirtide, indicating a hotspot from codons 36 to 38 of the HR1 domain[66],

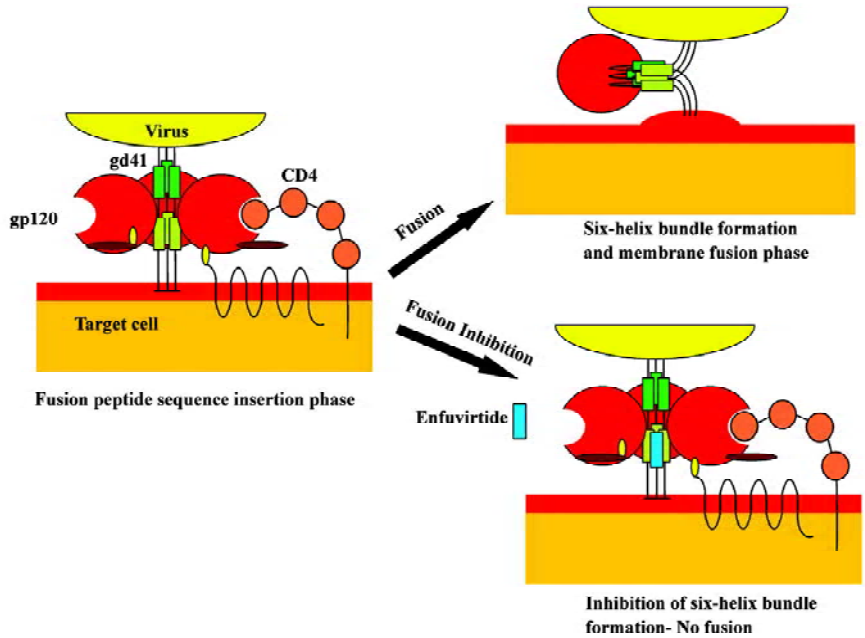

Figure 2. Mechanism of inhibition of HIV fusion to the cell membrane by enfuvirtide.

as well as other sites in gp41[67–69]. Additionally, primary resistance has been reported, which appears to be more frequent than predicted[70], indicating that more research is needed in this field.

Enfuvirtide obtained accelerated approval by the FDA in 2003 and became the 17th licensed ARV drug and the first to inhibit HIV entry. The drug is supplied as a lyophilized powder in single-dose vials containing 108 mg of the drug. Reconstitution of the powder in 1.1 mL sterile water for injection produces a single dose of 90 mg/mL[71] that is injected subcutaneously. Enfuvirtide has two currently known major drawbacks. First, being a peptide, it can only be administered by injection and not orally. This makes usage more difficult, because patients must be educated for self-administration. Second, the cost of enfuvirtide is high, because it is a synthetic peptide that is manufactured by a highly complicated process involving large amounts of raw materials[72,73]. It is estimated that the annual cost of enfuvirtide therapy is approximately US$20 000 per patient, and if taken in combination with other ARV drugs then the cost of therapy could approach US$30 000.

Potential drugs targeting entry and fusion

Attachment inhibitors Current novel antiretroviral drugs aim to interfere with the crucial HIV entry steps: viral attachment, co-receptor binding and fusion. One approach for interfering with viral attachment involves the use of a tetravalent fusion protein construct, consisting of a human IgG2 in which the Fv portions of both the heavy and light chains have been replaced with the D1 and D2 domains of human CD4[74,75]. This CD4-immunoglobulin fusion construct, called PRO 542, is suggested to bind to the viral gp120 and thus prevent the virus from interacting with CD4-bearing host cells. Phase I clinical trials indicated that PRO 542 has a half-life of 3–4 d when a relatively high dose was used (10 mg/kg), and no dose-limiting toxicities were observed[76]. In addition, in phase II clinical trials, 12 HIV-infected patients were treated with 25 mg/kg single-dose PRO 542. The drug was well tolerated and the acute reduction caused in the HIV-1 RNA was statistically significant, even in patients with advanced AIDS[77].

In the same way, several other compounds target either the gp120 or the CD4 receptor and interfere with HIV attachment. FP-21399 is a bis(disulfonapthelene) derivative that binds to gp120, most probably near the third variable domain, because interactions with antibodies against the V3 loop region were blocked[78]. A phase I study showed that it caused an increase in CD4 cell counts, and a significant decrease in viral load and minor side effects[79]. BMS-378806, a 4-methoxy-7-azaindole derivative, is a compound that can be administered orally, and was developed by Bristol Myers Squibb[80,81]. Despite the fact that phase I and II studies showed promising results, Bristol Myers Squibb decided to investigate similar drugs such as BMS-488043, an analogue of BMS-378806, in order to optimize its effectiveness[82]. A series of polyanionic compounds, for example dextrin-2-sulfate, Carraguard and PRO 2000 are in clinical trials, and

are designed to be topically administered[83–85]. Finally, TNX-355, a humanized anti-CD4 mAB that binds to CD4 without interfering with its biological function, significantly decreased viral load and increased CD4 cell counts in a phase I trial[86].

Co-receptor binding inhibitors The most interesting target in HIV entry is the co-receptor binding phase. Current drug research is focused on designing compounds that prevent the virus interacting with the chemokine receptors. The CCR5 receptor is the principal target, and a number of potential drugs are currently being studied. SCH-C is a small molecule that inhibits the binding of gp120 to CCR5, and initial *in vitro* experiments have indicated good inhibitory activity against R5 viruses as well as synergistic effects with several ARV drugs, including enfuvirtide[87,88]. Although it can be administered orally and clinical studies showed decreased viral loads, electrocardiographic anomalies due to arrhythmias were reported at high dosages[89]. Another compound, SCH-D, has been found to have greater *in vitro* and *in vivo* antiviral properties, with no apparent side effects. Clinical studies for this drug are still ongoing[90]. Interestingly, it was recently reported that V3-like peptides from X4 strains with more electropositive V3 domains were effective antagonists and potential infectivity blockers of R5 variants[91].

TAK-779 was the first non-peptidic molecule found to inhibit co-receptor attachment by binding to CCR5 at transmembrane helices 1, 2, 3, and 7[92,93]. It has the disadvantage of intravenous administration and because of irritations observed at the injection site, its development was discontinued. It was replaced by another compound, T-220, which can be administered orally, and shows promising anti-R5 HIV activity[94]. Similarly, UK-427,857 is a novel CCR5 inhibitor that has acceptable pharmacokinetic and metabolic rates in mice, rats, dogs and humans, and can be administered orally[95]. Finally, PRO 140 is one of the few monoclonal antibodies that has been used as an entry inhibitor and has been reported to block co-receptor attachment without down-modulating or inducing signaling of the CCR5 chemokine receptor[96,97].

CXCR4, the second major HIV co-receptor, is also a target for current drug research. AMD-3100, one of the first entry inhibitors, was found to inhibit viral entry well before the discovery of co-receptor usage by HIV[98]. It is a bicyclam compound of low molecular weight that inhibits the electrostatic interaction between CXCR4 and gp120 by ionic binding to the second extracellular loop (ECL2) and the adjacent membrane-spanning domain (TM4) of the CXCR4 receptor[99]. Despite the fact that in phase I and II clinical trials, intravenous administration of AMD-3100 significantly reduced the viral load[100], it was later replaced by an orally available compound, AMD-070. A non-peptidic compound, KRH-1636, which is absorbed through the duodenum, had similar efficacies to AMD-3100[101]. Finally, T-22 and ALX40-4C are positively charged peptides that occupy the V3 region and competitively inhibit binding of gp120 to the negatively charged amino acid residues on CXCR4[102–104].

In conclusion, the role of the V3 region in the mechanism of cell attachment and entry in relation to the major co-receptors is being actively pursued. In addition to biological studies, physicochemical studies on the interacting protein domains are being carried out in an attempt to decipher the interface conformations between the virus and the cell[105].

Fusion inhibitors Understanding the mechanism of fusion of the viral envelope with the host membrane played a crucial role in the development of new generation ARV drugs. This became apparent when enfuvirtide was licensed as the first viral entry inhibitor, and it is currently used in HAART. Resistance to enfuvirtide has been reported, which has led to the design of a second generation HR2 mimetic peptide. T-1249 is a 39-L-amino acid synthetic peptide that contains a pocket-binding sequence that makes the HR1 and HR2 interaction more stable. Studies on T-1249 showed that it has greater efficacy and longer half-life than enfuvirtide. Additionally, efficacy against enfuvirtide-resistant viruses has been reported, indicating that this second generation fusion inhibitor is a step forward[106,107]. However, Roche and Trimeris decided to halt clinical development due to formulation concerns[108].

5-Helix is a newly designed recombinant C-peptide that consists of 5 of the 6 helices that are formed during the fusion phase. A CHR domain is missing for the 6-helix bundle formation, and thus there is one exposed groove. This groove binds to a CHR domain in gp41 and inhibits fusion of the viral membrane to the host membrane[109]. Because it is a recombinant peptide, it has a much lower cost of production compared with the synthetic enfuvirtide. Initial studies demonstrated potent antiretroviral activity, with IC_{50} values in the low nanomolar range[110].

Finally, N-peptides represent another group of peptides with potential inhibitory effects against HIV entry. Initial studies have indicated that they are weaker inhibitors than the C-peptides, with IC_{50} values in the micromolar range. However, chimeric molecules composed of soluble trimeric coiled coils have shown promising results. IQN17 is one of the first such peptides with potent inhibitory effects, and the current most potent chimeric N-peptide, IQN23, is reported to have an IC_{50} value of 15 nmol/L[111].

Conclusion

Antiretroviral chemotherapy has recently acquired a new "weapon" in the fight against AIDS. Enfuvirtide is the first HIV entry inhibitor that was approved by FDA, and it is currently used in combination with other ARV drugs. Results from clinical trials indicated that it had potent activity against HIV strains that are resistant to other ARV drugs, although some resistance to enfuvirtide has been reported. The design of other entry inhibitors has moved forward, and every phase of HIV entry is actively pursued as a target for potential inhibitors. Probably the most exciting prospect is potential interference with co-receptor usage, particularly that of CCR5.

ARV drug development aims to produce drugs with potent antiretroviral activity, with IC_{50} values in the nanomolar range, with no or limited toxicity and that can be administered orally. Several compounds are currently in clinical trials, and we are optimistic that new, more effective drugs will be added to the ARV armory.

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