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Review
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Role of CXCR4 in HIV infection and its potential as a therapeutic target

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The chemokine receptors CCR5 and CXCR4 are the two major coreceptors for HIV entry. Numerous efforts have been made to develop a new class of anti-HIV agents that target these coreceptors as an additional or alternative therapy to standard HAART. Among the CCR5 inhibitors developed so far, maraviroc is the first drug that has been approved by the US FDA for treating patients with R5 HIV-1. Although many CXCR4 inhibitors, some of which are highly active and orally bioavailable, have also been studied, they are still at preclinical stages or have been suspended during development. Importantly, the interaction between CXCR4 and its ligand SDF-1 is involved in various disease conditions, such as cancer cell metastasis, leukemia cell proliferation, rheumatoid arthritis and pulmonary fibrosis. Therefore, CXCR4 inhibitors have potential as novel therapeutics for the treatment of these diseases as well as HIV infection.

Approximately 34 million people are currently living with HIV, and 2 million people died due to AIDS or AIDS-related diseases in 2008 [201]. After the introduction of HAART in 1996, which combines HIV-1 reverse transcriptase and protease inhibitors, the morbidity and mortality associated with HIV-1 infection decreased dramatically [1,2] due to sustained reductions in HIV-1 plasma levels and significant increases in the number of $CD4^+$ T cells [3–5]. However, there are still several remaining problems associated with HAART that need to be overcome, such as the emergence of drug-resistant mutants and drug-related side effects. One approach to overcome these issues is to develop new reverse transcriptase or protease inhibitors that are effective against known drug-resistant mutations. Indeed, a new protease inhibitor, darunavir (TMC114), has been approved by the US FDA for the treatment of HIV/AIDS patients harboring multidrug-resistant HIV-1 variants that do not respond to existing HAART regimens [6]. In addition, etravirine (TMC125), a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI), has been recently approved by the FDA for the treatment of HIV infection in adults when other antiretroviral drugs have failed. Multiple mutations are required for HIV-1 to become resistant to etravirine in comparison to first-generation NNRTIs [7]. An alternative approach is to discover new anti-HIV drugs that are directed against a novel target and have a unique mechanism of action. In 2003, the FDA approved enfuvirtide, previously known as T-20, which targets HIV-1 gp41. Enfuvirtide is classified as a fusion inhibitor, which blocks fusion between the HIV-1 envelope and the target cell membrane. Although the drug must be administered parenterally, it is also effective against a multidrug-resistant HIV-1 strain [8,9]. In addition, the FDA approved the integrase inhibitor raltegravir in 2007 for use in highly treatment-experienced patients [10]. In addition, other anti-HIV-1 drug candidates have been explored as well, such as the maturation inhibitor bevirimat (PA-457) [11].

After the chemokine receptors CCR5 and CXCR4 were found to be the major HIV-1 coreceptors together with the primary receptor CD4, numerous efforts were made to test whether chemokines, chemokine derivatives, or small-molecule inhibitors against chemokine receptors had the potential to be a new class of anti-HIV-1 agent. In particular, many receptor antagonists against CCR5 have been developed as anti-HIV-1 drugs. Among them, maraviroc was approved by the FDA in 2007 for the treatment of R5 HIV-1 in treatment-experienced adult patients and is used in combination with other antiretroviral treatments. Several classes of CXCR4 antagonists have also been developed, although all have yet to reach clinical testing. However, other clinical applications for CXCR4 inhibitors in addition to anti-HIV therapy have been considered, as the interaction between CXCR4 and its ligand SDF-1 is also involved in several other diseases, such as

Keywords

▪ anti-HIV therapy ▪ cancer metastasis ▪ CCR5 ▪ chemokine receptor ▪ CXCR4 ▪ CXCR4 antagonist ▪ hematopoietic stem cell mobilizer ▪ HIV infection ▪ rheumatoid arthritis

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cancer metastasis, rheumatoid arthritis (RA) and pulmonary fibrosis [12]. In addition, recent research on bicyclam AMD3100, a well-known CXCR4 antagonist, has revealed that it specifically increases CD34+ hematopoietic stem cell numbers in peripheral blood. A Phase III trial for AMD3100 as a stem cell mobilizer has been successfully completed [13]. In this review, we will highlight the role of CXCR4 in HIV infection and introduce the history and present status of various CXCR4 inhibitors. In addition, we will also describe the potential of CXCR4 as a therapeutic target in diseases other than HIV infection.

Identification of chemokine receptors as coreceptors for HIV-1 entry

Soon after the discovery that HIV-1 caused AIDS, CD4 was identified as the receptor for HIV-1 entry into target cells. However, it was recognized that a molecule was required in addition to CD4 to fully enable virus entry. One of the central observations that prompted this theory was the fact that human CD4 acts as a receptor only when expressed in human cells. It was then discovered that some chemokines, such as MIP-1α, MIP-1β and RANTES, inhibit HIV-1 infection, suggesting that the chemokines receptors may function as a coreceptor for HIV-1 entry [14]. Indeed, β-chemokine receptor CCR5, a member of the G protein-coupled receptor superfamily of seven-transmembrane domain proteins, was soon identified as the major coreceptor for M-tropic HIV-1 isolates, which can efficiently replicate in human macrophages and primary CD4+ T cells [15–18]. Shortly after the discovery of CCR5, the α-chemokine receptor CXCR4 was reported as the main coreceptor for T-tropic HIV-1 isolates, which can efficiently replicate in T-cell lines and primary CD4+ T cells [19]. Although the current consensus is that both CCR5 and CXCR4 are the major coreceptors for HIV-1 infection *in vivo*, additional chemokine receptors, including orphan receptors and others (e.g., CCR2b, CCR3, CCR8, CCR9, CXCR6 and CX_3CR1 [20]), have been reported to act as alternative coreceptors for CD4 when they are overexpressed.

The present model for HIV-1 fusion/entry is outlined in Figure 1 and is as follows:

- Binding of the gp120 trimer to CD4 induces conformational change(s) in gp120 that result in the exposure of high-affinity coreceptor binding sites;
- The interaction of gp120 with coreceptors then causes further conformational change(s) in gp120/gp41 that lead to exposure of the gp41 ectodomain;
- Extension of the gp41 fusion peptide causes a membrane fusion reaction between the viral membrane and the target cell membrane through formation of a six-helix bundle.

Determinants of HIV cell tropism & chemokine receptor usage

HIV-1 gp120 consists of five variable regions (V1–V5) and five constant regions (C1–C5). Cumulative evidence demonstrates that the V3 loop is a primary determinant of virus cell tropism [21–27] and coreceptor usage [16,28–31]. In addition, the V1/V2 region is also thought to regulate the efficiency of coreceptor-mediated HIV-1 entry [30,32]. Furthermore, conserved gp120/coreceptor binding sites have been proposed to exist mainly based on the fact that Env proteins from HIV-1, HIV-2 and SIV can interact with the same chemokine receptors (i.e., CXCR4 and CCR5).

Impact of nonfunctional chemokine receptor alleles on HIV resistance & disease progression

CCR5 δ32 is a mutant allele of CCR5 that is prevalent in European populations [33]. The gene product is nonfunctionally truncated such that the protein is unable to transport to the cell surface. Homozygotes for the δ32 allele exhibit a strong resistance to HIV infection, whereas heterozygotes show delayed progression to AIDS. Many other alleles are thought to affect the primary structure of CCR5 or its promoter, and some lead to nonfunctional receptors or otherwise influence AIDS progression [34,35]. For example, the CCR5 promoter polymorphism CCR5 59029G may affect viral transmission and disease progression due to its effect on the expression of CCR5. A single-nucleotide polymorphism (SNP) in the *CCR2b* gene has been identified as well. The presence of either one or two copies of this mutation, termed CCR-2b 64I, has been associated with delayed progression to AIDS and death in several cohorts [36]. Genome/polymorphism analysis of CXCR4 has so far failed to show a case equivalent to that of the δ32 allele. However, since SDF-1 interferes with X4-tropic HIV-1 infection, SDF-1 could be a genetic trait that might affect progression to AIDS. Indeed, a SNP in the 3′ untranslated region of SDF-1 was reported to be epidemiologically protective against HIV

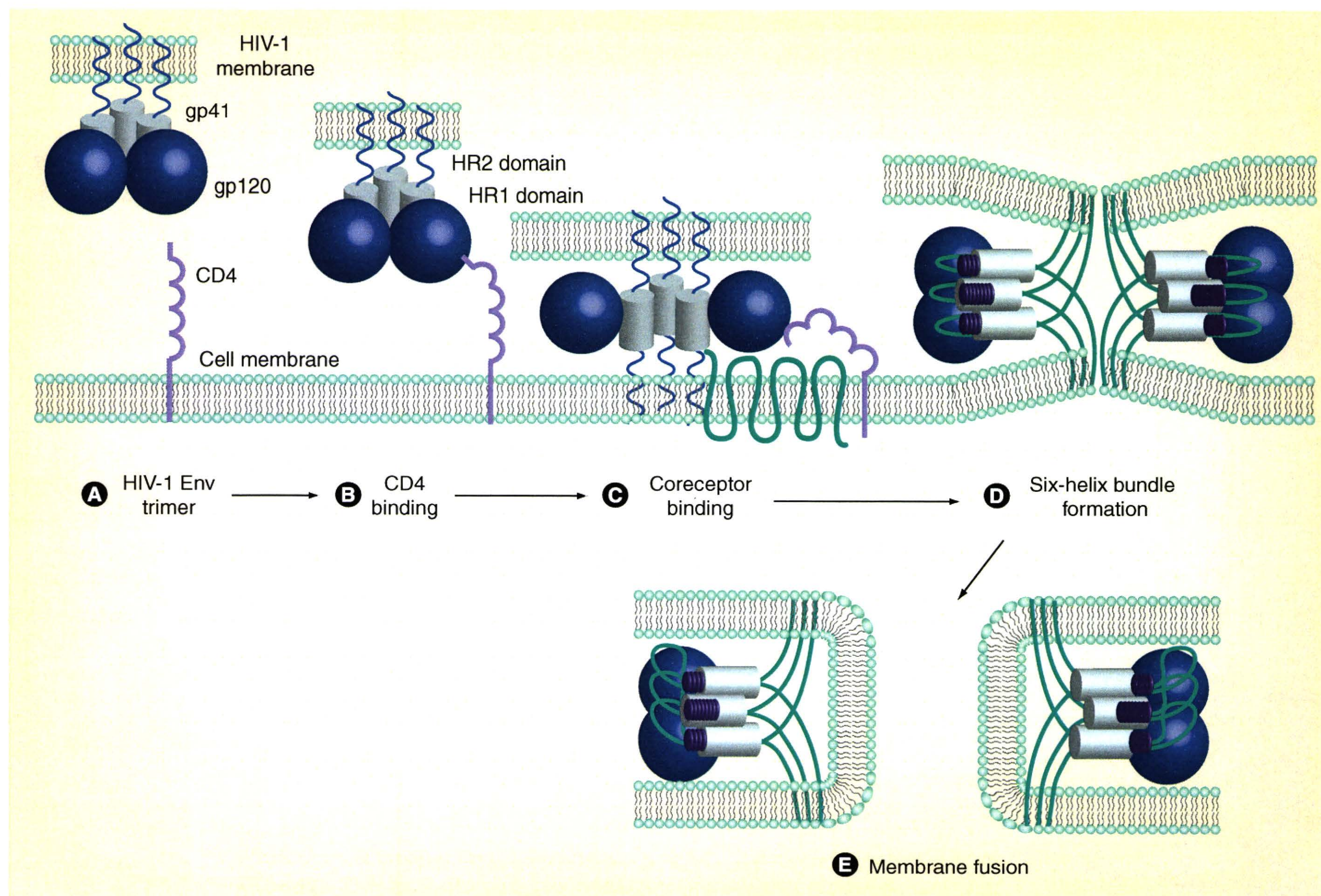

Figure 1. Proposed mechanism of HIV entry. The HIV Env protein is a homotrimeric protein that consists of surface gp120 and transmembrane gp41 proteins **(A)**. First, gp120 binds to the receptor CD4, and CD4 binding induces conformational changes in gp120 that lead to the exposure of a conserved gp120 domain and the gp41 ectodomain, which are important for coreceptor binding **(B)**. After binding to CD4, gp120 binds to a coreceptor, such as CXCR4 or CCR5, which is a member of the G-protein-coupled receptor superfamily of seven-transmembrane domain proteins **(C)**. Coreceptor binding can be blocked by many inhibitors that have been developed against CXCR4 and CCR5. Coreceptor binding causes further conformational changes in HIV Env that allow extension of the gp41 fusion peptide and generation of a six-bundle formation **(D)** following membrane fusion between the viral and target cell membrane **(E)**.

infection and progression [37]. In contrast to CCR5 δ32, this mutation is rather common in all geographical regions of the world. It has been suggested that this mutation may increase SDF-1 levels, thus competing with X4 HIV binding to cells. Those who are homozygous for this mutation demonstrate slow progression to AIDS without exhibiting decreased susceptibility to HIV infection [37]. Mutations in the *CXCR4* gene are generally rare and have not been implicated in HIV-1/AIDS pathogenesis.

Coreceptor-targeted anti-HIV therapy

Along with CCR5, CXCR4 is a major coreceptor in HIV infection. R5 HIV-1 is isolated predominantly during the acute and asymptomatic stages [38], whereas X4 HIV-1 strains emerge in approximately 50% of infected individuals, and their emergence is associated with rapid $CD4^+$ T cell loss and disease progression [39,40]. CXCR4, therefore, is a novel and attractive target for the development of new anti-HIV drugs. Significant efforts have been made to explore and identify small-molecule compounds that interfere with the interaction between gp120 and CXCR4. The structures of some of the well-studied CXCR4 inhibitors discussed below are shown in Table 1 & Figure 2.

AMD3100 & AMD070

Bicyclam AMD3100 (Table 1 & Figure 2) is a small molecule inhibitor that strongly restricts X4 HIV-1 infection [41]. The compound exhibits no antiviral activity against CCR5-utilizing HIV-1 strains. Correlation was observed between the inhibitory activity of AMD3100 on X4 HIV-1 replication, CXCR4 mAb binding and SDF-1α-induced signal transduction. Thus, AMD3100 is a specific antagonist of CXCR4. AMD3100 has proven effective not only in a severe combined

Table 1. CXCR4 and CCR5 inhibitors tested in HIV and other applications				
Compound	**Company**	**Stage of development**	**Disease**	**Note**
CXCR4 inhibitors				
ALX40-4C	NPS Allelix	Terminated (Phase I/II)	HIV	No apparent effect was observed on viral load
AMD3100	AnorMED	Terminated (Phase I/II)	HIV	Little effect was observed on viral load
AMD3100 (plerixafor)	Genzyme	Approved by US FDA	Stem cell mobilizer	Use in combination with G-CSF
AMD070	Genzyme	Suspended (Phase I/II)	HIV	A derivative of AMD3100 that can be orally administered. Liver histology changes were observed in long-term preclinical toxicity experiments.
T140	Kyoto University	Preclinical	HIV, cancer metastasis, leukemia, rheumatoid arthritis	A downsized analog of T22 peptide that specifically inhibits CXCR4
KRH-3955	Kureha	Preclinical	HIV, cancer metastasis	A highly potent, orally bioavailable CXCR4 antagonist
CCR5 inhibitors				
TAK-652 (TBR-652)	Takeda/Tobira	Phase II	HIV	A potent, orally bioavailable CCR5 antagonist
Aplaviroc	Ono	Terminated (Phase II/III)	HIV	Aplaviroc's development was stopped because of hepatotoxicity
Maraviroc	Pfizer	Approved by US FDA	HIV	The first FDA-approved CCR5 antagonist
PF-232798	Pfizer	Phase II	HIV	A second-generarion Pfizer oral CCR5 antagonist
Vicriviroc	Schering-Plough/Merck	Terminated (Phase III)	HIV	Vicriviroc did not meet the primary efficacy endpoint
INCB9471	Incyte	Phase II	HIV	A new class of oral CCR5 antagonist

G-CSF: Granulocyte colony-stimulating factor.

immunodeficiency (SCID)-hu/Thy/Liv mouse model [42] but also in a proof-of-concept study on a patient infected with X4 HIV-1. Development of the compound as an anti-HIV drug was suspended primarily due to its cardiotoxicity [43]. An effort to overcome the poor oral bioavailability and side effects of AMD3100 led to the generation of AMD070 (Table 1 & Figure 2), which has tetrahydroquinolineamine as its pharmacophore and which is a potent and specific X4 HIV-1 inhibitor with high oral bioavailability. Although AMD070 also showed potential activity against X4 HIV-1 in a clinical Phase Ib/IIa proof-of-concept study, the FDA halted development of this compound due to liver histology changes in long-term preclinical toxicity studies [44]. Further studies are needed to determine *in vivo* toxicity of AMD3100 and AMD070, is due to blocking CXCR4 functions.

T22, T134 & T140

Self-defense peptides isolated from the hemocytes of the Japanese and American horseshoe crab tachyplesins and polyphemusins have antibacterial and antiviral activities [45–47]. Several years prior to the discovery of the HIV-1 coreceptors, Yamamoto and colleagues were able to show that T22 ([Tyr5, 12, Lys7]-polyphemusin) (Table 1 & Figure 2), a synthetic polyphemusin analog with 18 amino acid residues and two disulfide bonds, inhibits HIV-1 replication [47,48]. The two disulfide bonds form an antiparallel β-sheet structure, which is important for the antiviral activity of the peptide. The peptide inhibits replication of T-tropic but not M-tropic HIV-1. The determinant of this specific antiviral activity was mapped to the V3 region of the HIV-1 Env protein [49], and the 50% effective concentration (EC_{50}) was 290 nM in an anti-HIV assay using MT-4 cells. Subsequent to the discovery of the CXCR4 and CCR5 coreceptors, we demonstrated that the T22 peptide specifically blocks virus–cell and cell–cell infection mediated through HIV-1 Env interaction with CXCR4 and CD4 [50], as also reported in other CXCR4 inhibitors such as ALX40-4C and AMD3100 [41,51]. It was also found that T22 suppresses Ca^{2+} mobilization induced by SDF-1α. Thus, T22 is a small CXCR4 antagonist that inhibits X4 HIV-1 infection via specific binding to the CXCR4 molecule.

Through an effort to downsize T22 by structure–activity relationship studies, it was found that T134 [52] and T140 [53] (Table 1 & Figure 2),

14-mer peptides with a single disulfide bond, had stronger anti-HIV-1 activity than T22. Although T140 lacks four amino acids and one disulfide bond, it maintains an antiparallel β-sheet structure. Through an Ala-substitution scanning study, it was demonstrated that Arg2, Nal3, Tyr5 and Arg14 form the pharmacophore of T140, which is useful information for the rational design of peptide derivatives with higher anti-HIV activity [54]. Downsizing T140 resulted in the development of a cyclic pentapeptide, FC131, which has strong CXCR4-antagonistic activity and could serve as a template for further modification [55]. Systemic toxicity of the T compounds was evaluated with TN14003, a derivative of T140 [56]. CB-17 SCID mice were injected with TN14003 at 100 ng/g body weight twice weekly for 45 days. Although no damage in the liver and kidney was observed by hematoxylin and eosin staining, further careful *in vivo* study is required for assessing the safety of the T compounds.

KRH-1636 & KRH-3955

In an attempt to discover a novel, small non-peptide CXCR4 antagonist Yamamoto and colleagues screened more than 1000 compounds from the Kureha Corporation (Tokyo, Japan) chemical library. This screen led to the identification of KRH-1636 (Table 1 & Figure 2), which strongly and specifically inhibits X4 HIV-1 replication, both *in vitro* and *in vivo* [57]. KRH-1636 blocks the replication of various X4 HIV-1 strains in a nanomolar range and has low cytotoxicity (CC_{50}: 400 µM in MT-4 cells). KRH-1636 also strongly inhibits both SDF-1α binding to CXCR4 and CXCR4-mediated Ca^{2+} signaling and blocks binding of monoclonal antibodies to CXCR4 without down-modulating the coreceptor. Importantly, KRH-1636 also inhibits X4 HIV-1 replication in a human peripheral blood lymphocyte (PBL)/ SCID mouse model. Furthermore, KRH-1636 was absorbed into the blood after intraduodenal administration in rats. KRH-1636 did not show severe toxicity in rats that received the compound (1.5 mg/kg per day) by intravenous administration for 4 days [57].

Vigorous efforts to search for more potent and orally bioavailable CXCR4 antagonists were undertaken through a combination of chemical modification of KRH-1636 and biological assays, leading to the identification of KRH-3955 (Table 1 & Figure 2) [58]. KRH-3955 very potently inhibits replication of X4 HIV-1 in activated peripheral blood mononuclear cells (PBMCs) from different

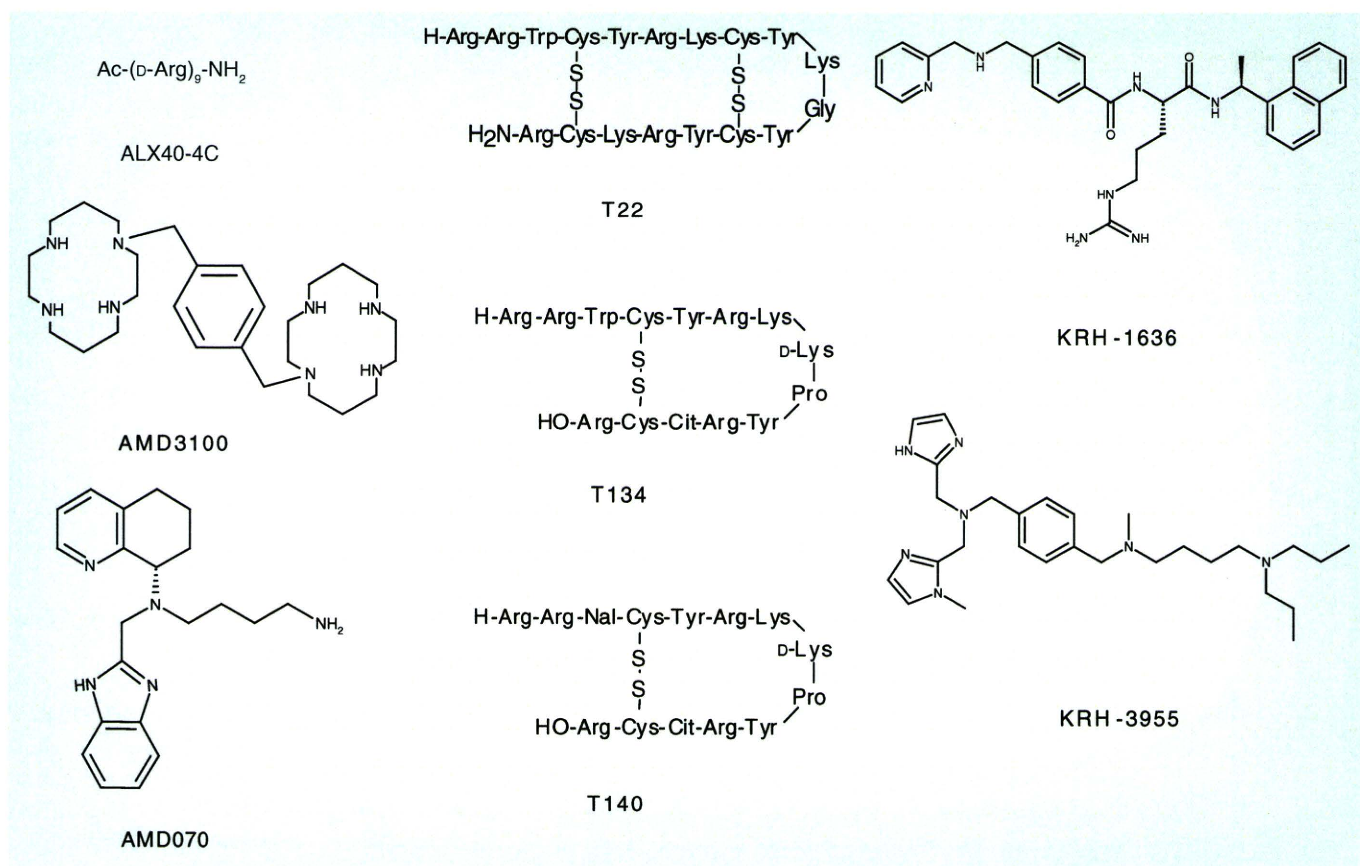

Figure 2. CXCR4 inhibitors.

donors and effectively restricts clinical HIV-1 isolates. The EC_{50} of KRH-3955 is approximately 1 nM, and it also blocks replication of recombinant X4 HIV-1 containing resistance mutations in reverse transcriptase and protease, as well as isolates with T-20-resistant mutations in the envelope protein. KRH-3955 inhibits both SDF-1α binding to CXCR4 and Ca^{2+} signaling through the coreceptor. Moreover, KRH-3955 does not induce CXCR4 internalization but inhibits the binding of anti-CXCR4 monoclonal antibodies that recognize the second or third extracellular loops of the receptor. The compound shows an oral bioavailability of 26% in rats and oral administration blocks X4 HIV-1 replication in the human PBL/SCID model. Thus, KRH-3955 is a strong CXCR4 antagonist and seems to be a new promising therapeutic agent against HIV-1 infection and AIDS although further studies are definitely needed on *in vivo* safety of the compound.

Other CXCR4 inhibitors

In addition to the compounds above, there are several other CXCR4 inhibitors. These include: ALX40-4C (Table 1 & Figure 2) [51,59] and Arg-mimetic conjugates [60,61], POL3026 (β-hairpin mimetic) [62,63] and CGP64222 [64]. ALX40–4C, a polypeptide of nine D-Arg residues stabilized by terminal protection, inhibits X4 HIV-1 infection as well as R5X4 (dual-tropic) HIV-1 infection in the context of CXCR4 use [51]. The peptide also blocks binding of SDF-1α and the anti-CXCR4 monoclonal antibody 12G5 to CXCR4. Although no significant reduction in viral load was observed, ALX40–4C was well tolerated by 39 out of 40 HIV-infected individuals for a 1-month treatment period in Phase I/II clinical trials [59]. The β-hairpin motif in the polyphemusin and T22, which are described in the previous section, was used to design a β-hairpin mimetic POL3026. The cyclic peptide specifically inhibits X4 HIV-1 infection [62]. Importantly, POL3026 showed excellent stability in human plasma and favorable pharmacokinetics when administered subcutaneously in dogs [63].

Molecular interactions between CXCR4 inhibitors & the CXCR4 receptor

The molecular interactions between CXCR4 inhibitors or antagonists with the CXCR4 receptor have been examined primarily using three methods. The first method involves examining the effect of CXCR4 inhibitors on the binding of several anti-CXCR4 monoclonal antibodies that recognize various regions of the receptor. In the second, the effect of mutations in CXCR4 on the inhibitory activity of CXCR4 antagonists is investigated together with binding of certain anti-CXCR4 monoclonal antibodies, such as 12G5, or the ligand SDF-1α to the receptor. Thirdly, radio or photo-labeled inhibitors are used to identify the binding sites of inhibitors directly.

Using various CXCR4 mutants, the determinants of AMD3100 sensitivity were identified as four Asp residues at positions 171, 182, 193 and 262, as well as Glu288, indicating the importance of the second extracellular loop (ECL)2 and ECL3 and their connected transmembrane domains (Figure 3) [65–68]. Reduced binding of radiolabeled T140 was observed when CXCR4 was mutated at Asp171, Arg188, Tyr190, Gly207 and Asp262, again suggesting the contribution of ECL2 to inhibitor binding [69]. Furthermore, T140 photolabeling recently revealed that the peptide actually interacts with the fourth transmembrane domain of CXCR4 [70]. To determine the binding site(s) of KRH-3955, its effect on the binding of four different anti-CXCR4 monoclonal antibodies was examined, and the data obtained suggested that KRH-3955 binds a region composed of all three CXCR4 ECLs. Further studies were performed using various CXCR4 point mutants to narrow down the binding site(s) of KRH-3955. While AMD3100 lost its activity when Asp171, Asp262 or Glu288/Leu290 was mutated, unexpectedly, His281 was the only residue whose mutation affected the inhibitory activity of KRH-3955 (Figure 2) [58]. Together, these data indicate that several acidic amino acid residues in CXCR4, such as Asp171 and Asp262, are crucial for the binding of most of the CXCR4 inhibitors.

CCR5-targeted anti-HIV therapy

Initially, natural CCR5 ligands, such as MIP-1α and RANTES, or their protein (or peptide)-based inhibitors, were studied as potential CCR5 inhibitors. However, these molecules were not developed further as HIV therapeutics due to their poor pharmacokinetics and bioavailability. Several small molecule CCR5 antagonists have been reported to date (Table 1): TAK-779 [71], TAK-652 (TBR-652) [72] and TAK-220 [73] (Takeda; Osaka, Japan), AK602 (presently termed aplaviroc; Ono; Osaka, Japan) [74], SCH-C [75] and SCH-D (renamed vicriviroc) [76] (Schering-Plough/Merck), UK-427,857 (presently termed maraviroc) [77], PF-232798 [78] (Pfizer; Sandwich, UK) and INCB9471 (Incyte, DE, USA) [79]. Among them, the FDA approved maraviroc in 2007, for

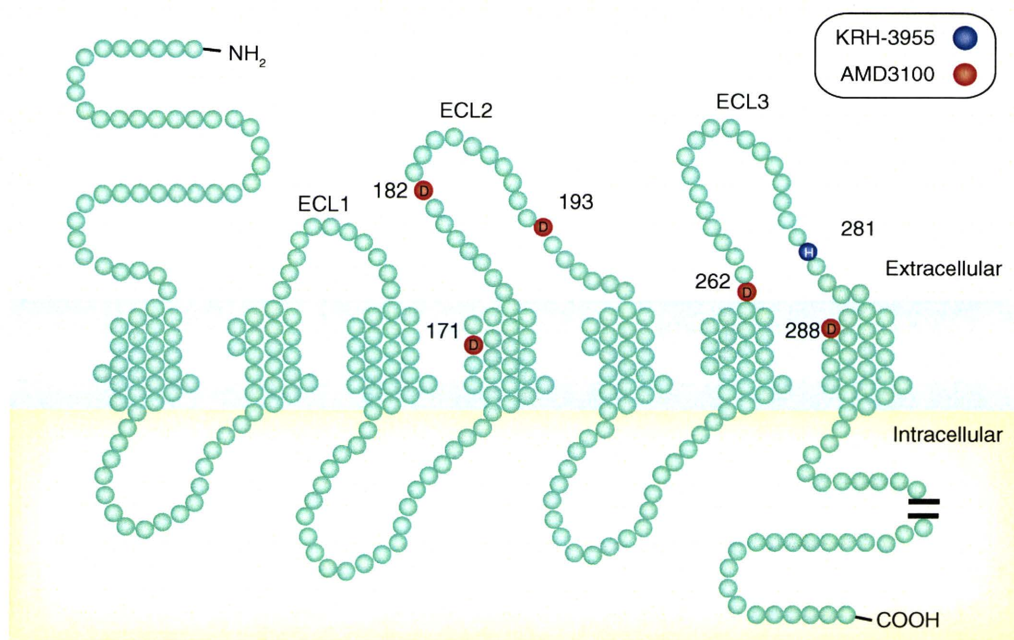

Figure 3. Serpentine diagram of the CXCR4 receptor. White letters in blue circles and black letters in red circles represent amino acid residue substitutions that severely reduce the inhibitory activities of AMD3100 and KRH-3955, respectively.
ECL: Extracellular loop.

treatment-experienced adult patients, in combination with other antiretroviral drugs. Vicriviroc is being terminated in Phase III clinical trials.

Resistance to coreceptor inhibitors

Theoretically, HIV-1 acquires resistance to coreceptor inhibitors, including coreceptor ligands or anti-coreceptor antibodies, either by changing the way it uses coreceptors or switching coreceptor usage, such as shifting from CCR5 to CXCR4. Experiments to select viruses resistant to coreceptor inhibitors have been mainly performed in two ways. In one approach, a selection experiment is performed using cell lines that express only CXCR4 or CCR5 [80–84]. In this case, resistant viruses did not show coreceptor switching, but rather started to use the same coreceptor in a drug-bound form. Many mutations in resistant HIV-1 were found in the gp120 region of HIV-1 Env, especially in the V2 and V3 regions. It is generally considered that it is much more difficult for the virus to gain resistance to coreceptor inhibitors than to HIV-1 inhibitors that target viral proteins such as reverse transcriptase. Indeed, 145 passages over 1.5 years were required to obtain T134-resistant NL4–3, which shows modest resistance to the T134 peptide (15-fold increase) [84]. The T134-resistant HIV-1 is also resistant to AMD3100 as well (15-fold increase). Interestingly, effective concentration of T134 against AMD3100-resistant HIV-1 is almost the same as that against the wild-type strain. In the second resistant virus generation strategy, selection experiments are performed using a cell that expresses both CXCR4 and CCR5, such as human PBMC or PBL [85–95]. Sequence analysis of resistant variants revealed different patterns of amino acid changes in the envelope regions. In some cases, resistant mutations were mainly located in the V3 region of the HIV-1 envelope. Amino acid changes also occurred throughout gp160 in the absence of changes in the V3 loop [89]. Furthermore, detailed analysis revealed that three physically proximal mutations in the gp41 fusion peptide region are responsible for viral resistance to vicriviroc [93]. It is of note that most HIV-1 variants with resistance to CCR5 inhibitors remain CCR5-tropic, although emergence of CXCR4-using variants from SF162 was also reported in cell culture passage in the presence of maraviroc [94].

Another important issue in viral drug resistance is whether resistance mutations affect virus fitness and/or sensitivity to neutralizing antibodies. Reduced fitness was reported for HIV-1 isolates resistant to CXCR4 inhibitors such as AMD3100 [96]. On the other hand, resistance to the CCR5 inhibitor AD101 (SCH-30851, a precursor of vicriviroc) is not associated with a fitness loss [87]. Escape mutants from two

different CCR5 inhibitors, AD101 and vicriviroc, were examined for their sensitivity to well-known neutralizing monoclonal antibodies, such as b12, 2G12, 2F5 and 4E10, as well as to sera from HIV-1-infected individuals [92]. The rationale for this study is that at least some of the escape mutants against CCR5 inhibitors change amino acids in their envelope region(s), which can be targets for neutralizing antibodies. Interestingly, the escape mutants were more sensitive than the parental isolates to a subset of the neutralizing antibodies and some sera from HIV-1-infected individuals, indicating that the humoral immune response could exert selection pressure *in vivo*. Further studies will be required to more completely examine how drug resistance alters virus fitness and/or susceptibility to the immune system.

Several studies have been reported on clinical resistance to CCR5 inhibitors. Coreceptor tropism was examined of 64 HIV-1-infected patients who were given short-term monotherapy of maraviroc. Phylogenic analysis of CXCR4-using variants selected only in two patients revealed that those variants were most likely derived from a CXCR4-using reservoir [97]. Clinical resistance to vicriviroc was examined using both subtype C and subtype D of HIV-1. In the case of an HIV-1 subtype C-infected subject, amino acid changes within the V3 loop were sufficient to confer vicriviroc resistance [98]. On the other hand, amino acid changes in the V3 loop as well as the C4 domain are fully responsible for vicriviroc resistance in HIV-1 subtype D [99].

Other applications of CXCR4 inhibitors

Anticancer

CXCR4 is expressed constitutively in a wide variety of normal tissues, including lymphoid tissues, thymus, brain, spleen, stomach and the small intestine [100]. The SDF-1/CXCR4 interaction is critical for normal lymphocyte trafficking and homing and retention of hematopoietic stem cells within the bone marrow, and is essential in fetal hematopoiesis [101]. Interaction of SDF-1 with CXCR4 activates effector molecules that regulate cell survival, proliferation, chemotaxis, migration and adhesion. Growing evidence suggests that a number of chemokine receptors, most notably CXCR4, are also deeply involved in cancer pathogenesis, such as cancer growth, metastasis and angiogenesis [102]. Abundant CXCR4 expression is reported in tumor cells in more than 23 various types of human cancers of epithelial, mesenchymal and hematopoietic origin [103]. This includes tumors such as breast cancer, ovarian cancer, hepatocellular carcinoma and hematologic malignancies, as well as a number of other malignancies, including lung, brain and prostate cancer.

Cancer metastasis is the result of several sequential steps and represents a highly organized, nonrandom and organ-selective process [104]. Activation of CXCR4 not only results in cytoskeletal changes leading to cell migration, but also induces the production of matrix metalloproteinases at the primary tumor site [105]. This is important for detachment of the tumor cells and their migration through the extracellular matrix into the circulation. Cancers can also have diverse metastatic patterns involving the regional lymph nodes, bone marrow, lung and liver. Muller *et al.* were the first to show that CXCR4 is highly expressed in human breast cancer cells, malignant breast tumors and metastatic tumors, and that in mice CXCR4-expressing breast cancer cells aggressively metastasized to secondary organs where SDF-1 expression is significantly higher [106]. Blocking SDF-1/CXCR4 interaction with a neutralizing antibody significantly inhibited the metastasis of breast cancer cells to distant organs [106]. Other studies have shown that a CXCR4 antagonist peptide (CTCE-9908) reduces metastasis consistently in many different murine cancer models [107,108]. Similar *in vivo* observations have been made with CXCR4 blocking small molecules, such as AMD3100 (ovarian carcinoma) [109], peptides (melanoma) [110], TN14003 (head and neck cancer) [111], antibodies (prostate cancer) [112] and a siRNA (breast cancer) [113]. Indeed, AMD3100 (Genzyme Corporation; MA, USA) is currently being tested for hematologic malignancies in a Phase I/II trial in combination with other treatments such as chemotherapy. To define the spontaneous metastasis of breast cancer cells, we also conducted histological examinations using various organs of NOD/SCID/γ(null) (NOG) mice inoculated with CXCR4-low-expressing MCF-7 and CXCR4-high-expressing MDA-231 cell lines [102]. Results from this study showed that NOG mice inoculated with CXCR4-low-expressing MCF-7 cells barely exhibited organ metastasis, whereas spontaneous metastasis of tumor cells was found in the lungs of NOG mice inoculated with CXCR4-high-expressing MDA-231 cells. These results suggest that CXCR4-high-expressing tumor cells could spontaneously metastasize to secondary organs.

We also examined the effect of KRH-1636 and its derivatives on SDF-1α-mediated chemotaxis of cancer cells to address whether these molecules could be applied to abrogate cancer metastasis [114]. We found that two derivatives, KRH-2731 and KRH-3955, potently antagonize SDF-1α-mediated chemotaxis, exhibiting an EC_{50} of less than 10 nM, which was more than a 1000-fold improvement in efficacy over the prototype KRH-1636. As far as we analyzed the KRH compounds tested, there was a correlation of inhibition among SDF-1α binding, SDF-1α-mediated chemotaxis and HIV-1 infection [58,114]. Therefore, the KRH-1636 derivatives KRH-2731 and KRH-3955 may be promising as novel anticancer metastasis drugs in addition to potential HIV-1 therapeutics.

Although less well studied compared with metastasis, the effects of CXCR4 on survival, proliferation, angiogenesis and vasculogenesis of cancer cells are also important. SDF-1 produced by stromal cells may induce a survival or antiapoptotic signal in tumors that reduces their susceptibility to current treatments, such as chemotherapy [108]. It has been reported that in ovarian, glioma, small-cell lung, renal and thyroid cancers, SDF-1 can stimulate the proliferation and/or survival of CXCR4-expressing cancer cells when they are grown under suboptimal conditions, such as low serum concentrations [102,115]. To define the role of SDF-1/CXCR4 interaction in breast cancer cell proliferation and apoptosis, we cultured the breast cancer cell line MDA-321 in serum-depleted media. MDA-321 cells depend on serum for maximal growth in culture. A total of 24 h after serum withdrawal, MDA-321 cells exhibited a significant decrease in cell number and underwent apoptosis. However, when serum was withdrawn in the presence of SDF-1, there was almost no decline of cell number and no apoptotic cells were observed. Two breast cancer cell lines (CXCR4-low-expressing MCF-7 and CXCR4-high-expressing MDA-231 cells) were inoculated subcutaneously in the postauricular region of NOG (NOD/SCID/gc null) mice. CXCR4-low-expressing MCF-7 cells formed a small tumor at the inoculation site in NOG mice after 8–9 weeks. By contrast, CXCR4-high-expressing MDA-231 cells produced a large tumor within 2–3 weeks in NOG mice [Dewan *et al.*, Unpublished Data]. These data suggest that SDF-1/CXCR4 interaction might play an important role in breast cancer cell proliferation and prevention of apoptosis and in primary tumor growth in SCID mice at the inoculation site. The effects of CXCR4 on cancer growth can be modulated, as inhibition of CXCR4 with the CXCR4 antagonist AMD3100 reduced proliferation and increased apoptosis of human brain cancer cells [116]. In this study, systemic administration of AMD3100 inhibited the growth of intracranial glioblastoma and medulloblastoma xenografts and increased tumor cell apoptosis within 24 h [116]. Thus, CXCR4 signaling may promote cancer through a wide range of mechanisms, including proliferation and survival of cancer cells, angiogenesis and chemoinvasion of cells at primary and metastasis sites.

Antiarthritis & allergy

In addition to HIV infection, cancer cell metastasis and leukemia cell proliferation, the SDF-1–CXCR4 system has been implicated in several other pathological conditions, such as RA, allergy and pulmonary fibrosis. AMD3100 significantly improves the symptoms of these diseases [13,43], and the 14-mer peptide T140 and its analogs, initially characterized as potent anti-HIV inhibitors, were also found to be anti-RA agents [117].

Hematopoietic stem cell mobilization

Xiao *et al.* tested whether stem cell number and function were related to *CXCR4* gene variation. Stem cell number was inversely associated with SDF-1α levels, and an *SDF-1* gene variation (rs2297630) influenced SDF-1α levels and circulating stem cell number. Moreover, the plasma SDF-1α level is a predictor of stem cell number [118]. Transplantation of stem cells harvested from peripheral blood is an important treatment option for hematological malignancies. Though the cytokine granulocyte colony-stimulating factor (G-CSF) has frequently been used to mobilize stem cells, it is not sufficient alone for effective autologous transplantation. Preclinical studies have demonstrated that AMD3100 (now known clinically as plerixafor; Genzyme Corporation) can rapidly and reversibly mobilize hematopoietic stem cells into peripheral circulation in synergy with G-CSF [13,119]. These data suggest that plerixafor could potentially be useful in treating hematological disease. At the end of 2008, the FDA approved a plerixafor solution for subcutaneous injection (Mozobil™) for use in combination with G-CSF to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin lymphoma and multiple myeloma.

Conclusion

The discovery of HIV coreceptors, such as CCR5 and CXCR4, has prompted a search for inhibitors against these receptors as an alternative therapy

to HAART. Although new anti-HIV drugs that target HIV gp41 and integrase have recently been approved, inhibitors against CCR5 and CXCR4 represent the first anti-HIV agents that target host proteins rather than viral enzymes or proteins. The FDA approved the CCR5 inhibitor maraviroc in 2007 in combination with other antiretroviral drugs for use in treatment-experienced adult patients with R5 HIV-1. Several types of CXCR4 inhibitors have also been developed, such as the bicyclam AMD3100, peptide T140 and nonpeptide small molecules such as KRH-3955, and these molecules have been extensively studied. Some of these CXCR4 inhibitors are not only highly active against HIV but are also orally bioavailable; however, they are still in preclinical stages or have been suspended during development because of unforeseen side effects. The interaction between CXCR4 and its ligand SDF-1 also plays important roles in the migration of progenitor cells during embryonic development of various systems, such as the hematopoietic system. Indeed, AMD3100 has been shown to induce the rapid and reversible mobilization of hematopoietic stem cells into peripheral circulation in synergy with G-CSF. Furthermore, the SDF-1–CXCR4 axis has been found to play critical roles in various disease conditions, such as cancer cell metastasis and growth, RA, allergy and pulmonary fibrosis. These results indicate that CXCR4 inhibitors could be used to treat not only HIV infection, but also various diseases associated with CXCR4.

Future perspective

There are at least two important issues that will need to be addressed when coreceptor inhibitors are used as clinical drugs. First, it is necessary to determine which combination of entry inhibitors should be used once CXCR4 inhibitors are available for clinical use. In this scenario, it is reasonable to use CCR5 inhibitors, such as maraviroc, in combination with CXCR4 inhibitors because:

- It is not neccessary to assess the amount of R5, R5X4 and X4 HIV in the patients before initiating treatment with coreceptor inhibitors;
- Combination of a CCR5 inhibitor with CXCR4 inhibitors shows more potent synergism compared with the synergy obtained with other anti-HIV drugs, although the precise mechanism for this synergy will need to be clarified [120].

In addition, this combination could limit evolution and switching of coreceptor usage in both R5 and X4 HIV strains. Alternatively, coreceptor inhibitors could be used with fusion inhibitors such as enfuvirtide. In fact, synergistic inhibition has been reported when enfuvirtide and CCR5 inhibitors are combined [121,122].

The other important issue is the long-term safety of CXCR4 inhibitors. As mentioned above, the interaction between CXCR4 and its ligand SDF-1 plays important roles in the migration of progenitor cells during embryonic development of the cardiovascular, central nervous and hematopoietic systems. In addition, the SDF-1–CXCR4 axis has been found to be involved in various disease conditions such as cancer cell metastasis and RA. At the same time, however, inhibiting SDF-1α–CXCR4 interactions may evoke severe adverse effects. It has been reported that knocking out either the *SDF-1α* or the *CXCR4* gene in mice causes marked defects, such as abnormal hematopoiesis and cardiogenesis in addition to vascularization of the gastrointestinal tract [123–125]. Thus, an important point to be considered is whether blocking CXCR4 functions can affect animals at postdevelopmental stages, although approval of plerixafor (AMD3100) as a stem cell mobilizer indicates that at least short-term blockade of SDF-1/CXCR4 is safe in humans. Further careful studies for long-term use of CXCR4 inhibitors will certainly be required to properly address this question.

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Executive summary

Identification of chemokine receptors as coreceptors for HIV-1 entry

- CXCR4 and CCR5 are the two major coreceptors for HIV-1 infection.
- Coreceptors are responsible for a crucial step between Env binding to the receptor CD4 and membrane fusion mediated by the gp41 fusion peptide.

Determinants of HIV cell tropism & chemokine receptor usage

- The V3 loop of HIV-1 gp120 is a primary determinant of cell tropism and chemokine receptor usage, whereas the V1/V2 region is involved in effective entry of the virus.

Impact of nonfunctional chemokine receptor alleles on HIV resistance & disease progression

- Homozygotes and heterozygotes for CCR5 δ32, which are prevalent in European populations, exhibit a strong resistance to HIV infection and delayed progression to AIDS, respectively.
- A single-nucleotide polymorphism in CCR2b, one of the minor coreceptors for HIV, is associated with delayed progression to AIDS in either homozygote or heterozygote individuals.

Coreceptor-targeted anti-HIV therapy

- CXCR4 inhibitors:
  - The bicyclam AMD3100 is a well-known small molecule CXCR4 antagonist that strongly and specifically inhibits X4 HIV-1 infection. Tetrahydroquinoline-based AMD070 is a potent and specific X4 HIV-1 inhibitor with high oral bioavailability;
  - T22, an 18-mer anti-X4 HIV peptide, was designed from self-defense peptides isolated from horseshoe crabs. T140, a downsized analog of T22, has stronger anti-HIV activity than T22;
  - KRH-1636, which strongly and specifically inhibits X4 HIV-1 infection, is a novel small nonpeptide CXCR4 antagonist. KRH-3955, a derivative of KRH-1636, is much more potent than KRH-1636 and is orally bioavailable.
- CCR5 inhibitors:
  - Among the CCR5 inhibitors that have been developed, maraviroc is the only one that has been approved for use in treatment-experienced adult patients with R5 HIV-1 infection in combination with other antiretroviral drugs.

Resistance to coreceptor inhibitors

- Generation of HIV-1 strains resistant to coreceptor inhibitors *in vitro* seems difficult and time-consuming compared with generation of strains resistant to antiretrovirals that target viral proteins such as reverse transcriptase.
- Experiments using cells that expresses both CXCR4 and CCR5, such as human peripheral blood mononuclear cells, rarely result in the selection of drug-resistant viruses that change coreceptor usage.

Other applications of CXCR4 inhibitors

- Anticancer:
  - Many CXCR4 inhibitors have been shown to significantly reduce metastasis of various cancers such as ovarian carcinoma, melanoma, prostate cancer and breast cancer;
  - The CXCR4 inhibitors KRH-1636 and KRH-3955 are highly potent in antagonizing SDF-1α-mediated chemotaxis of cancer cells.
- Antiarthritis & allergy:
  - CXCR4 inhibitors AMD3100 and T140 are effective in mitigating rheumatoid arthritis and/or pulmonary fibrosis in animal models for these diseases.
- Hematopoietic stem cell mobilization:
  - AMD3100 (now known clinically as plerixafor) induces the rapid and reversible mobilization of hematopoietic stem cells from bone marrow into the circulating blood in synergy with granulocyte colony-stimulating factor.

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Remodeling of Dynamic Structures of HIV-1 Envelope Proteins Leads to Synthetic Antigen Molecules Inducing Neutralizing Antibodies

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A synthetic antigen targeting membrane-fusion mechanism of HIV-1 has a newly designed template with C3-symmetric linkers mimicking N36 trimeric form. The antiserum produced by immunization of the N36 trimeric form antigen showed structural preference in binding to N36 trimer and stronger inhibitory activity against HIV-1 infection than the N36 monomer. Our results suggest an effective strategy of HIV vaccine design based on a relationship to the native structure of proteins involved in HIV fusion mechanisms.

INTRODUCTION

Antibody-based therapy is one of the promising treatments for AIDS. In recent years, AIDS antibodies have been produced by immunization (*1*) and by de novo engineering of monoclonal antibodies (mAb) with molecular evolution tactics such as phage display (*2*). Despite enormous efforts, however, only a limited number of highly and broadly HIV-neutralizing human mAbs have been isolated and characterized. These antibodies include gp41 Abs, 2F5 (*3–6*) and 4E10 (*5–7*), and gp120 Abs, 2G12 (*8*) and b12 (*9*). gp41 is a transmembrane envelope glycoprotein, which is divided into an endodomain and an ectodomain by the transmembrane region; the latter contains a hydrophobic amino-terminal fusion peptide, followed by amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat domains with helical structures (HR1 and HR2, respectively). In the membrane fusion process of HIV-1, these subunits form a "pre-bundle" complex. The HR1 and HR2 regions are termed the N-terminal helix (N36) and C-terminal helix (C34), respectively. These helices form a six-helical bundle consisting of a central parallel trimeric coiled-coil of N36 surrounded by C34 in an antiparallel hairpin fashion. In design of immunogens that elicit broadly neutralizing antibodies, a useful strategy is to produce molecules that mimic the natural trimer on the virion surface. Previous studies show that these molecules could be proteins expressed as a recombinant form or on the surface of particles such as pseudovirions or proteoliposomes (*10–12*). The X-ray crystallographic study of gp41 shows that the distances between any two residues at the N-terminus of N-region are almost equal at approximately 10 Å (Figure 1A). A chemically synthetic template could be useful in connection with the design of a peptidomimetic corresponding to the native structure of gp41. To date, several gp41 mimetics have been synthesized as inhibitors or antigens and subjected to inhibition or neutralization assays (*13–16*). However, the templates for assembly of these helical peptides contain branched peptide linkers, which are not exactly equivalent in length (*14*). The N-terminal peptides constrained by another threefold linker showed high affinity for C-terminal peptides, although its biological advantages have not been determined (*15*). The mimicry can be estimated using the broadly neutralizing mAbs; suitable mimetics will bind neutralizing mAbs efficiently, but they will bind non-neutralizing mAbs poorly. In the present study, we designed and synthesized a novel three-helical bundle mimetic, which corresponds to the trimeric form of N36. We investigated whether mice immunized with the equivalent trimeric form of N36 mimetic can produce antibodies with stronger binding affinity for N36 trimer than for N36 monomer. This approach demonstrates the possibility of producing structure-specific antibodies by immunization of synthetic antigens corresponding to the natural form of viral proteins.

EXPERIMENTAL PROCEDURES

Conjugation of N36REGC and the Template to Produce triN36e. Compound **6** (100 μg, 0.174 μmol) and N36REGC (3.4 mg, 0.574 μmol) were dissolved in a mixture of 300 μL of 200 mM acetate buffer (pH 5.2) and 300 μL of TFE under a nitrogen atmosphere, then TCEP·HCl was added. The reaction was stirred for 72 h at room temperature and monitored by HPLC. The ligation product (triN36e) was separated as an HPLC peak and was characterized by ESI-TOF-MS, *m/z* calcd for $C_{690}H_{1160}N_{226}O_{201}S_3$ 15933.1, found 15933.8. The purification was performed by reverse phase HPLC (YMC-Pack ODS-A column, 10 × 250 mm). Elution was carried out with a 40–50% linear gradient of acetonitrile (0.1% TFA) over 50 min. Purified triN36e, obtained in 16% yield, was identified by ESI-TOF-MS. The detailed synthesis of peptides is described in the Supporting Information (SI).

CD Spectra. CD measurements were performed with a J-720 circular dichroism spectrolarimeter equipped with a thermoregulator (JASCO). The wavelength dependence of molar ellipticity [θ] was monitored at 25 °C from 190 to 250 nm. Peptides were dissolved in 20 mM acetate buffer (pH 4.0) containing 40% MeOH (*23, 24*). The experimental helicity was calculated as reported previously (*17–19*).

Immunization and Sample Collection. Six-week-old male BALB/c mice were purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan) and maintained under specific pathogen-free conditions in an animal facility. The experimental protocol was approved by the ethical review committee of Tokyo Medical and Dental University. Freund incomplete adjuvant and PBS

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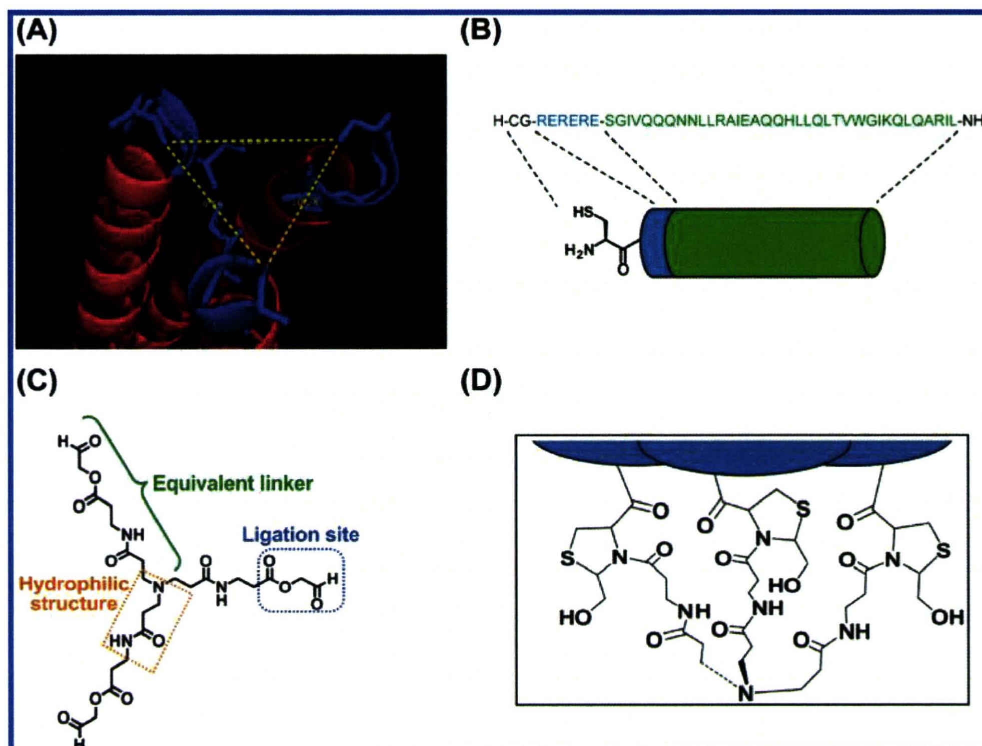


Figure 1. (A) Distances between hydrogen atoms for hydroxyl groups in N-terminal serine residues of N36 helices in trimeric form. The distances were evaluated by PyMOL (*21*). (B) Cartoon presentation of each N36 derived peptide, N36REGC. (C) Design of a C3-symmetric template. The amino acid residues are described in single letters. (D) Conjugated structure of trimeric N36 after thiazolidine ligation.

were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMSO (endotoxin free) was purchased from Sigma-Aldrich (St. Louis, MO).

All mice were bled one week before immunization. One hundred micrograms of antigen was dissolved in 1 μL of DMSO. The solution was mixed with 50 μL of PBS and 50 μL of Freund incomplete adjuvant. The mixture was injected subcutaneously under anesthesia on days 0, 14, 28, 42, and 58. Mice were bled on days 21, 35, 49, and 65. Serum was separated by centrifugation (15 000 rpm) at 4 °C for 15 min and inactivated at 56 °C for 30 min. Sera were stored at −80 °C before use.

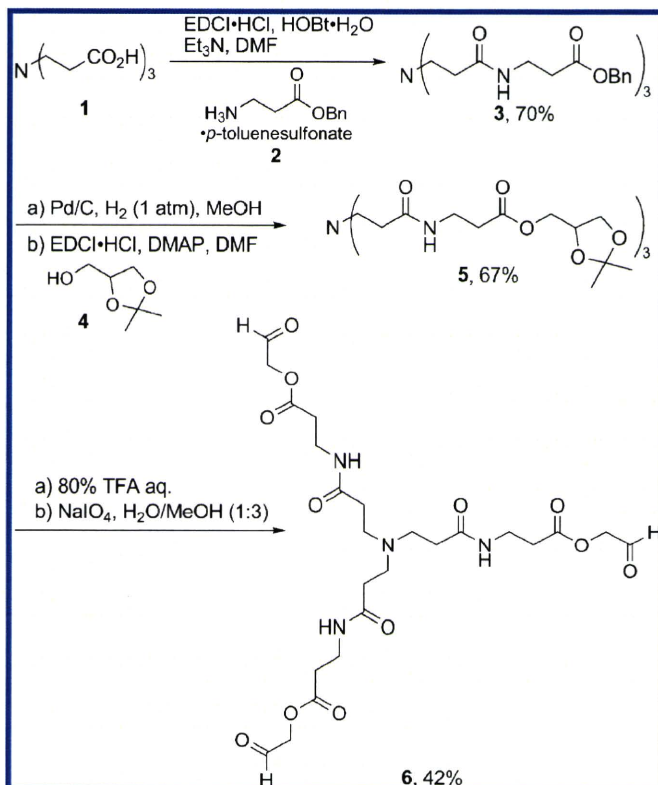
Serum Titer ELISA. Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako. ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was purchased from Sigma-Aldrich. Antimouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninty-six-well microplates were coated with 25 μL of a synthetic peptide at 10 μg/mL in PBS at 4 °C for overnight. The coated plates were washed 10 times with deionized water and blocked with 150 μL of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °C for 1 h. The plates were washed with deionized water 10 times. Mice sera were diluted in 0.02% PBST with 1% skim milk, and 50 μL of 2-fold serial dilutions of sera from 1/200 to 1/102400 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were washed 10 times with deionized water. Twenty-five microliters of HRP-conjugated antimouse IgG, diluted 1:2000 in 0.02% PBST, was added to each well. After 45 min incubation, the plates were washed 10 times and 25 μL of HRP substrate, prepared by dissolving 10 mg ABTS to 200 μLof HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H_2O_2 (3 μL), and H_2O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μL/well 0.5 M H_2SO_4, and optical densities were measured at 405 nm.

Virus Preparation. The pNL4-3 construct (8 μg) was transfected into 293T cells by Lipofectamine LTX (Invitrogen, Carlsbad, CA) followed by changing medium at 12 h after transfection. At 48 h after changing medium, the supernatant was collected, passed through a 0.45 μm filter, and stored at −80 °C as HIV-1_{NL4-3} strain before use. For titration, MT-4 cells were infected with serially 3-fold diluted virus from 1/10 to 1/196830, and cultured for 7 days. HIV-1 p24 levels in supernatants were measured, and then the titer of virus solution was calculated.

Anti-HIV Assay. Virus was prepared as described above except that the transfection of pNL4-3 was performed by the calcium phosphate method. Anti-HIV-1 activity was determined on the basis of protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of AZT, N36RE, and triN36e (The starting concentrations are 100, 10, and 1 μM, respectively) were added to HIV-1-infected MT-4 cells (MOI = 0.01) by 2-fold serial dilution and placed in wells of a flat-bottomed microtiter plate (2.0 × 10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (EC_{50}). Cytotoxicity of compounds was determined on the basis of viability of mock-infected cells using the MTT method (CC_{50}). Each experiment was performed three times independently.

Neutralizing Assay. MT4-cells (1 × 10^5 cells/100 μL) were incubated in 100 μL medium containing 10 μL sera from immunized or preimmunized mice for 1 h at 37 °C, then pretreated MT-4 cells were infected with HIV-1_{NL4-3} (MOI = 0.05). At 3 days after infection, cells were collected by centrifuge at 4000 rpm for 10 min at 4 °C. After discarding supernatant, pellets were lysed with 30 μL of lysis buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% NP-40), then 30 μL of 2 × SDS buffer (125 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-ME, 0.004% BPB) were added and boiled for 10 min. The samples (5 μL) were subjected to SDS-page to perform Western blotting. The HIV-1 gag p24 was detected by using Western lightning ECL kit (PerkinElmer, MA) according to manufacturer's instruction after treatment of HIV-1 p24

Scheme 1. Synthesis of the Equivalently Branched Template 6



antibody (2C2; 1:2000 dilution) (*20*) and anti- mouse IgG (H+L)-HRP (Millipore, MA). The band intensity of p24 was calculated with post/pre-immunized samples by using *ImageJ* image analyzing software.

RESULTS AND DISCUSSION

The N-region of gp41 is known to be an aggregation site involving a trimeric coiled-coil conformation. In design of an N36-derived peptide (N36RE), the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase the solubility in buffer solution (Figure 1B). In order to form a triple helix corresponding precisely to the gp41 prefusion form, we designed the novel C3-symmetric template depicted in Figure 1C. This designed template linker has three branches of equal length and possesses the hydrophilic structure and ligation site for coupling with N36RE. The template was synthesized from the commercially available 3-[bis(2-carboxyethyl)amino]propanoic acid **1** as shown in Scheme 1. Coupling of **1** with β-alanine benzyl ester **2** gave the corresponding triamide **3** in 77% yield. Cleavage of three benzyl esters by hydrogenation and coupling with solketal **4** produced the corresponding triester **5**. Deprotection of the acetonides with aqueous 80% TFA followed by oxidative cleavage of diol group led to the desired template **6**. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected N36RE (N36REGC) with a three-armed aldehyde scaffold producing triN36e (Figure 1D). Thiazolidine ligation is a peptide segment coupling strategy which does not require side chain protecting groups (*22*−*26*). The reaction consists of three steps: (i) aldehyde introduction, in which a masked glycolaldehyde ester is linked to the carboxyl terminus of an unprotected peptide by reverse proteolysis; (ii) ring formation, in which the unmasked aldehyde reacts at acidic pH with the α-amino group of an N-terminal cysteine residue of the second unprotected peptide forming a thiazolidine ring; and (iii) rearrangement at higher pH in which O-acyl ester linkage is converted to an N-acyl amide linkage forming a peptide bond with a pseudoproline structure (Figure 2).

Circular dichroism (CD) spectra of triN36e and N36RE, which is a monomer form without N-terminal Cys-Gly residues, are shown in Figure 3A. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, suitable for measurement of CD spectra of membrane proteins (*27, 28*). Both spectra display double minima at 208 and 222 nm and showed high molar ellipticity as absolute values (Table 1). The results indicate that these peptides form a highly structured α-helix and that the helical content of the trimer triN36e is higher than that of the monomer N36RE. Furthermore, to assess the interaction of triN36e with C34, CD spectra of the peptide mixture with C34-derived peptide, C34RE, were measured (Figure 3B,C). The spectrum of triN36e and C34RE mixture showed high molecular ellipticity as an absolute value comparable with that of triN36e alone. This supports the conclusion that C34RE interacts with tri36e and thereby induces a higher helical form as shown previously (*29*).

Mice were immunized with these synthetic gp41 mimetics and antibody production was successfully induced (the detailed titer increase in 5 weeks' immunization is given in the Supporting Information). Two out of three mice showed induction of antibodies against either antigen (N36RE or triN36e). Antibody titers and selectivity of antisera isolated from mice immunized with N36RE or triN36e were evaluated by serum titer ELISA against coated synthetic antigens. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Figure 4). The N36RE-induced antibody showed approximately 5 times higher affinity for N36RE than for triN36e, as 50% bound serum dilutions are 3.88×10^{-4} and 2.14×10^{-3} to N36RE and triN36e, respectively. It is noteworthy that the triN36e-induced antibody showed approximately 30 times higher preference in binding affinity for triN36e antigen than for N36RE (serum dilutions at 50% bound are 3.83×10^{-3} to N36RE and 1.33×10^{-4} to triN36e). Although this evaluation was not determined with purified mAbs, it is clear that the antibodies produced exploit a structural preference for antigens. The mechanism of induction

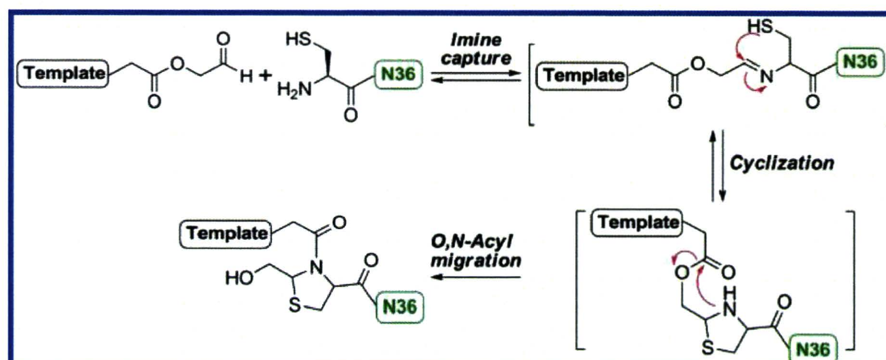


Figure 2. Reaction mechanisms of thiazolidine ligation utilized for assembly of N36RE helices on the template.

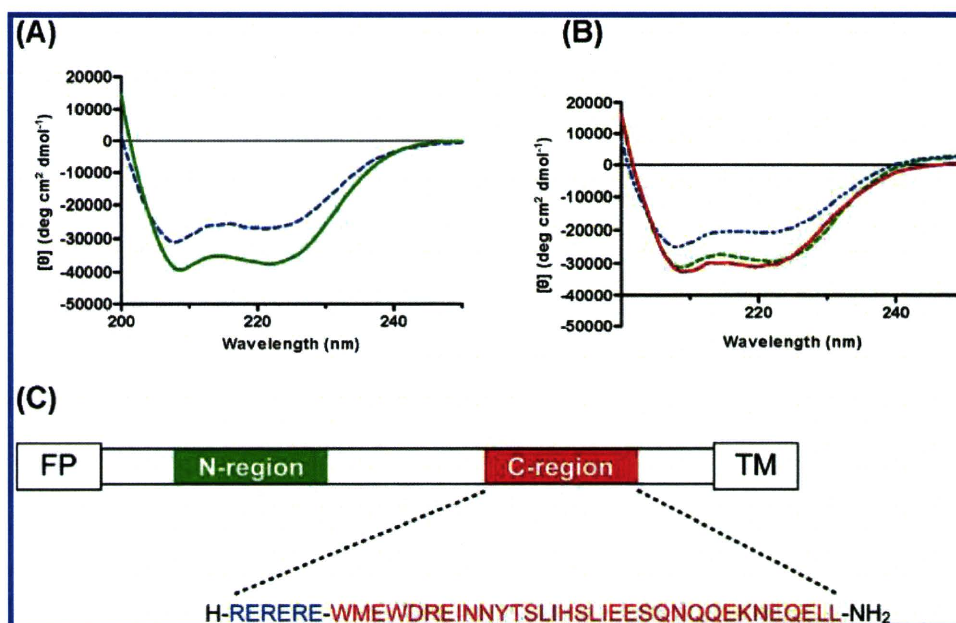


Figure 3. (A) Circular dichroism (CD) spectra of N36RE and triN36e. In the spectra, a blue dashed line and a green line show N36RE (monomer) and triN36e (trimer), respectively. Concentrations of the peptides are 10 and 3.3 μM for N36RE and triN36e, respectively. (B) CD spectra in the presence or absence of C34RE peptide. The spectra show the following: a dashed green line, triN36e; a dashed blue line, C34RE; a red line, triN36e+C34RE, respectively. The concentrations of peptides were as follows: triN36e (2.3 μM), C34-derived peptide C34RE (7 μM), and mixture of both peptides (3.5 μM each). (C) The amino acid sequence of C34RE described in single letters. FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively.

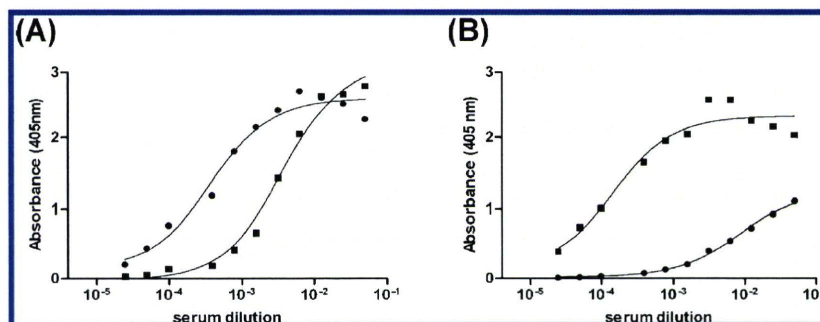


Figure 4. Serum titers of antibodies produced by N36 monomer and conformationally constrained N36 trimeric antigen. The titers were evaluated against N36RE (monomer) (A) and triN36e (trimer) (B). The plots indicate the results of sera obtained from N36RE-immunized mouse (•) and triN36e-immunized mouse (■).

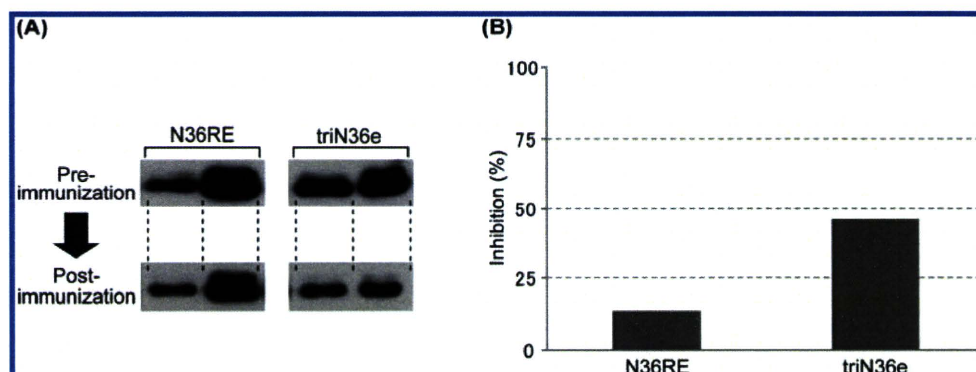


Figure 5. Determination of neutralization activity of the antibodies produced by immunization of peptidomimetic antigens. (A) Results of p24 assay to evaluate inhibition for HIV-1 infection by produced antibodies. Preimmunization sera were used as control. Experiments were duplicated. (B) Average % inhibition of p24 production calculated from the band intensities in panel (A).

of structure-specific antibody is still not clear, but the results could suggest the efficacy of producing antibodies with structural specificity and that the synthesis of structure-involving antigens is an effective strategy when higher specificity is required.

Neutralizing activity of sera against HIV-1 infection was assessed by p24 assays utilizing antisera from two mice that showed antibody production for each antigen (Figure 5). Sera

Table 1. Differences of α-Helicities between N36RE and triN36e Calculated from CD Spectra in Figure 3

	$[\theta]_{222}$	$[\theta]_{222}/[\theta]_{208}$	α-helicity
N36RE	−30 957	0.87	73%
triN36e	−38 998	0.96	95%