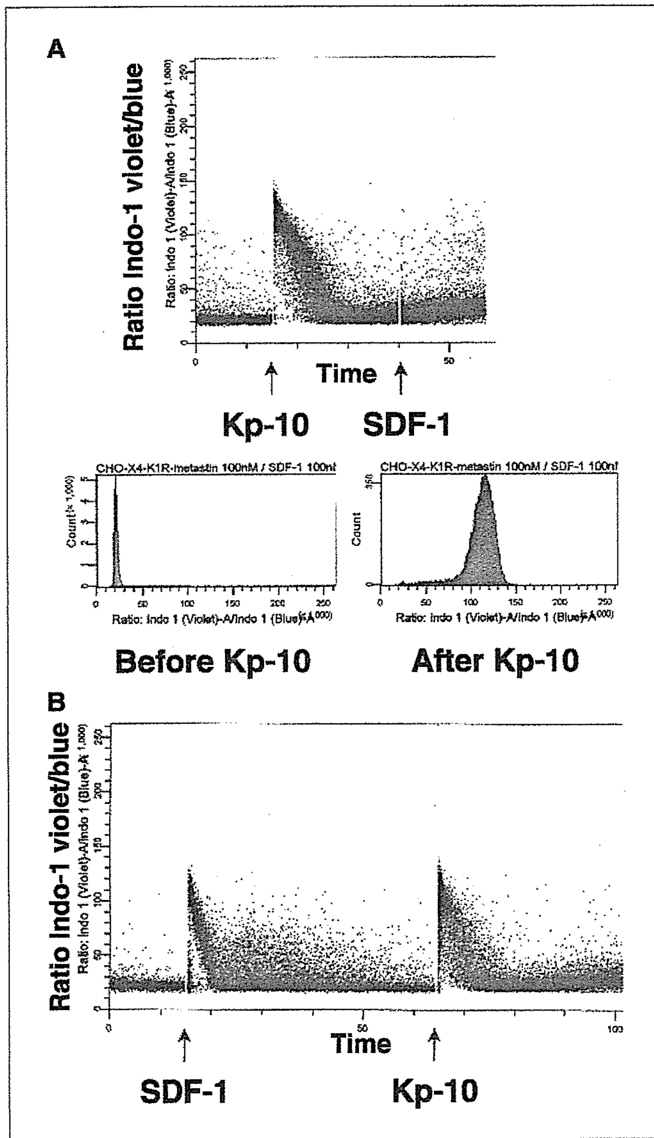

Figure 3. Calcium mobilization induced by SDF-1 and Kp-10 in CHO cells expressing CXCR4 and GPR54. *A,* the cells were sequentially exposed to 100 nmol/L of Kp-10 than 100 nmol/L of SDF-1. Time gating before and immediately after addition of Kp-10 (*bottom*) shows that over 99% of the cells responded to Kp-10 as indicated by a change of the ratio between the violet (Ca^{2+} bound) and blue (free) fluorescence of Indo-1. *B,* when the cells were sequentially exposed to SDF-1 then Kp-10, the entire cell population (>99%) could respond to both ligands.

resulting on the modulation of the signaling of one GPCR by another one (16). Serial additions of SDF-1 and Kp-10 to CHO-CXCR4/GPR54 transfectants resulted in mobilization of cytoplasmic calcium ions by both ligands, as expected from two independent receptors in the absence of cross-desensitization (Fig. 2*A*). Prestimulation with SDF-1 did not reduce the amplitude of the response to 100 nmol/L of Kp-10 (compare with Fig. 2*E*). In contrast, following a calcium flux response to Kp-10 at concentrations ranging from 1 to 100 nmol/L, exposing CHO-CXCR4/GPR54 transfectants to SDF-1, failed to induce a significant response, as shown in Fig. 2*B-E*. Even exposure to 1 nmol/L of Kp-10 that induced a smaller and slower (with decreased rates of both release and recapture of cytoplasmic calcium) almost abolished the response to SDF-1. These results suggest a rapid and profound cross-desensitization of CXCR4 by the signaling of the GPR54. Because the activation of GPR54 by Kp-10 generated a slow and delayed increased in cytoplasmic calcium (probably related to calcium influx), a very small response to SDF-1 could be masked and cannot be completely excluded. Wash-out experiments were done in which cells were exposed for 3 minutes to 100 nmol/L of Kp-10, washed thrice, and allowed to recover for various periods of time before being challenged with SDF-1. The recovery of the response to SDF-1 was partial 15 minutes after removal of Kp-10 and complete after 30 minutes (data not shown), indicating that the effect is reversible and requires continuous presence of Kp-10. Exposure of target cells lacking GPR54 expression to Kp-10 induced no calcium mobilization and no cross-desensitization of CXCR4 signaling (data not shown). The specificity of this cross-desensitization was further shown by pretreating the cells with 5-hydroxytriptamine, which has an endogenously expressed Gi-coupled receptor in CHO cells. The stimulation with 5-hydroxytriptamine induced a calcium flux that did not modify the amplitude of the subsequent response to SDF-1 (Fig. 2*F*). This result illustrates the absence of desensitization between the 5-hydroxytriptamine receptor and CXCR4, although the receptors couple to the same G-protein. Because such a profound effect of GPR54 on the signaling of CXCR4 may be surprising, we tested the homogeneity of the cell response to both ligands by flow cytometry. When CHO cells expressing CXCR4 and GPR54 were exposed to Kp-10, all the cells responded by releasing calcium into their cytoplasm (Fig. 3*A*). Again, subsequent addition of SDF-1 did not have any effect (Fig. 3*A*). When SDF-1 was added to the cells before Kp-10, all the cells responded to both ligands (Fig. 3*B*).

Negative regulation of CXCR4 signaling does not alter receptor biology. The negative regulation of GPR54 activation on the induction of signaling by SDF-1 could result from direct effects on CXCR4 impairing its membrane expression, binding to SDF-1, or coupling to G-proteins. The first hypothesis was tested in down-modulation experiments. A late consequence of the activation of a GPCR by its cognate agonist is the phosphorylation of cytoplasmic domains of the receptor by GPCR-regulated kinases (GRK) and protein kinase C. Interaction with β-arrestins and internalization of the receptor follow resulting in the desensitization of the receptor. In some cases, activation of one receptor can result in the cross-phosphorylation and desensitization of another one. The possibility of such a mechanism was investigated. As shown in Fig. 4*A*, exposure of CHO-CXCR4/GPR54 transfectants to SDF-1 resulted in rapid down-modulation of CXCR4 levels on the plasma membrane. In contrast, exposure of these cells to Kp-10 did not alter levels of cell surface CXCR4.

Ligand binding experiments were done to establish whether the decreased response of CXCR4 was the result of decreased affinity for SDF-1. As shown in Fig. 4*B*, pre-exposure of CHO-CXCR4/GPR54 transfectants to Kp-10 did not result in a significant alteration of the affinity of CXCR4 binding to SDF-1.

The earliest event in GPCR signaling is the activation of Gα subunits of heterotrimeric G-proteins. The effect of GPR54 activation on the induction of Gα subunit binding to GTP by SDF-1 activation of CXCR4 was tested using $\gamma[^{35}S]$GTP. Exposure of membrane fractions from CHO-CXCR4/GPR54 transfectants to SDF-1 resulted in a significant increase in $\gamma[^{35}S]$GTP over basal levels (Fig. 4*C*). In contrast, incubation with Kp-10 did not increase $\gamma[^{35}S]$GTP binding. Preincubation of membrane fractions with Kp-10 before exposure to SDF-1 did not alter $\gamma[^{35}S]$GTP binding

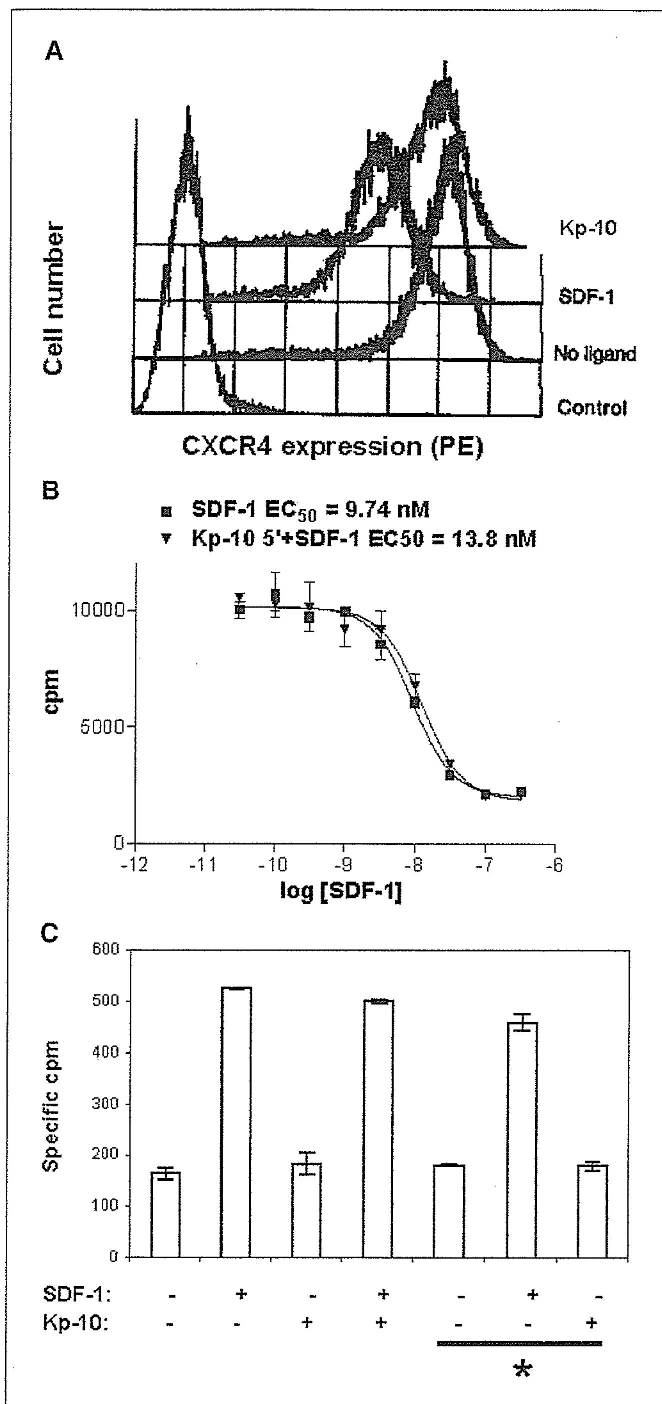

Figure 4. Stimulation of GPR54 by Kp-10 does not directly affect CXCR4. *A,* Kp-10 does not induce cross-internalization of CXCR4. CHO transfectants were either left unstimulated (no ligand) or exposed for 30 minutes to 100 nmol/L of SDF-1 or Kp-10 to allow internalization to occur. The cells were then stained for detection of surface expression of CXCR4 by flow cytometry. Representative of two identical experiments. *B,* prestimulation of CHO transfectants with Kp-10 before preparation of the plasma membrane fraction does not affect the binding of SDF-1 to CXCR4 as measured by radioactive ligand binding assay. *C,* stimulation of GPR54 by Kp-10 does not prevent the activation of G-proteins by CXCR4 upon binding to SDF-1. Prestimulation with 100 nmol/L of Kp-10 for 5 minutes was done either on the membrane fraction used for the assay or on the whole cells before the preparation of the membrane fraction (*). The latter experimental condition reproduces exactly the condition where cross-desensitization of CXCR4 is complete as assessed by Ca flux and Western blot experiments. One representative experiment of five similar experiments with duplicate samples.

seen with SDF-1 alone. To exclude the possibility that exposure of membrane fractions to Kp-10 would not allow the cross-desensitization mechanism to be preserved, an alternative experiment was also done where whole cells were exposed to Kp-10 before preparation of the membrane fraction and activation by SDF-1 (Fig. 4*C*).

We finally investigated whether the normal mechanism of desensitization of CXCR4 after exposure to SDF-1, which results in the internalization of the receptor, was preserved when the cells were pre-exposed to Kp-10. As shown in Fig. 5, pretreatment with Kp-10 only partially reduced the ability of SDF-1 to induce the internalization of CXCR4 (50-60% internalization with both ligands versus 75-80% with SDF-1 alone), suggesting that although part of the signaling of CXCR4 is interrupted, the activated conformation of the receptor is still permissive to the interaction with the GRK.

GPR54 activation inhibits induction of Akt phosphorylation by SDF-1. CXCR4 signaling has previously been shown to induce activation of both the MAPK pathway and Akt. Time course experiments done both in CHO (Fig. 6*A*) and HeLa (Fig. 6*B*) transfectants showed that the activation of MAPK by the GPR54 extended beyond the extinction of its activation by CXCR4. Whereas activation of CXCR4 by SDF-1 resulted in phosphorylation of Akt on Ser^{473} and Thr^{308} in CHO, activation of GPR54 by Kp-10 did not (Fig. 6*C*). Furthermore, the costimulation of the cells with Kp-10 abolished the effect of SDF-1, showing that the Akt pathway is a target of the crosstalk mechanism between the two receptors.

Discussion

The growth and metastasis promoting role of CXCR4 has been well documented (3). However, it is unclear whether regulatory

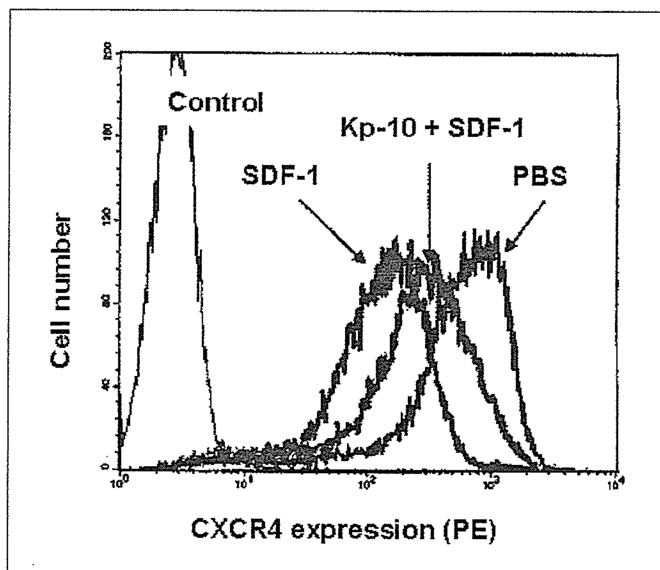

Figure 5. Internalization of CXCR4 in CHO cells after activation of the GPR54 with Kp-10. Cells were exposed to 100 nmol/L Kp-10 for 5 minutes at 37°C before addition of 100 nmol/L SDF-1 and incubation for 30 minutes at 37°C to allow internalization of CXCR4. After an acid wash, the cells were stained to detect the membrane expression of CXCR4 by flow cytometry. In the PBS control, cells were only exposed to PBS-BSA. In the SDF-1 control, the cells were incubated with PBS-BSA for 5 minutes then with SDF-1 for 30 minutes. Left histogram (*Control*) shows the fluorescence of cells stained without CXCR4 antibody.

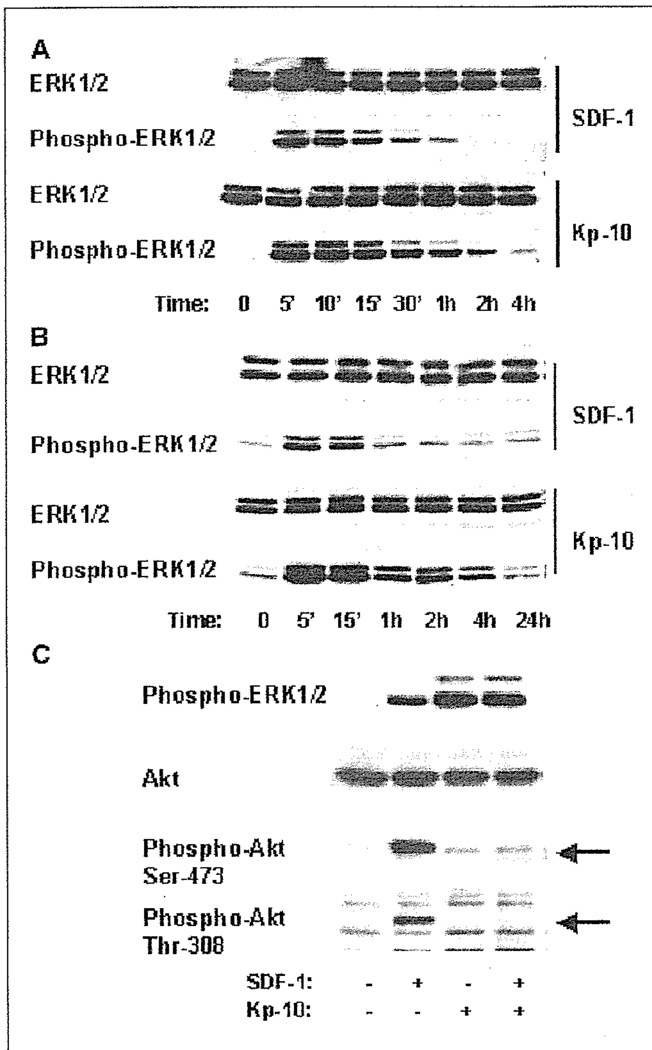

Figure 6. Signaling mechanism of GPR54 by Western blot. *A,* CHO cells expressing CXCR4 and GPR54 were exposed to 100 nmol/L of SDF-1 or Kp-10, and lysates were prepared at the indicated times from 5 minutes to 4 hours. *A,* long-lasting activation of ERK1/2 MAPK by GPR54 that is clearly detectable at 4 hours, whereas the effect of SDF-1 in the same cells cannot be detected beyond 1 hour. *B,* similar results were obtained in HeLa cells in which the signaling of GPR54 is maintained for at least 4 hours in contrast to 1 hour for CXCR4. Suggest a slow desensitization of the signaling of GPR54. *C,* activation of GPR54 by Kp-10 inhibits the phosphorylation of Akt induced by the stimulation of CXCR4 by SDF-1. CHO cells were exposed for 5 minutes to either 100 nmol/L of SDF-1, 100 nmol/L of Kp-10, or both. The efficiency of the stimulation by each ligand was evaluated by detection of phospho-ERK1/2. Both Ser^{473} and Thr^{308} of Akt were phosphorylated upon activation by SDF-1, whereas Kp-10 did not phosphorylate Akt. Costimulation of CXCR4 and GPR54 resulted in the abrogation of the activating effect of SDF-1. Representative of at least five experiments with similar results.

(potentiating or inhibitory) mechanisms exist besides the expression of the receptor by tumor cells and of its ligand by target organs. We hypothesized that an antagonism between the metastasis suppressor receptor GPR54 and CXCR4 within tumor cells could modulate the metastatic potential of CXCR4. Here, we show that Kp-10 and its receptor negatively regulate the chemotactic activity of CXCR4. The effect is immediate and sustained but does not influence levels of CXCR4 expression on the cell surface, binding to ligand, or activation of Gα subunits. GPR54 blocked distal CXCR4 signaling events, including calcium mobilization and Akt phosphorylation. The suppression of Akt phosphorylation by Kp-10 mimics the effects of phosphatidylinositol 3-kinase (PI3K) inhibitors, which also block chemotaxis. This raises the possibility that there is equilibrium between signaling pathways of metastasis promoting and suppressor genes through reciprocal crosstalk.

KiSS-1 is one of 14 genes that have been shown to suppress metastasis of malignant cells and the only one to bind a GPCR (4). Activation of the GPR54 has been shown to have a variety of effects on tumor cell biology, including suppression of motility induced by FBS, culture scratch repair, proliferation, metastasis of B16 melanoma cells, and invasion *in vitro*. However, the precise mechanism for the antimetastatic function of Kp-10 is unclear.

PI3K and its downstream effector Akt/protein kinase B can be activated by tyrosine kinase receptors. Multiple GPCRs have also been reported to modulate the activity of Akt, although the exact mechanism is not completely understood. The polarized activation of PI3K and Akt at the leading edge of migrating cells has been described as a key event in chemotaxis (17, 18). If Gi-coupled receptors are generally recognized as activators of PI3K and Akt, the effect of Gq-coupled receptors is more controversial. Several Gq-coupled receptors activate PI3K and Akt, which can result in inhibition of apoptosis (19–22). For instance, it was shown that the activation of Gq-coupled M1, M3, and M5 muscarinic receptors but not Gi-coupled M2 and M4 receptors could protect cells from apoptosis induced by etoposide or UV (20, 23, 24). Other reports suggest instead that signaling of Gq, either activated by a GPCR or in the form of a constitutively active mutant (CAM), can inhibit the activation of Akt induced by growth factors and trigger apoptosis (25–29). Additional studies showed an opposite effect for Gq-coupled receptors (phosphorylation of Akt and inhibition of apoptosis) and CAMs of Gq (reduction of phosphorylation of Akt and apoptosis; ref. 30). These apparently contradictory observations may be reconciled by a model in which Gβγ subunits would activate PI3K and Akt whereas Gαq would inhibit them (30, 31), hypothesis supported by the fact that Gαq was shown to interact with PI3K (28).

The suppression of calcium release and the inhibition of Akt activation are at least two ways the KiSS-1 receptor uses to prevent the chemotaxis normally induced by the interaction between SDF-1 and CXCR4. Additional possible factors have already been described, like reorganization of actin fibers and focal adhesion (8, 10). We ignore at this time if the phenomenon is specific for some subtypes of PI3K or Akt or what the exact effect is on the complex signaling network around Akt in tumor cells (32). However, because the antibodies against phosphorylated Akt used here react with all three forms of the kinase and because some of the Western blot experiments were done with total SDS cell lysates, it is likely that any subtype of Akt activated by CXCR4 irrespectively of its subcellular localization will be affected by the GPR54. It should also be noted that the inhibitory effect of the GPR54 on CXCR4-mediated chemotaxis noted in CHO and HeLa cells was reproduced in Jurkat cells, again without any effect of the ligand in the absence of the receptor. This rules out the possibility that the effect could only exist in one particular cell type. It is possible however that the amplitude of the effect depends in part on the expression level of GPR54. It is likely that the remarkable efficacy of the KiSS-1 receptor ultimately results from several coordinated impairments of prochemotactic cellular mechanisms. It is interesting to note that the KiSS-1 receptor completely silences CXCR4 without affecting the receptor itself and without employing

the canonical mechanisms of cross-desensitization between GPCRs (i.e., cross-phosphorylation; data not shown) and down-modulation.

Finally, Akt activity being essential to cell survival, it is likely that the inhibition of its phosphorylation by the KiSS-1 receptor would result in apoptosis, either by itself or in conjunction with other proapoptotic signals. The signaling of the GPR54 and other Gq-coupled GPCRs includes both proapoptotic and antiapoptotic events. Depending on the cellular environment and the subtypes of signaling molecules involved, one or the other may prevail. However, very recently (33), new information showed that the signaling of the GPR54 in breast tumor cells could specifically promote the expression of an array of proapoptotic genes, suggesting that it may cross the line between metastasis suppressors and tumor suppressors.

Acknowledgments

Received 5/20/2005; revised 8/22/2005; accepted 9/13/2005.

Grant support: Philip Morris USA, Inc.; Philip Morris International; and Georgia Cancer Coalition.

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Development of Anti-HIV Agents Targeting Dynamic Supramolecular Mechanism: Entry and Fusion Inhibitors Based on CXCR4/CCR5 Antagonists and gp41-C34-Remodeling Peptides

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Abstract: A molecular mechanism involved both in HIV-entry and -fusion steps has been disclosed in detail: The interaction of an HIV envelope protein, gp120, with chemokine receptors, CXCR4 and CCR5, which were identified as major co-receptors in association with CD4, triggers conformational changes in the gp120-gp41 (another envelope protein) complex, and subsequently forms the trimer-of-hairpins structure of gp41 followed by virus-cell membrane fusion. The elucidation of the above dynamic supramolecular mechanism in HIV-entry and -fusion has provided insights into new type of drugs that can block HIV infection. Based on this, we have developed not only coreceptor antagonists (1) but also fusion inhibitors (2). (1) Potent CXCR4 antagonists, T22 and T140, have been developed through the structure-activity relationship studies on tachyplesins and polyphemusins that are horseshoe crabs' antimicrobial peptides. T22, which was initially found to bind gp120 and CD4, and T140 selectively suppress T-cell line-tropic HIV-1 (X4-HIV-1) entry based on their specific binding to CXCR4. Furthermore, molecular-size reduction of T140 using cyclic pentapeptide templates brought us to find low molecular weight CXCR4 antagonists, such as FC131. (2) Artificial remodeling of a gp41 fragment, C34, has led to development of strong inhibitors of HIV-fusion into cells. These fusion inhibitors effectively block the formation of the trimer-of-hairpins structure of gp41. HIV-entry/fusion inhibitors such as CXCR4 antagonists and C34 analogs would improve the clinical chemotherapy of AIDS and HIV-infected patients. This review article focuses on our recent research on the development of the above two types of inhibitors, including comparative studies with several CXCR4 antagonists besides T22/T140-related compounds and other fusion inhibitors such as Fuzeon (T-20).

Keywords: AIDS, chemokine, low molecular weight CXCR4 antagonist, X4-HIV-1 entry, cancer metastasis, rheumatoid arthritis, fusion inhibitor, artificial remodeling.

INTRODUCTION

Highly active anti-retroviral therapy (HAART), which utilizes a combination of HIV protease inhibitors and reverse transcriptase inhibitors, has brought us a great success and hope in the clinical treatment of HIV-infected patients [48]. However, HAART involves serious clinical problems such as the emergence of multi-drug resistant (MDR) HIV-1 strains, significant side effects, nonetheless high costs, etc. These drawbacks encouraged us to develop brand-new drugs with novel action mechanisms, such as HIV-entry and -fusion inhibitors. Recently, the molecular mechanism concerning the HIV-1 replication has been elucidated in detail, especially for a dynamic supramolecular mechanism relevant to HIV entry/fusion steps: At first, an HIV envelope protein gp120 interacts with a cell surface protein CD4, which leads to a conformational change in gp120 followed by subsequent binding of gp120 to the second cellular receptors, such as CCR5 [1,12,17,20,21] and CXCR4 [22]. These are the major co-receptors for the entry of macrophage-tropic (R5-) and T cell line-tropic (X4-) HIV-1, respectively, whereas these play a physiologically important role as the receptors for endogenous ligands, chemokines. Next, the interaction of gp120 with CCR5 or CXCR4 triggers penetration of another envelope protein gp41 to the cell membrane from the *N*-terminus end and formation of the gp41 trimer-of-hairpins structure in the middle region, which causes fusion of HIV/cell-membranes and results in completion of the infection [11]. Elucidation of the above dynamic molecular machinary drove many researchers to develop effective inhibitors blocking HIV-entry/fusion steps targeting the second receptors, CCR5 and CXCR4, and the dynamic process involving the gp41 structure change.

This article reviews our recent approaches into the development of CXCR4 antagonists and gp41-fragment-remodeling peptides targeting the dynamic supramolecular mechanism.

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1. CXCR4 and CCR5 Antagonists

1-1. Biostable Lead Compounds Derived from T140

R5-HIV-I strains, which use CCR5 as a co-receptor, constitute majority in the early stage of HIV infection,

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whereas X4-HIV-1 strains, which use CXCR4 as a co-receptor, are major species in the late stage of HIV infection and AIDS. Our research has focused on drug discovery targeting CXCR4. Tachyplesins and polyphemusins are 17-mer and 18-mer antimicrobial peptides contained in horseshoe crabs, respectively. Structure-activity relationship (SAR) studies on these peptides led to the discovery of an 18-mer peptide, T22 ([$Tyr^{5,12}$, Lys^{7}]-polyphemusin II) that was initially found to interact with gp120 and CD4 [89], which were not the real targets for the expression of the activity, and its downsized 14-mer peptide, T140 [26,45,79,91] (Fig. (**1**)). T22 and T140 proved to strongly block an X4-HIV-1 entry through their specific binding to CXCR4 [52,53,101]. Four residues in T140, Arg^{2}, L-3-(2-naphthyl)alanine $(Nal)^{3}$, Tyr^{5} and Arg^{14}, are indispensable for high potency (Fig. (**1**)) [88], and T140 forms an antiparallel β-sheet structure that is maintained by a disulfide bridge between Cys^{4} and Cys^{13} and connected by a type II' β-turn with Lys^{7}-D-Lys^{8}-Pro^{9}-Tyr^{10} at the i – (i+3) site [90]. Examination of biostability *in vitro* disclosed that T140 is not stable in mouse serum or in rat liver homogenate due to degradative deletion of Arg^{1}, Arg^{2}, Nal^{3} and Arg^{14} from *N*-/*C*-terminus, which causes drastic diminution in the T140 activity [81,87]. *N*-Terminal benzoylation and *C*-terminal amidation of T140 analogs suppressed their biodegradations leading to development of novel effective compounds, which showed increased bio-stability and even higher CXCR4-antagonistic activity. Through intensive SAR studies on *N*-terminal benzoylation, we found that an aromatic ring having an electron-withdrawing substituent, such as a *p*-fluorobenzoyl moiety, at the *N*-terminus constitutes a novel pharmacophore for strong anti-HIV activity [83]. 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 are biostable analogs, which have two orders of magnitude higher anti-HIV activity than T140 (Fig. (**1**)). Both peptides are promising lead compounds of 14-mer peptides for clinical development.

1-2. Low Molecular Weight CXCR4 Antagonists Based on Cyclic Pentapeptides

The four indispensable residues of T140, Arg^{2}, Nal^{3}, Tyr^{5} and Arg^{14}, are shown to be in close proximity to each other in the spatial structure by conformational analysis (Fig. (**1**)) [90]. Thus, the pharmacophore-guided approach based on these four residues might lead to the development of low molecular weight CXCR4 antagonists. Cyclic pentapeptides have been utilized as conformationally restricted templates disposing functional groups in medicinal chemistry [28,31,32,61,73,98], e.g. in efficient discovery of bioactive lead compounds such as integrin antagonists [31,32,98] and endothelin antagonists [28,73]. Thus, the library of cyclic pentapeptides containing these four residues of T140 (Arg^{2}, Nal^{3}, Tyr^{5} and Arg^{14}) was constructed. Initially, we devised possible library (total 192 compounds) using two L/D-Arg, L/D-Nal, L/D-Tyr and Gly (a spacer) to dispose indispensable functional groups of the T140 side-chain in space. Utilization of two focused libraries consisting of conformation-based and sequence-based libraries (total 60 compounds) led to rapid and efficient discovery of a hit compound, FC131, which has strong CXCR4-antagonistic activity comparable to that of T140 [27] (Fig. (**1**)). NMR and simulated annealing molecular dynamics (SA-MD) analysis of FC131 showed a backbone structure with a nearly symmetrical pentagonal shape. The pharmacophore-guided approach using cyclic pentapeptide templates proved to be useful for the lead discovery of low molecular weight CXCR4 antagonists.

We also wish to advance a research project to develop non-peptidic CXCR4 antagonists. Initially, we tried to investigate contributions of each amide bond in FC131 to the biological activity in order to develop FC131-derived pseudopeptides, in which the peptide character is reduced to access more drug-like structures. The practical utility of (*E*)-alkene dipeptide isosteres (EADIs) and reduced amide-type dipeptide isosteres (RADIs) has been intensively investigated in their introduction into biologically active peptides (Fig. (**2**)) [14,25,34,38,82,86,100]. Backbone replacements of amide bonds in peptides by EADIs and RADIs provide information on the contributions of the corresponding amide bonds to biological activity toward development of peptide-lead drugs. Thus, to identify the biological importance of these amide bond in FC131, EADIs and RADIs of Arg-Nal and Nal-Gly were synthesized

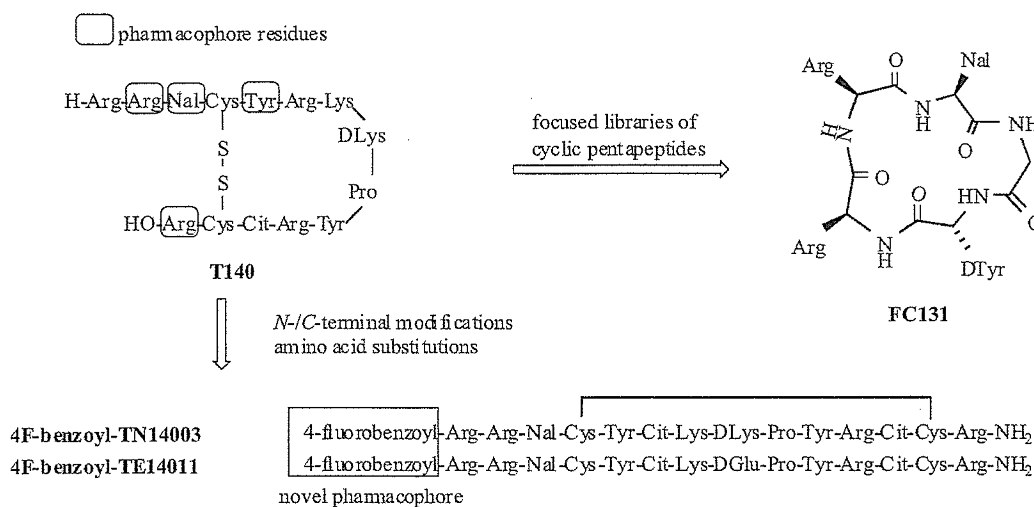


Fig. (1). Development of bio-stable T140 analogs and a low molecular weight CXCR4 antagonist, FC131, based on cyclic pentapeptide templates. Cit = L-citrulline.

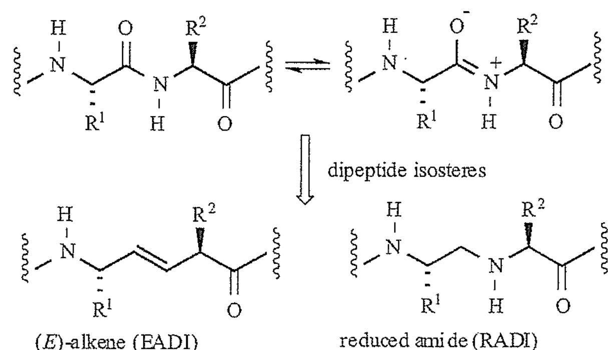


Fig. (2). Structures of (*E*)-alkene dipeptide isosteres (EADIs) and reduced amide-type dipeptide isosteres (RADIs).

[84], since the amide bonds between Arg^2 and Nal^3 and between Nal^3 and Cys^4 in T140 were cleaved by treatment with rat liver homogenates [81,87]. (Arg-L/D-Nal)-type EADIs, **5** and **8**, were synthesized by the combination of stereoselective aziridinyl ring-opening reactions and organozinc-copper-mediated *anti*-S_N2' reactions toward a single substrate of γ,δ-*cis*-γ,δ-epimino (*E*)-α,β-enoate **1** (Fig. (3)) [92,93]. A (Nal-Gly)-type EADI **11** was also synthesized by the samarium diiodide (SmI_2)-induced reduction of a γ-acetoxy-α,β-enoate **9** (Fig. (4)) [36,58]. RADIs of Arg-Nal and Nal-Gly were prepared by a standard method of reductive amination. Then, several FC131 analogs, in which the above isosteres were introduced, were prepared by the synthetic strategy of cyclic pentapeptides as reported in the previous paper (Table 1) [27]. (*E*)-Alkene substitutions, which can fix amide bonds in a plane form, cause the conformational restriction of the backbones. NMR and SA-MD analysis showed that the parent peptide, FC131, and these EADI-introduced pseudopeptides have nearly equal distances between any two β-carbons in all of the side chains: these compounds maintain similar dispositions of pharmacophores, suggesting that the biological differences among these compounds are derived only from the (*E*)-alkene–amide bond unit replacement. Substitutions of (Arg-L/D-Nal)-type EADIs for Arg-Nal caused a remarkable decrease in anti-HIV activity (Table 1, FC13110 and FC13414): The amide bonds of the Arg-L/D-Nal sequences were necessary for high potency, suggesting that either a deletion of the hydrogen bonding interaction with CXCR4 by the EADI introduction or an increase in hydrophobicity might be unsuitable. An (Arg-Nal)-type RADI-containing FC131 analog did not show significant anti-HIV activity (FC13126), suggesting that the planar nature of the amide bond is critical to maintain the pentagonal shape conformation for high anti-HIV activity. As in the case of Arg-Nal, an importance of the amide bond of the Nal-Gly sequence was indicated (FC13122 and FC13130). These results will provide useful information for the design of non-peptide CXCR4 antagonists derived from FC131.

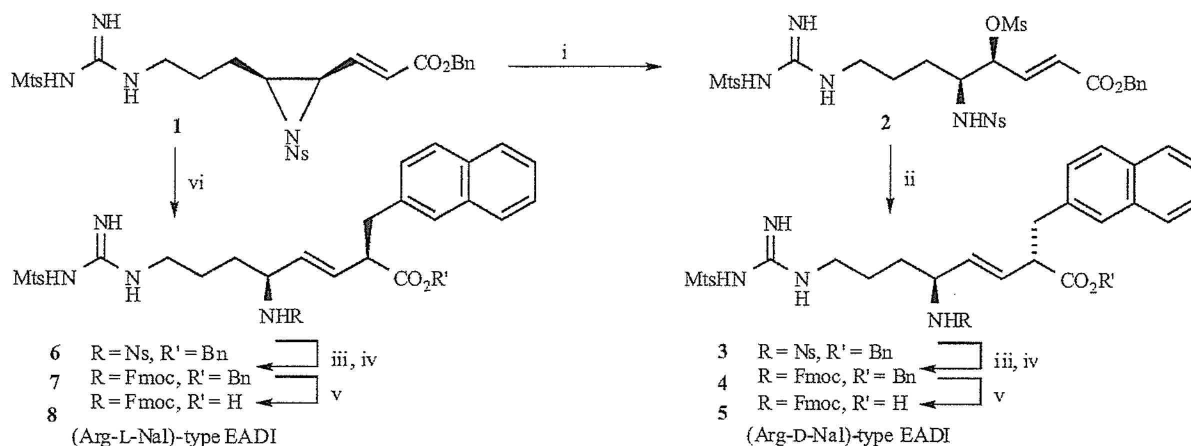


Fig. (3). Synthesis of (Arg-L/D-Nal)-type EADIs by the combination of stereoselective aziridinyl ring-opening reactions and organozinc-copper-mediated *anti*-S_N2' reactions toward a single substrate of γ,δ-*cis*-γ,δ-epimino (*E*)-α,β-enoate **1**. Mts = 2,4,6-trimethylphenylsulfonyl; Ns = 2-nitrobenzenesulfonyl. *Reagents:* (i) MsOH; (ii) 2-naphthylmethylCu(CN)ZnBr·BF_3; (iii) PhSH, K_2CO_3; (iv) Fmoc-OSu, Et_3N; (v) thioanisole, TFA; (vi) 2-naphthylmethylCu(CN)ZnBr·2LiCl.

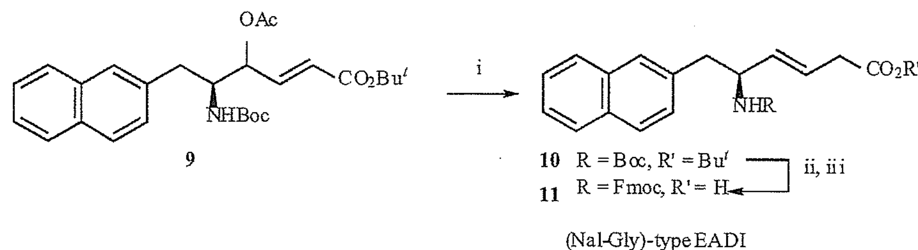
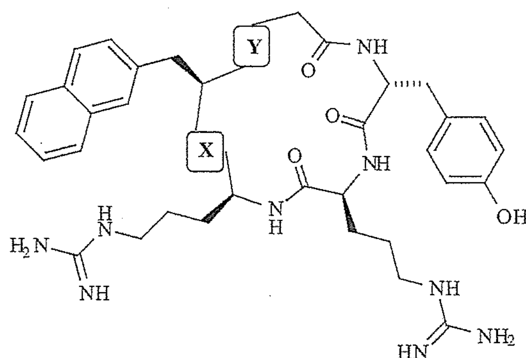


Fig. (4). Synthesis of a (Nal-Gly)-type EADI **11** by the samarium diiodide (SmI_2)-induced reduction of a γ-acetoxy-α,β-enoate **9**. *Reagents:* (i) SmI_2, ^{t}BuOH; (ii) anisole, TFA; (iii) Fmoc-OSu, Et_3N.

Table 1. Anti-HIV Activity of FC131 Analogs Containing Dipeptide Isosteres



Compd.	Sequence (cyclo)	X	Y	EC_{50} (μM)
FC131	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CO-NH-	-CO-NH-	0.073
FC13110	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CH=CH-	-CO-NH-	2.4
FC13126	(-D-Tyr-Arg-Arg-Nal-Gly-)	$-CH_2-NH-$	-CO-NH-	> 100
FC13122	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CO-NH-	-CH=CH-	2.4
FC13130	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CO-NH-	$-CH_2-NH-$	0.98
FC134	(-D-Tyr-Arg-Arg-D-Nal-Gly-)	-CO-NH-	-CO-NH-	1.9
FC13414	(-D-Tyr-Arg-Arg-D-Nal-Gly-)	-CH=CH-	-CO-NH-	9.1

1-3. Low Molecular Weight CXCR4 Antagonists Based on Structural Tuning of Cyclic Tetrapeptide-scaffolds

The cyclic pentapeptide, FC131, has a Gly residue as a spacer for cyclization. To diminish the ring size, the Nal-Gly sequence of FC131 was replaced by 4-amino-5-naphthalen-2-yl-pentanoic acid (γ-Nal), 4-amino-5-naphthalen-2-yl-pent-2-enoic acid (γ-(*E*)-Nal), etc. Among these γ-amino acid-containing cyclic tetrapeptides, an analog with substitution of γ-Nal for Nal-Gly, FC151, showed high CXCR4-antagonistic activity (IC_{50} = 54 nM) (Fig. (**5**)). This suggests that the Gly residue and the amide bond of the Nal-Gly sequence are not necessary for high activity. On the other hand, to optimize the ring structures of FC131-derived compounds, the utility of templates different from that of cyclic pentapeptides was investigated. Since the four essential amino acid residues of T140 are disposed in close vicinity each other due to the disulfide bridge and cyclic peptides having the Arg-Arg-Nal sequence, such as FC131, showed high CXCR4-antagonistic activity, we designed and prepared disulfide-bridged cyclic peptide libraries involving the *N*-3-guanidinopropanoyl-L/D-Cys(S-)-L/D-Arg-L/D-Nal-L/D-Cys(S-)-NH_2 (or –tyramine) sequence (total 32 compounds). Among these compounds, FC205 [*N*-3-guanidinopropanoyl-Cys(S-)-Arg-Nal-D-Cys(S-)-NH_2] and FC225 [*N*-3-guanidinopropanoyl-Cys(S-)-Arg-Nal-D-Cys(S-)-tyramine] exhibited significant CXCR4-antagonistic activity (IC_{50} = 690 and 530 nM, respectively) (Fig. (**6**)).

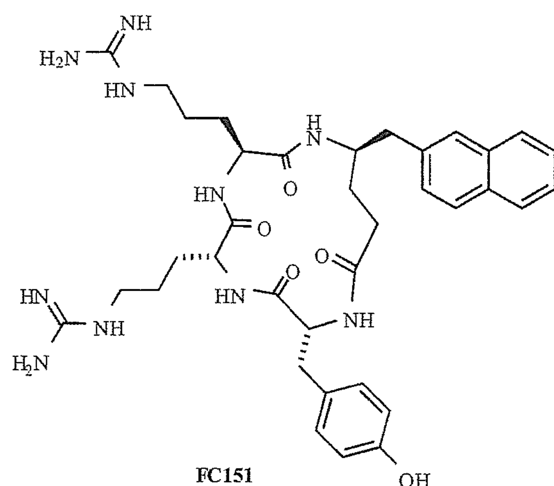


Fig. (5). Structure of a γ-Nal-containing cyclic tetrapeptide, FC151.

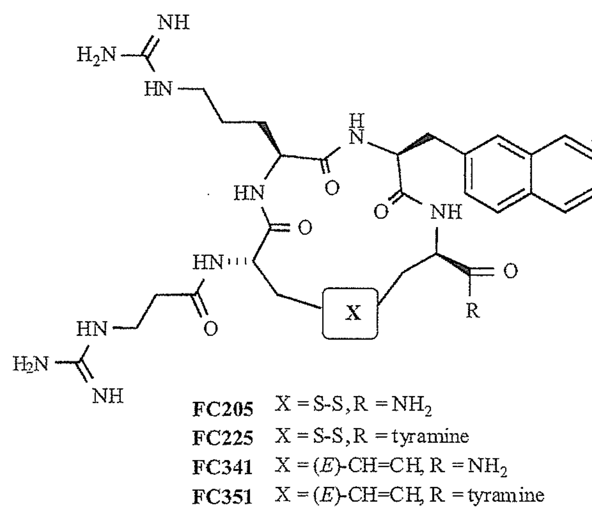


Fig. (6). Structures of cyclic analogs of FC131 that were bridged by a disulfide or an olefin.

FC205 and FC225 have a common combination of chiralities of composed amino acids, suggesting that these compounds form similar conformations. Furthermore, cyclic analogs that were bridged by an olefin instead of a disulfide in FC205 and FC225 were synthesized. These olefin-bridged peptides, FC341 and FC351, showed moderate CXCR4-antagonistic activity, which is lower (IC_{50} = 1-10 μM) compared to that of FC205 and FC225 (Fig. (**6**)). Exploratory studies on further downsizing and reduction of peptide character, including the discovery of useful scaffolds besides cyclic tetra- and pentapeptides, are now in progress. In addition, we have also developed other small-sized CXCR4 antagonists involving the novel pharmacophore such as a *p*-fluorobenzoyl moiety (data will be published).

1-4. Anti-cancer-metastasis and Anti-cancer Cell Progression Activities of CXCR4 Antagonists

CXCR4 is a seven transmembrane (7TM) GPCR, which transduces signals of its endogenous ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12 [6,54,56,94]. The interaction of CXCL12 and CXCR4 plays an important role in the migration of progenitor cells during embryologic development of the cardiovascular, hemopoietic and central nervous systems. Recently, this interaction has been shown to be relevant to several problematic diseases, such as cancer cell metastasis/progression [5,7,29,37,40,41,49,50,51,62-64,67-69,77,78,85,96] and rheumatoid arthritis (RA) [55], in addition to HIV infection. Malignant cells from at least 23 different types of cancer express CXCR4 [3]: e.g. B cell chronic lymphocytic leukemia (CLL) [96], pre-B acute lymphoblastic leukemia (ALL) [37], non-Hodgkin lymphoma [5], multiple myeloma [64], pancreatic cancer [41,49], prostate cancer [77], breast cancer [50,85], ovarian cancer [68,69], neuroblastoma [29], kidney cancer [67], small cell lung cancer (SCLC) [7,40], melanoma [51,62,78] and brain cancer [63]. A. Müller *et al.* reported that CXCR4 is highly expressed on the surface of human breast cancer cells, while CXCL12 is highly expressed in lymph nodes, bone marrow, lung and liver, which constitute the common metastasis destinations of breast cancer [50]. Pulmonary metastasis of breast cancer cells was significantly inhibited by neutralizing anti-CXCR4 antibody [50] and 4F-benzoyl-TN14003 [85] in SCID mice, suggesting that the suppression of CXCL12/CXCR4 interaction may represent a novel therapeutic strategy against breast cancer metastasis that involves this ligand-receptor system. Furthermore, another biostable T140 analog, 4F-benzoyl-TE14011, significantly suppressed pulmonary metastasis of melanoma cells by using a sustained drug release formulation of biodegradable poly D,L-lactic acid (PLA) microcapsules [78]. In addition, T140 analogs exhibited significant inhibitory effects on the progression of pancreatic cancer cells [41,49], small cell lung cancer cells [7], pre-B ALL cells [37], CLL B cells, etc. These results suggest that CXCR4 antagonists, especially inverse agonists that have no CXCL12-like agonistic activity, have the potential of promising agents for cancer chemotherapy.

1-5. Anti-rheumatoid Arthritis (RA) Activity of CXCR4 Antagonists

Rheumatoid arthritis (RA) is an annoying disorder, which is mainly caused by the $CD4^+$ memory T cell accumulation in the inflamed synovium. T. Nanki *et al.* found that CXCL12 which is released in the RA synovium stimulates migration of the memory T cells that highly express CXCR4, and thereby inhibits T cell apoptosis [55]. This suggests that the CXCL12/CXCR4 interaction plays an important role in T cell accumulation in the RA synovium. 4F-benzoyl-TN14003 significantly suppressed the delayed-type hypersensitivity (DTH) response induced by sheep red blood cells (SRBC) and collagen-induced arthritis (CIA), which represent *in vivo* mouse models of this pathology [80]. These findings suggest that the CXCL12/CXCR4 axis may become a useful therapeutic target for RA chemotherapy, and that CXCR4 antagonists also have great promise as anti-RA agents. Actually, P. Matthys *et al.* reported the first paper to show that a CXCR4 antagonist, AMD3100, which is described in the next section, had anti-RA activity [46].

1-6. Other CXCR4 Antagonists

In 1997, three CXCR4 antagonists, the bicyclam AMD3100 (AnorMED, Inc.) [65] (Fig. (**7**)), T22 [52] and ALX40-4C (Ac-[D-Arg]$_9$-NH_2; NPS Allelix) [19], were incidentally reported at the same time. These compounds have a common character: high basicity. The amino acid residues in CXCR4 used for interaction with T140 and AMD3100 were comparatively studied using Ala-scanning mutagenesis and computation docking simulation analyzed by J. O. Trent *et al.* [95]. Critical residues for both T140 and AMD3100 bindings mainly exist in the second extracellular loop (ECL2) of CXCR4, but these are slightly different, indicating that the mechanisms of these antagonists are different. In association with AMD3100, an *N*-pyridinylmethylene cyclam (monocyclam) AMD3465 (AnorMED, Inc.) [16], a non-cyclam AMD8665 (AnorMED, Inc.) [70] and AMD070 (AnorMED, Inc.) [97] were found as new CXCR4 antagonists [41] (Fig. (**7**)). Twin functional agents based on AMD3100 and galactosylceramide (GalCer) analog conjugates were reported [15]. Another low molecular weight CXCR4 antagonist, KRH-1636 (Kureha Chemical & Sankyo), which might be derived from intensive modification of the *N*-terminal tripeptide of T140, Arg-Arg-Nal, was reported as an orally bioavailable agent [35]. AMD3465, AMD8665 and KRH-1636 have a 4-[[[pyridin-2-yl-methyl]amino]methyl]phenyl group as a common substructure unit, which might be a critical pharmacophore. Distamycin analogs, such as NSC651016 [33], and a flavonoid compound, ampelopsin [42], were found to be CXCR4 antagonists that have different structures. Several Arg-mimetic conjugates, CGP64222, R3G and NeoR, were also reported as cationic CXCR4 antagonists [8,9,13]. These low molecular weight antagonists including our lead compounds seem to be promising agents for chemotherapy of AIDS, cancer, RA, etc.

1-7. Advantageous Characters of T140-derived CXCR4 Antagonists

The emergence of MDR HIV-1 variants is one of the most serious problems in the clinical treatment of HIV-1-infectious and AIDS patients. H. Nakashima *et al.* found that T140 showed remarkable delaying effect against the generation of drug-resistant strains *in vitro* [39]. The difficulty of the generation of drug-resistant HIV-1 strains might be a great advantage of T140-derived compounds.

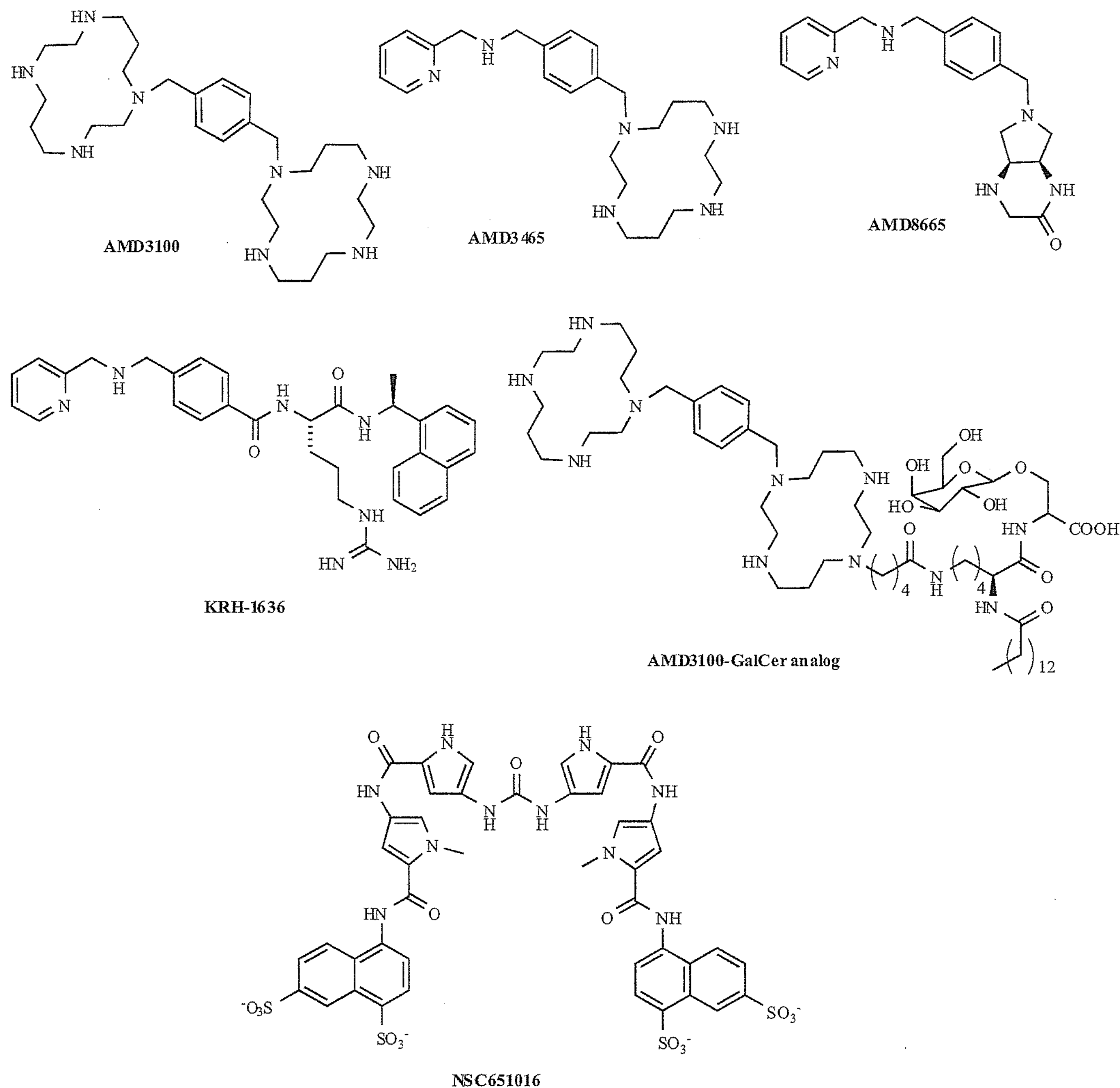


Fig. (7). Other CXCR4 antagonists.

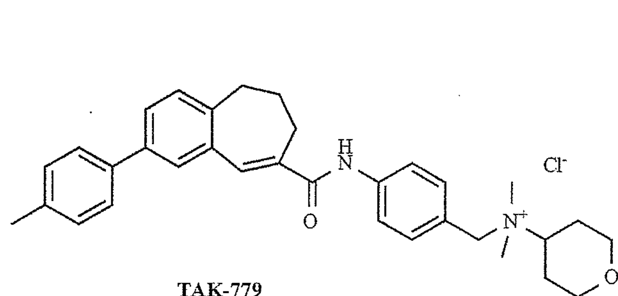
Antagonists are normally classified into inverse agonists that show no agonistic activity and partial agonists that show weak agonistic activity. Partial agonists of CXCR4 have CXCL12-like agonistic activity through CXCR4 and might activate cancer cells and memory T cells that highly express CXCR4. Especially, in terms of cancer and RA chemotherapy, inverse agonists have a clinical advantage, since they do not show any activating effects on CXCR4. S. C. Peiper et al. revealed that T140 is an inverse agonist, whereas AMD3100 and ALX40-4C are partial agonists, based on evidences that T140 treatment of CXCR4 wild type and constitutively active mutant (CAM), which were coupled to the pheromone response pathway in yeast, reduced autonomous signaling, while AMD3100 or ALX40-4C treatment induced the partial G protein activation in a dose-dependent manner [102]. This difference of the actions of AMD3100 and T140 toward CXCR4 might be caused by the difference of binding sites of these agents on CXCR4 (Section 1-6).

Cell adhesion-mediated drug resistance (CAM-DR) represents one of the serious problems in a clinical use of several anti-cancer drugs. T140 analogs showed significant effects overcoming CAM-DR in *in vitro* CLL, ALL and SCLC experiments [7,37].

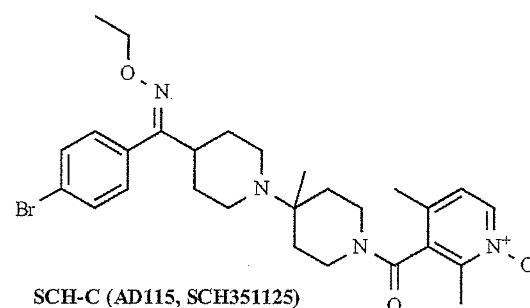
1-8. CCR5 Antagonists

Several chemokine antagonists against another co-receptor CCR5, which is used by primary HIV (R5-HIV-1) strains, have been developed. The validity of this research for development of CCR5 antagonists is based on the

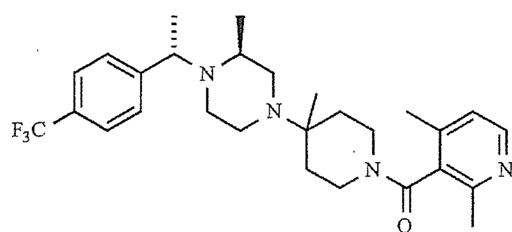
finding that individuals who have the CCR5 32 deletion mutation are healthy and strongly protected from HIV-1 infection [4]. CCR5 antagonists that have been reported to date are follows (Fig. (**8**)): a quaternary ammonium anilide, TAK779 [2], its orally bioavailable derivative, TAK220 (Takeda), a piperidino-piperidine, SCH-C (AD115, SCH351125) [59,74], piperidinopiperazine series AD101 (SCH350581), AD114 (SCH350634) [76], SCH-D (SCH417690) [75] (Schering-Plough), a spiro-diketopiperazine, AK602/ONO-4128/GW873140 (Ono & GlaxoSmithKline) [44], a 2-phenyl-4-(piperidin-1-yl)butanamine compound, a 1,3,4-trisubstituted pyrrolidine, MRK-1, its cyclopentane analog, CMPD 167 (Merck) [24], UK-427,857 (Pfizer) [59], AMD887 (AnorMED, Inc.) [66], synthetic RANTES analogs, AOP-, NNY-, and PSC-RANTES [30], etc. Individuals who have this CCR5 mutation completely lack functional CCR5 but are healthy. Since it suggests that blocking the function of CCR5 might not have any significant side-effects, CCR5 antagonists are thought to be useful as anti-HIV agents. However, CCR5 antagonists cannot suppress the emergence of the more pathogenic HIV-1 (X4-HIV-1) strains that contribute to the accelerated decrease in $CD4^+$ T cells. In a combinational use with the above CCR5 antagonists and CXCR4 antagonists, no viral replication with any HIV-1 strains was observed *in vitro* [66]. CCR5 antagonists also have the potential of promising agents for AIDS chemotherapy, especially in their combinational use.



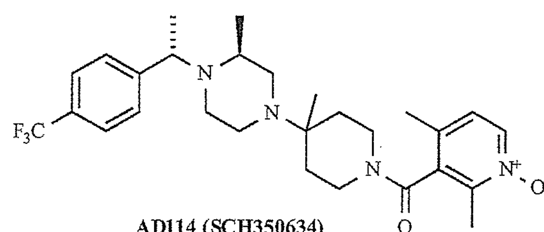
TAK-779



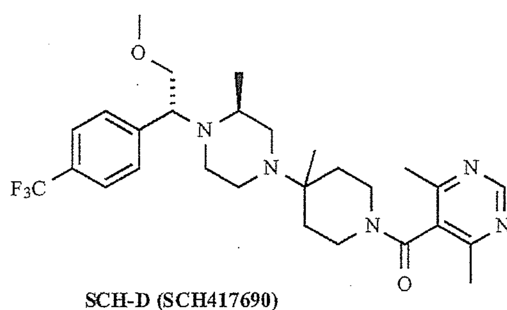
SCH-C (AD115, SCH351125)



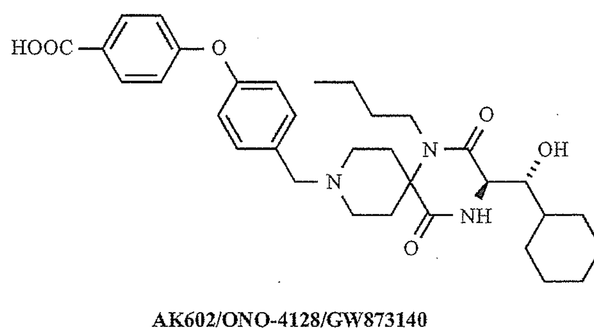
AD101 (SCH350581)



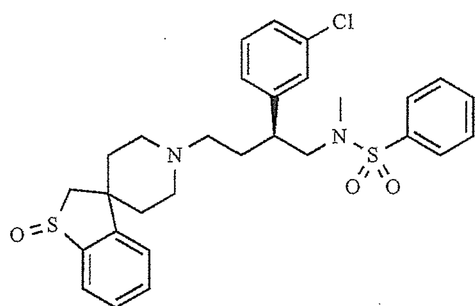
AD114 (SCH350634)



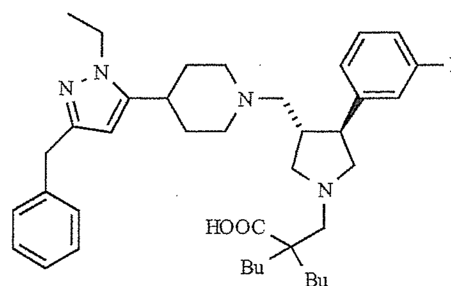
SCH-D (SCH417690)



AK602/ONO-4128/GW873140



2-phenyl-4-(piperidin-1-yl) butanamine compound



MRK-1

(Fig. 8). contd.....

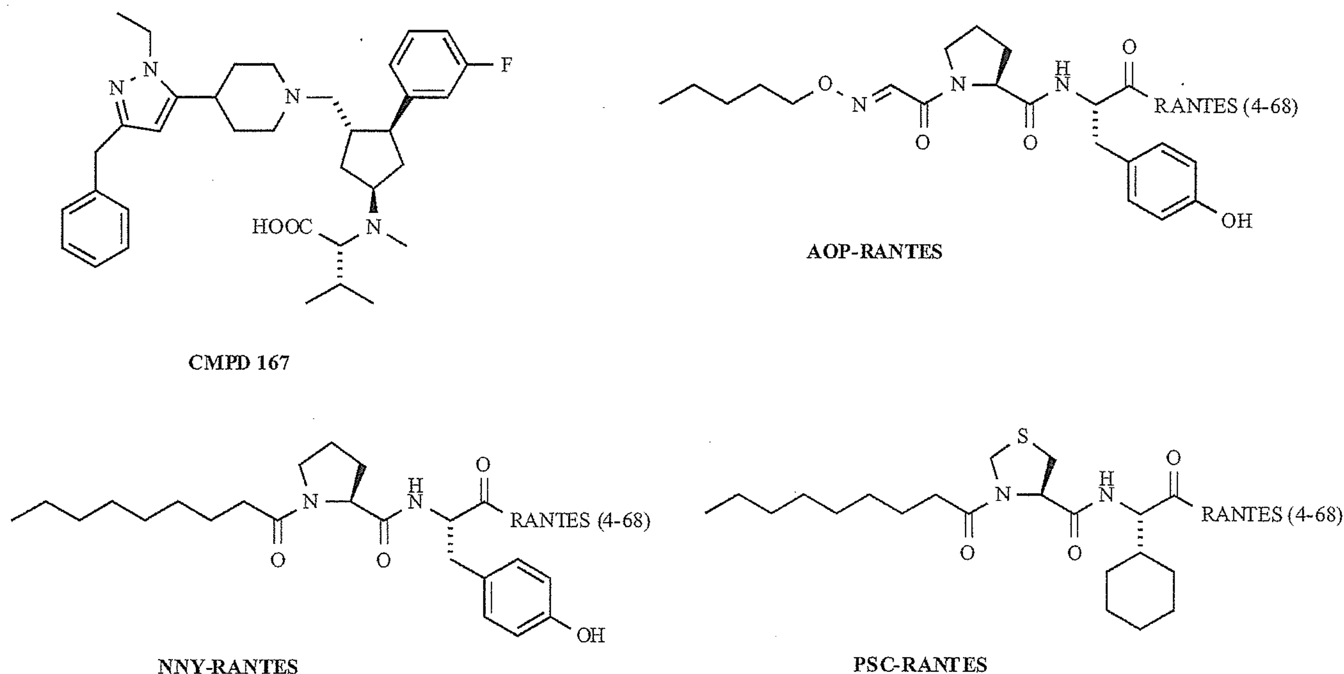


Fig. (8). CCR5 antagonists.

2. Fusion Inhibitors Targeting the Dynamic Supramolecular Mechanism

2-1. Gp41-Fragment-remodeling Peptides

The binding of gp120 to CCR5/CXCR4 triggers the formation of the trimer-of-hairpins structure of gp41 and the subsequent fusion of HIV/cell membranes, as described in the introduction. Thus, a dynamic supramolecular mechanism involving membrane fusion becomes rational targets for inhibitors against HIV-1 replication. The trimer-of-hairpins structure of gp41 is formed as a bundle of six α-helices, which involve antiparallel packing by both inner three-stranded coiled coils derived from the gp41 *N*-terminal helical region and the outer three-stranded coiled coils derived from the *C*-terminal helical region (Fig. (**9**)) [11]. The subdomain is composed of two peptides, N51 and C43, which are *N*-region 51 residue and *C*-region 43 residue peptides, respectively [43]. According to previous papers, several *C*-peptides derived from *C*-terminal helical region inhibited bundle formation of six α-helices and thereby HIV-1 infection. A *C*-peptide, C34, which has the native sequence of a gp41 fragment, exhibited potent inhibitory activity against HIV-1 fusion [10]. However, C34 has a defect in solubility in aqueous media. Thus, we developed highly soluble C34 analogs (SC peptides) by artificial remodeling of C34 (Fig. (**9**)) [57]. In the helical wheel diagram of C34, the amino acid residues at *a*, *d*, and *e* positions, which are essential for interaction with the inner coiled-coil strand formed by an *N*-region peptide (N36), were maintained without any substitutions, whereas non-

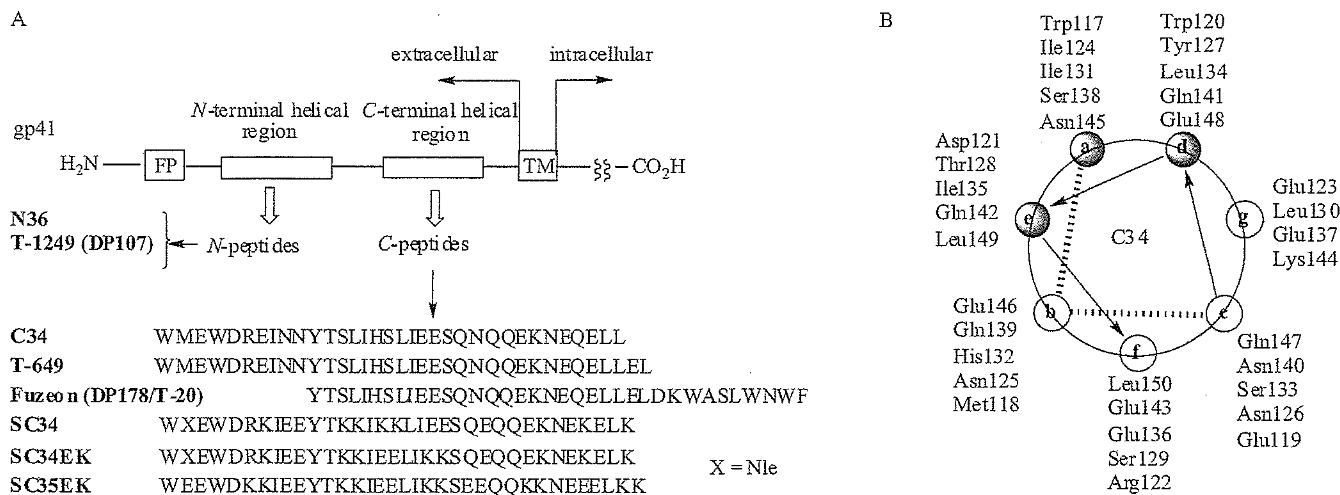


Fig. (9). A: Schematic representation of gp41 and sequences of *C*-peptides. FP = Fusion peptide; TM = transmembrane domain; B: Helical wheel representation of C34. Residues are numbered based on HIV-1 NL4-3 gp41.

conserved residues at *b*, *c*, *f*, and *g* positions, which are located in solvent-accessible region, were replaced by Glu or Lys. Several side-chain ion pairs of Glu-Lys formed between i and i+4 positions is thought to enhance solubility and α-helicity of C34 analogs (Fig. (**10**)). The aqueous solubility of SC peptides, SC34, SC34EK and SC35EK, was increased by more than 3 orders of magnitude, compared to that of C34. Analytical ultracentrifugation sedimentation of the N36/SC peptide complexes indicated that each N36/SC peptide forms a six-molecule complex consisting of three molecules each of N36 and SC peptide. Comparison of melting temperatures of the complexes based on the changes in $[\theta]_{222}$ of CD analysis as a function of temperature revealed that stabilities of the N36/SC peptide complexes were remarkably increased, compared to that of the N36/C34 complex. The six-helix bundle structures of the N36/SC peptide complexes were confirmed by X-ray analysis. Anti-HIV activities of these SC peptides were superior or comparable to that of C34, and ten-fold stronger than that of Fuzeon (DP178, T-20, Trimeris & Roche) [99] in the multinuclear activation of the galactosidase indicator assay (MAGI assay). Furthermore, SC peptides were active even against a Fuzeon-resistant strain. As a result, highly soluble and potent fusion inhibitors, SC34, SC34EK and SC35EK, have been developed by the remodeling of C34 based on introduction of Glu-Lys pairs into the solvent-accessible surface of the six-helix bundle. Studies on a further increase in helicity and anti-HIV activity, downsizing and reduction of peptide character are now in progress.

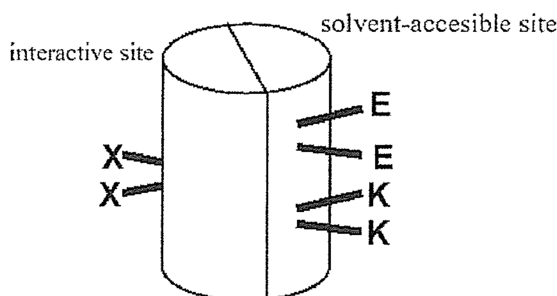


Fig. (10). Formation of side-chain ion pairs of Glu-Lys between i and i+4 positions and appropriate disposition of X-residues by α-helix formation.

2-2. Other Fusion Inhibitors

Approval of FDA to clinical use of Fuzeon in March, 2003, has brought us a great hope toward fusion inhibitors as a new class of anti-HIV drugs against MDR HIV-1 strains. C34, T-649 [18], Fuzeon, SC peptides are all 34—36-mer peptides derived from *C*-terminal helical region of gp41, as described in the above section. T-1249 (DP107, Trimeris & Roche) [47], which is a 38-mer peptide derived from *N*-terminal helical region of gp41, is Trimeris & Roche's second inhibitor active against Fuzeon-resistant isolates. Several researchers have tried to discover small non-peptide inhibitors that block gp41 activation [60,71,72]. Membrane fusion is a valid target for inhibition of an HIV-1 entry due to clinical use of Fuzeon. However, low molecular weight inhibitors, which are highly potent and really useful, have not yet been discovered. S. C. Harrison, S. L. Schreiber et al. identified non-natural binding elements that contribute to the formation of a stable complex with the inner coiled-coil strand and to inhibition of membrane fusion using a biased combinatorial chemistry library [23]. Researches on development of useful fusion inhibitors (small organic compounds) are ongoing in many laboratories.

CONCLUSION

The recent researches on the development of anti-HIV agents are assorted into two orthogonal approaches in general terms: 1) the improvement of conventional drugs, such as reverse transcriptase inhibitors and protease inhibitors, which are classified in known drug categories; and 2) the discovery of new drugs with novel action mechanisms. This review article has focused on the latter issues (2). CXCR4 antagonists derived from T140 including its low molecular weight analogs were developed as HIV co-receptor inhibitors. Furthermore, since the CXCL12/CXCR4 system is involved in progression and metastasis of several types of cancer cells and migration of the memory T cells, these CXCR4 antagonists are also useful compounds for cancer and RA chemotherapy. In association to the appearance of Fuzeon, highly soluble and potent fusion inhibitors, a series of SC peptides, have been developed by the remodeling of C34 based on introduction of Glu-Lys pairs. These therapeutic candidates that block the early stage of the HIV replication would be idealistic in the complement of HAART.

ACKNOWLEDGEMENTS

This work was supported in part by a 21st Century COE Program "Knowledge Information Infrastructure for Genome Science", a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and the Japan Health Science Foundation. The authors thank our collaborators: Profs. Naoki Yamamoto (National Institute of Infectious Diseases/Tokyo Medical and Dental University), Hideki Nakashima (St. Marianna University), Hiroaki Mitsuya (Kumamoto University), Toshio Hattori (Tohoku University), Michinori Waki (Kyushu University), Masao Matsuoka (Kyoto University), Yuji Kobayashi (Osaka University), Tsutomu Murakami (National Institute of Infectious Diseases), Ryuichiro Doi (Kyoto University), Masayuki Imamura (Kyoto University), John O. Trent (University of Louisville) and Stephen C. Peiper (Medical College of Georgia), Drs. Zixuan Wang (Medical College of Georgia), Jan A. Burger (Freiburg University), Shuichi Kusano (St. Marianna University), Shigemi Terakubo (St. Marianna University), Rie Igarashi (St. Marianna University), Mitsuko Takenaga (St. Marianna University), Eiichi Kodama (Kyoto University) and Shinya Oishi (University of Sizuoka) and Mr. Kenichi Hiramatsu (Kyoto University), Satoshi Ueda (Kyoto University) and Takanobu Araki (Kyoto University) and Ms. Akane Omagari (Kyoto University) and Miki Nakamura (Kyoto University).

ABBREVIATIONS

HIV = Human immunodeficiency virus

X4-HIV-1	=	T cell line-tropic HIV-1
HAART	=	Highly active anti-retroviral therapy
MDR	=	Multi-drug resistant
R5-HIV-1	=	Macrophage-tropic HIV-1
AIDS	=	Acquired immunodeficiency syndrome
SAR	=	Structure-activity relationship
T22	=	[Tyr^{5,12}, Lys^{7}]-Polyphemusin II
Nal	=	L-3-(2-Naphthyl)alanine
SA-MD	=	Simulated annealing molecular dynamics
EADI	=	(*E*)-Alkene dipeptide isostere
RADI	=	Reduced amide-type dipeptide isostere
7TM GPCR	=	7-Transmembrane segment G-protein-coupled receptor
SDF-1	=	Stromal cell-derived factor-1 = CXCL12
RA	=	Rheumatoid arthritis
CLL	=	Chronic lymphocytic leukemia
ALL	=	Acute lymphoblastic leukemia
SCLC	=	Small cell lung cancer
SCID	=	Severe combined immunodeficient
PLA	=	Poly D,L-lactic acid
DTH	=	Delayed-type hypersensitivity
SRBC	=	Sheep red blood cells
CIA	=	Collagen-induced arthritis
ECL	=	Extracellular loop
GalCer	=	Galactosylceramide
CAM	=	Constitutively active mutant
CAM-DR	=	Cell adhesion-mediated drug resistance
RANTES	=	Regulated on activation, normal T cell expressed and secreted
CD	=	Circular dichroism
MAGI	=	Multinuclear activation of the galactosidase indicator
FDA	=	Food and Drug Administration

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Received: 31 December, 2004 **Accepted: 28 May, 2005**

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QSAR & Combinatorial Science QCS

A simple, Automated Quasi-4D-QSAR, Quasi-multi Way PLS Approach to Develop Highly Predictive QSAR Models for Highly Flexible CXCR4 Inhibitor Cyclic Pentapeptide Ligands Using Scripted Common Molecular Modeling Tools

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Keywords: CXCR4 inhibitors, automated quasi-multi-way PLS, Sybyl Programming Language (SPL), conformers, alignment

Received: October 5, 2004; Accepted: January 5, 2005

Abstract
A methodology for developing highly predictive ($r^2 > 0.9$) 3D-QSAR models ($q^2 > 0.7$) based on sixteen flexible CXCR4 cyclic pentapeptide inhibitors is reported. The effective automated use of common molecular modeling tools such as Macromodel and Sybyl is demonstrated. The recently developed multi-way Partial Least Square (PLS) approach for discovering the bioactive conformers and alignment was used in a quasi-multi-way PLS approach. Twenty-five conformers for each compound were generated by Monte Carlo conformational searches and alignments (seventy five in total) were based on the templates from the three most active compound conformers. These were aligned in Sybyl Molecular Databases and Sybyl Molecular Spreadsheets. All repetitive tasks were automated by use of simple Unix shell, python and Sybyl Programming Language (SPL) scripts. This efficient protocol furnished three 3D-QSAR models with q^2 values of 0.714, 0.734 and 0.657 and predictive r^2 values of 0.951, 0.990, and 0.956 respectively. The best 3D-QSAR model predicted the biological activities of nine test compounds from all activity ranges within 0.5 log units.

1 Introduction

The human chemokine receptor CXCR4 is the stromal cell-derived factor (SDF-1α) chemokine receptor. Several diseases have been reported to be linked to CXCR4 such as AIDS [1, 2], cancer metastasis and progression [3], and rheumatoid arthritis [4]. Masuda *et al.*[5] reported an eighteen-residue peptide T22, to be a CXCR4 inhibitor. T140, a fourteen residue peptide, was reported by Tamamura *et al.* [6] as a more potent CXCR4 inhibitor. Fujii *et al.* [7] have recently employed orthogonal combination of conformations with sequence-based libraries for the discovery of potent cyclic pentapeptides as CXCR4 inhibitors. The biological data reported by Fujii *et al.* [7] is used in this QSAR study.

The Quantitative Structure Activity Relationship (QSAR) is among the most widely used techniques in rational drug design. Following the pioneering work of Hansch *et al.* [8] in 2D-QSAR, several techniques like Comparative Molecular Field analysis (CoMFA) [9], Molecular Shape Analysis MSA [10], distance geometry [11], molecular similarity matrices [12], Comparative Molecular Similarity Index Analysis (CoMSIA) [13], Condensed-phase Optimized Molecular Potentials for Atomistic Simulation Studies (COMPASS) [14], and Hypothetical Active Site Lattice (HASL) [15] have been developed for three dimensional QSAR (3D-QSAR). Among these techniques, CoMFA has been widely used [16] as it provides for the visual display of electrostatics and steric fields of the regions important for biological activity. However, CoMFA models have been reported to be very sensitive to the chosen bioactive conformations [17], selection of alignment rules [18], spatial orientation and grid sizes [19]. The issue of the choice of the bioactive conformation has been addressed with techniques such as conformational averaging (conformational ensembles) [20], employing several conformers in a multi-way data array [21], multi-conformational ligand representation [22], tensor decomposition [23], and three-way-PLS analysis [24]. The second important issue of alignment rule selection has been addressed by the Field Based Similarity Searching (FBSS) program for automated alignment [25], automated alignment using

 DOI: 10.1002/qsar.200430912 © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

the GOLD program [26], multiple orientation ligand representation [22], use of docking algorithms and protocols for aiding in alignment selection [27], use of Generalized Procrustes Analysis (GPA) for consensus molecular alignment [28], use of local structure analysis (molecular footprints) for partial molecular alignment [29] and cross-validated R^2 guided region selection (q^2-GRS) for CoMFA [19]. The development of 3D-QSAR models for highly flexible ligands is challenging [30]. The choice of the active conformer and the selection of the alignment rule may be guided by available experimental data like the X-ray crystal structure of ligand-receptor complexes or the solution NMR structure of active analogs.

The cyclic pentapeptide CXCR4 inhibitors [7] are highly flexible, with hundreds of possible conformers within a few kcal/mol range of the global minimum. There are many reports on sophisticated techniques to handle the problem of choosing the active conformation, such as conformation ensembles and multi-way data arrays. There are also several ways to handle the problem of alignment in CoMFA based 3D-QSAR such as using FBSS or GOLD. However, to our knowledge, a simple, efficient method, employing regular molecular modeling tools, to develop CoMFA based 3D-QSAR models for highly flexible ligands has not been reported. We therefore, developed the methodology reported here to provide such a method.

2 Material and Methods

All computation work was done on Silicon Graphics Octane workstations and an Origin 2000 server running the IRIX 6.5 operating system. The molecular modeling software used were Macromodel version 7.0 [31] and Sybyl version 6.9.1 [32]. Python programs were run using Python version 2.3 on a Dell PC running the Windows XP operating system.

2.1 Data Set

We chose a subset of twenty five cyclic pentapeptide inhibitors of CXCR4 whose IC_{50} values were determined by displacement binding of [^{125}I]SDF-1 to CHO transfectants stably expressing CXCR4 [7]. In brief, the CXCR4 transfectants were incubated with [^{125}I]SDF-1 (0.15 nM) on a shaker at 4 °C for one hour in the presence and absence of the cyclic pentapeptides. The unbound isotope was separated by centrifugation and counted. The inhibitory ability of the cyclic pentapeptides was analyzed in triplicate at 0.01, 0.10, 1.0, and 10.0 μM concentrations. The specific IC_{50} values were determined by Scatchard analysis [7].

The cyclic pentapeptide sequences and their respective bioactivities are presented in Table 1. We divided this set into a training set of sixteen compounds and test set of nine compounds. The training set comprises of the cyclic peptide sequence Tyr-Arg-Arg-Nal-Gly. The training set had the unnatural amino acid Nal (NapthylAlanine) placed in all possible positions around the ring.

The NMR structure of the most active compound FC131 has been reported by Fujii *et al.* [7], and it was made available to us by Trent *et al.* [33]. In the absence of the X-ray crystal structure of the ligand-receptor complex, the use of the NMR based structure for the bioactive conformation, has been reported [34]. We, therefore, used the NMR structure of FC131 as the template, by keeping the cyclic pentapeptide backbone identical for all of the training and test data set molecules.

2.2 Conformer selection

The Maxwell-Boltzmann distribution gives the population of various conformers at any given temperature. This has been used for conformer population ratio studies of epothilones [35]. For highly flexible molecules, like the cyclic pentapeptides, there are hundreds of possible conformers within a 5 kcal/mol energy range of the global minima. We chose the twenty five lowest energy conformers of every training set compound for the QSAR study. These conformers were within 3–5 kcal/mol energy range of the respective global minimas (see Table 1).

2.3 Molecular structure building and conformational search

The structures of all the cyclopentapeptides were built in Macromodel and minimized using the AMBER* force field [36]. The Polak–Ribiere conjugate gradient method [37] was employed with a gradient convergence criteria of 0.01 kcal/Å-mol. The conformation of the central core cyclic pentapeptide ring was preserved, by employing positional constraint of 239.23 kcal/mol-Å on the fifteen backbone ring atoms. The conformational search was performed in Macromodel using the Monte Carlo Multiple Minimum method [38] (10,000 steps, 11.96 kcal/mol energy window, with subsequent of minimization of 10,000 steps to ensure convergence). Water solvation was simulated using the Generalized Born (GB/SA) implicit solvation method [39]. All the backbone bonds of the of the central pentapeptide ring (N-Cα and Cα-C) were fixed with a torsion constraint of 2,392.34 kcal/mol-Å.

Macromodel creates a single output file containing the conformations, starting with the global minimum conformer, followed by the rest of the conformers in the order of ascending energy. The individual data files of the conformers (twenty five each) can be created in Macromodel, by sequentially reading the conformers and then saving each of them. However, since we would have had to repeat this process (16×25) four hundred times, we automated this task by using a python script [40]. All the data files were converted into mol2 files using BABEL, a file format conversion utility [41]. The repetitive task of converting four hundred files was performed using a Unix shell script [42].

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