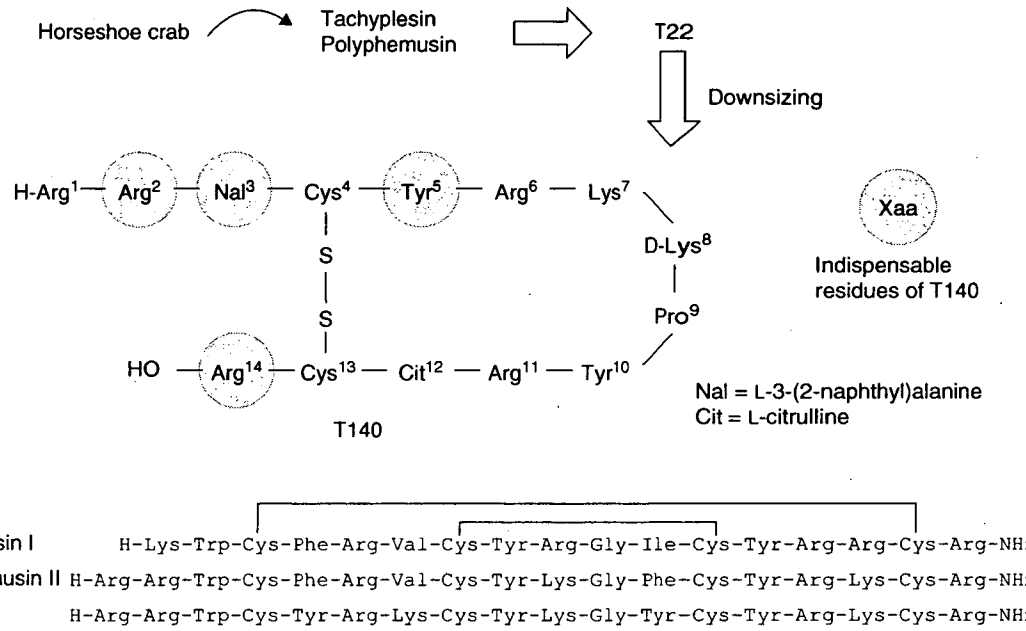


Figure 2. Development of the CXCR4 antagonist T140 based on self-defence peptides of horseshoe crabs. Two disulfide bridges of tachyplesin I, polyphemusin II and T22 are shown by solid lines.

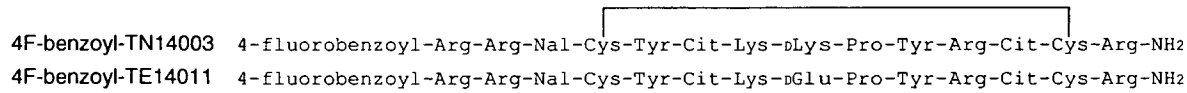


Figure 3. Structures of biostable T140 derivatives, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011.

Nuclear magnetic resonance (NMR) and Ala-scan studies revealed that T140 forms an antiparallel β-sheet structure that is maintained by a disulfide bridge between Cys4 and Cys13 and connected by a Type II β-turn with Lys7-D-Lys8-Pro9-Tyr10 at the i – (i+3) site [46], and that four residues in T140, Arg2, L-3-(2-naphthyl)alanine (Nal)3, Tyr5 and Arg14, are indispensable for high potency (**Figure 2**) [47].

3. Development of biostable T140 derivatives

Examination of biostability *in vitro* revealed that T140 is not stable in mouse/feline serum or in rat liver homogenate [48,49]. Degradative deletion of indispensable residues (Arg14 in serum; Arg2, Nal3 and Arg14 in liver homogenate) from N-/C-terminus drastically diminished the efficacy of T140. N- and C-terminal modifications of T140 analogues suppressed the biodegradations, leading to development of novel effective compounds, which showed highly CXCR4-antagonistic activity and increased biostability, in association with substitution of several amino acids. Through the N-terminal modification studies, the authors found that an electron-deficient aromatic ring at the N-terminus is preferable for strong anti-HIV activity, and that a substituted benzoyl group such as a p-fluorobenzoyl moiety at the N-terminus constitutes a novel pharmacophore for strong anti-HIV activity [50]. p-Fluorobenzoyl moiety-containing analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, are promising lead compounds to date, which have two orders of magnitude higher anti-HIV activity than T140 and enhanced stability in serum/liver homogenate (**Figure 3**).

4. The difficulty of the generation of a T140-resistant HIV strain

The generation of drug-resistant viral strains is one of the most serious problems in clinical AIDS chemotherapy. An entry/fusion inhibitor, enfuvirtide (DP178, T-20, Fuzeon, Trimeris & Roche), has attracted a great deal of attention as the third generation of anti-HIV drugs, because it is effective against MDR strains and is thought not to tend to generate

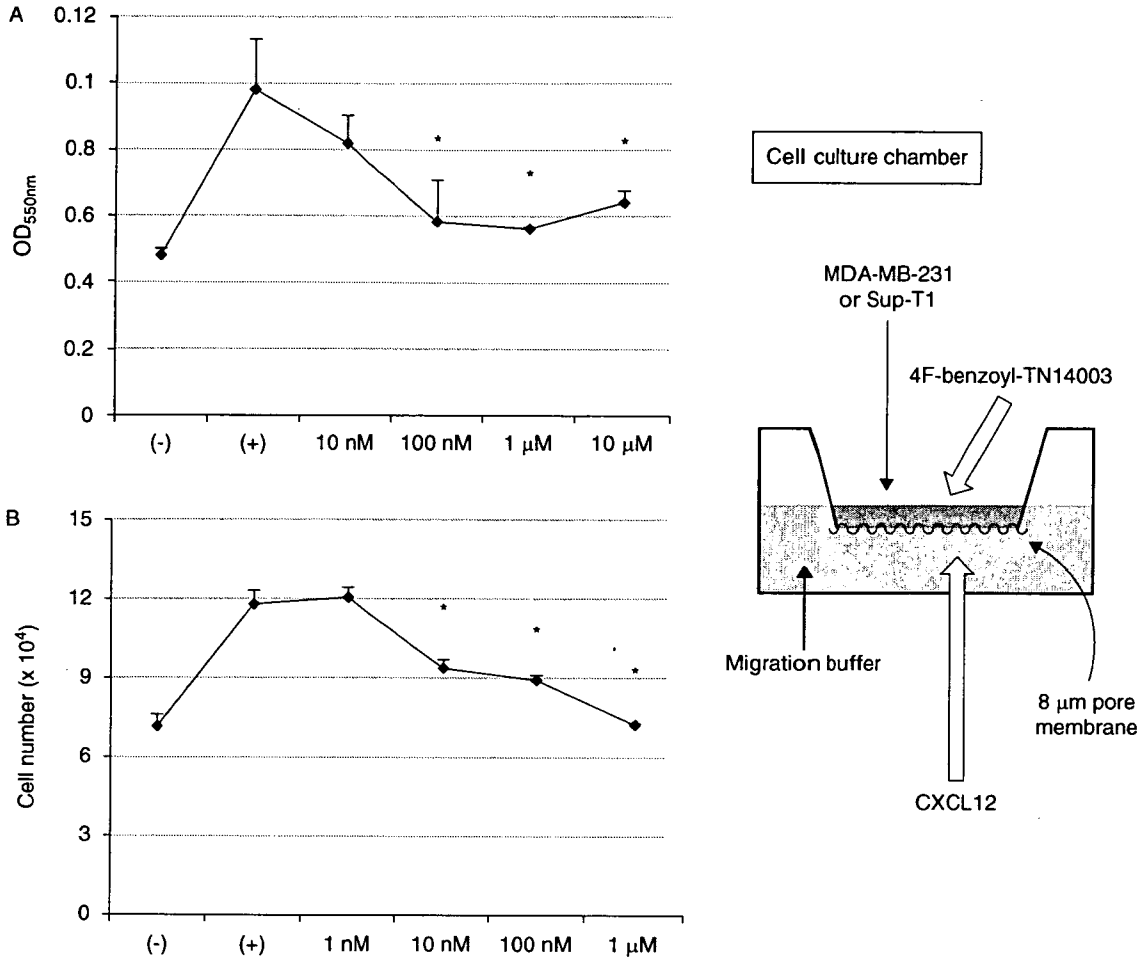


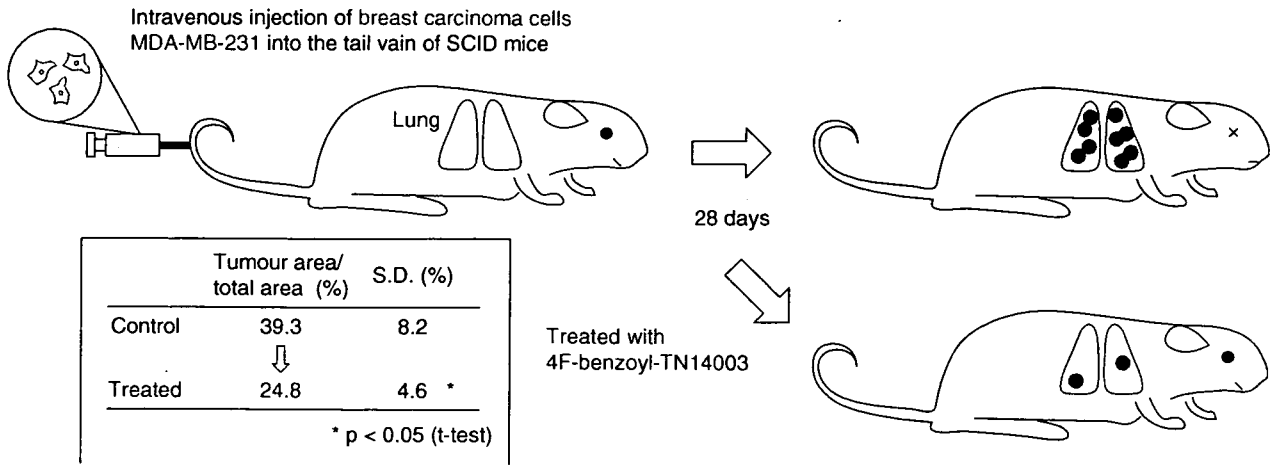
Figure 4. Effects of 4F-benzoyl-TN14003 on CXCL12-induced migration of MDA-MB-231 (A) and Sup-T1 cells (B). The effects were investigated using cell-culture chambers. MDA-MB-231 cells were treated by CXCL12 (100 nM) and various concentrations of 4F-benzoyl-TN14003 (A). Sup-T1 cells were treated by CXCL12 (30 nM) and various concentrations of 4F-benzoyl-TN14003 (B). Control migrating cells in the absence and presence of CXCL12 are shown as (-) and (+), respectively. Data are expressed as means ± SD (n = 2). * $p \leq 0.025$ (Williams' test).
SD: Standard deviation.

drug-resistant strains [51]. The T140 analogue exhibited remarkable delaying effect against the generation of drug-resistant strains in *in vitro* passage experiments using cell cultures [52]. The difficulty of the generation of drug-resistant strains would be a useful advantage for development of T140 analogues in clinical chemotherapy.

5. T140 analogues and cancer

5.1 Antimetastatic activity of a biostable T140 analogue against breast cancer

In order to evaluate the potency of small-molecule CXCR4 antagonists as anticancer-metastatic agents, the authors investigated whether or not T140 analogues inhibit migration of breast cancer cells *in vitro* and metastasis of breast cancer cells *in vivo* [25]. In cell migration assays using cell culture chambers, CXCL12 doubled the migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231. T140 analogues, including 4F-benzoyl-TN14003, inhibited CXCL12-induced migration of MDA-MB-231 in dose-dependent manners. At a concentration of 100 nM, these peptides caused 60 – 80% reductions of MDA-MB-231 chemotaxis induced by 100 nM of CXCL12 (data of 4F-benzoyl-TN14003: **Figure 4A**). In addition, these compounds reduced CXCL12-induced migration of a human leukaemia cell line Sup-T1 (data of



	Tumour area/ total area (%)	S.D. (%)
Control	39.3	8.2
⇩		
Treated	24.8	4.6 *

* $p < 0.05$ (t-test)

Figure 5. Effects of 4F-benzoyl-TN14003 against pulmonary metastasis of breast cancer MDA-MB-231 cells in SCID mice. SCID mice were injected intravenously into the tail vein with MDA-MB-231 breast carcinoma cells (10^6 cells). The day before transplantation, an Alzet pump (duration, 14 days, pumping rate, 0.25 $\mu l h^{-1}$) containing 80 mg/ml of 4F-benzoyl-TN14003 (100 µl in saline) or vehicle was implanted subcutaneously. On day 14, the Alzet pump containing the same amounts of peptide was additionally implanted subcutaneously. On day 28, mice were killed, and 0.2% Evans blue solution was injected through trachea to stain the lung. Ratios of tumour area to total area on the lung surface were calculated from difference of colour between tumour and normal lung area using image analysing techniques. Mean values of four treated and seven control mice are shown. Results of *in vivo* metastasis assays were assessed with Student's t test. The level of significance was defined as $p < 0.05$.
SCID: Severe combined immunodeficient.

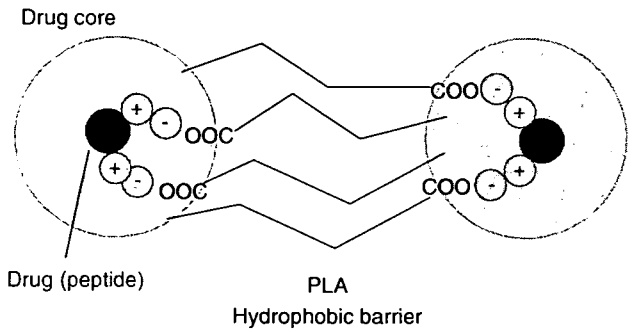


Figure 6. A simple model of the structure of biodegradable PLA microcapsules.
PLA: Poly D,L-lactic acid.

4F-benzoyl-TN14003: Figure 4B) and a human endothelial cell line HUVEC at a concentration of 10 nM. Next, the effect of the biostable CXCR4 antagonist, 4F-benzoyl-TN14003, was investigated using experimental metastasis models of breast cancer, in which MDA-MB-231 cells were injected intravenously (i.v.) into the tail vein of SCID mice and trapped in the lung through heart and pulmonary artery (Figure 5). Mice were administered 4F-benzoyl-TN14003 by subcutaneous (s.c.) injection using an Alzet osmotic pump (DURECT Corp., Cupertino, CA, USA) beginning from the day preceding transplantation of MDA-MB-231. Agent-treated mice showed an effective suppression of tumour accumulation on lung surface derived from the MDA-MB-231 metastasis. Quantitative analyses based on calculations of ratios of tumour area to total lung surface area revealed that 4F-benzoyl-TN14003 significantly reduced pulmonary metastasis of MDA-MB-231 cells in mice. This result strongly suggests that small-molecule CXCR4 antagonists, such as T140 analogues, could replace neutralising anti-CXCR4 antibodies as antimetastatic agents.

5.2 Antimetastatic activity of a bio-stable T140 analogue against melanoma

Next, a controlled release by biodegradable poly D,L-lactic acid (PLA) microcapsules (Figure 6) containing 4F-benzoyl-TE14011 was performed in experimental metastasis models of melanoma [10]. 4F-benzoyl-TE14011 can be steadily released from 4F-benzoyl-TE14011-PLA for a long period *in vivo*, leading to maintenance of the concentration in bloods. A single subcutaneous administration of 4F-benzoyl-TE14011-PLA significantly and drastically reduced the number of colonies derived from pulmonary metastasis of CXCR4-positive B16–BL6 melanoma cells (Figure 7). This result also suggests that a sustained and controlled release of CXCR4 antagonists might contribute to the effective suppression of cancer metastasis. This is supported by Murakami's study showing that daily intraperitoneal treatment with T22 did not reduce pulmonary metastasis in mice following inoculation of B16 melanoma cells, but in mice following inoculation of CXCR4-transduced B16 cells [53].

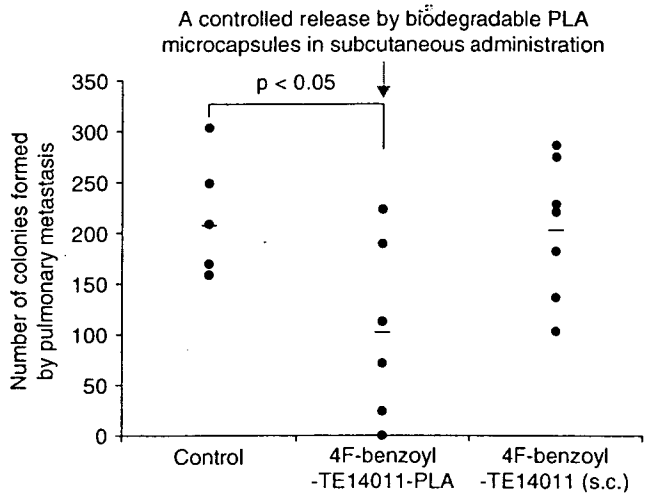


Figure 7. Effects of 4F-benzoyl-TE14011-PLA against experimental pulmonary metastasis of B16-BL6 melanoma cells in mice. B16-BL6 cells (1 x 10^4/0.2 ml) were injected into the tail veins of mice. 4F-benzoyl-TE14011-PLA, 4F-benzoyl-TE14011 (bolus injection, 3 mg drug) or vehicle was administered through a subcutaneous route 30 min prior to tumour cell inoculation. On day 14, mice were sacrificed, and tumour nodules on the surface of the lungs were counted. Data represent the mean ± SE. n = 5 – 8.
PLA: Poly D,L-lactic acid; SE: Standard error.

5.3 Effect of T140 analogues against pancreatic cancer

The authors have found that CXCL12 mRNA is expressed in pancreatic cancer tissues, whereas CXCR4 mRNA is expressed both in pancreatic cancer tissues and in pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1, HPAC and PANC-1), indicating that the CXCL12-CXCR4 interaction is involved in pancreatic cancer cell progression [7]. CXCL12 stimulated both migration and invasion of pancreatic cancer cells, AsPC-1, PANC-1 and SUIT-2, in dose-dependent manners *in vitro*. CXCL12-induced migration and invasion of these cancer cells were completely blocked by 100 nM of T140 analogue [8] (**Figure 8**). The treatment of PANC-1 cells with CXCL12 caused a significant and drastic increase of actin polymerisation (cytoskeleton), resulting in the invasion of malignant cells into tissues and subsequent metastasis. This phenomenon was also effectively inhibited by T140 analogue.

5.4 Effect of T140 analogues against small cell lung cancer

SCLC, which constitutes 20 – 25% of lung cancer, is the leading cause of death in Western countries [54]. SCLC is a highly aggressive cancer involving early and widespread metastasis and development of drug resistance. Thus, new efficient therapeutic strategies towards SCLC metastasis and drug resistance are urgently required. CXCL12 is constitutively secreted by marrow stromal cells and plays a critical role in homing of haematopoietic cells to the marrow. Primary tumour samples from SCLC patients express high levels of CXCR4. Burger *et al.* found that CXCL12 stimulated SCLC cell invasion into extracellular matrix and firm adhesion to marrow stromal cells, which were inhibited by T140 *in vitro*, demonstrating involvement of the CXCL12–CXCR4 interaction in SCLC metastasis [16]. Adhesion of SCLC cells to extracellular matrix or accessory cells within the tumour microenvironment confers cell adhesion-mediated drug resistance (CAM-DR) to chemotherapy via integrin signalling. CXCL12 was found to induce activation of α_2, α_4, α_5 and β_1 integrins through CXCR4, which was inhibited by T140 analogue. They also found that stromal cells protected SCLC cells from anticancer drug-induced apoptosis, and that this protection was inhibited by T140 analogue [55]. Thus, T140 analogues in combination with anticancer drugs, such as etoposide, might overcome CXCL12-mediated CAM-DR in SCLC.

5.5 Effect of T140 analogues against osteolytic bone disease in multiple myeloma patients

It is suggested that CXCL12 plays a potential role in the recruitment of osteoclast precursors to the bone marrow and activation, and that the CXCL12 level is correlated to the expression of multiple radiological bone lesions in individuals with multiple myeloma. 4F-benzoyl-TE14011 was found to significantly inhibit both CXCL12-mediated and the myeloma plasma cell line (RPMI-8226) conditioned medium-stimulated osteoclast activity and formation *in vitro* [20]. Thus, blockade of the CXCL12/CXCR4 axis might be an effective remedy against osteolysis in multiple myeloma patients.

5.6 Effect of T140 analogues against Epstein-Barr virus-associated lymphoproliferation

Intraperitoneal injection of peripheral blood mononuclear cells (PBMCs) from Epstein-Barr virus (EBV)-seropositive donors into SCID mice causes lymphomas. The precise mechanisms have not yet become clear, but implications of the CXCL12/CXCR4 axis are suggested. For CXCR4 neutralisation, SCID mice were administered 4F-benzoyl-TN14003 by s.c. injection using Alzet pumps beginning from the day preceding transplantation of lymphoma cells. Whereas all control mice (saline-treated mice) succumbed within 75 days after tumour cell transplantation, 50% of mice treated with 4F-benzoyl-TN14003 were alive after 120 days, suggesting that CXCR4 neutralisation delays lymphoma development, and that the CXCL12/CXCR4 axis may be associated to EBV-mediated lymphomagenesis [26].

5.7 Effect of T140 analogues against chronic lymphoblastic leukaemia

B cell CLL, which is the most common leukaemia in adults in Western countries, is caused by the accumulation of long-lived, monoclonal B malignant cells in the blood, secondary lymphoid organs and bone marrow. CLL B cells express high levels of CXCR4. Marrow stromal cells or nurse-like cells constitutively release CXCL12, thereby activate

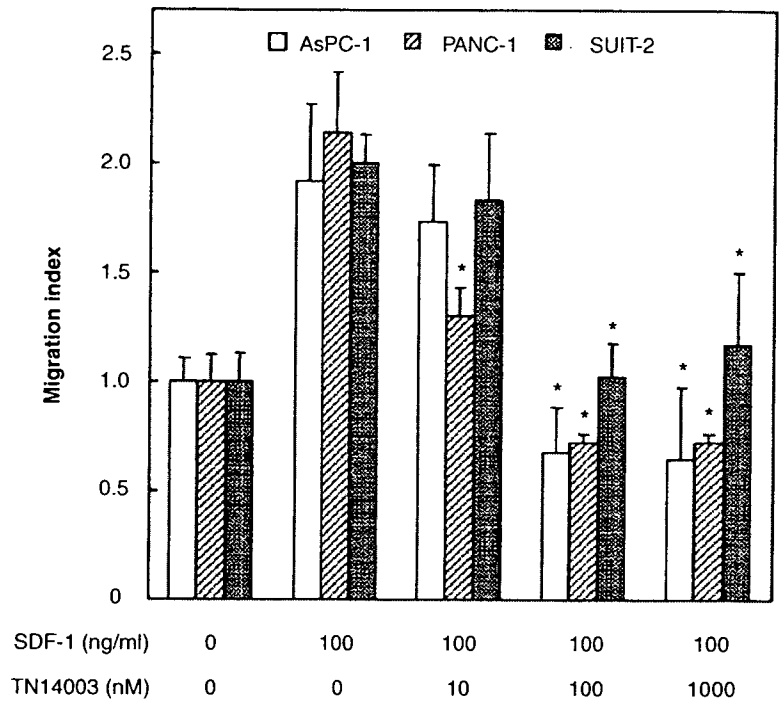


Figure 8. Effect of TN14003 on CXCL12-stimulated migration of pancreatic cancer cells. Pancreatic cancer cells were stimulated by CXCL12 at 100 ng/ml and various concentrations of TN14003. The CXCL12-stimulated migration was inhibited by TN14003 and was completely eradicated by TN14003 at 100 nM. Columns show the mean of three separate experiments in triplicate wells; bars = SE.
*$p < 0.05$ compared with 100 ng/ml CXCL12.
SE: Standard error.
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CLL B cells, and rescue the cells from apoptosis, leading to their accumulation. Thus, the CXCL12–CXCR4 interaction would represent a useful therapeutic target for B cell CLL [21]. T140 analogues inhibited CXCL12-induced chemotaxis of CLL cells, their migration beneath marrow stromal cells and actin polymerisation, *in vitro* [22]. Furthermore, T140 analogues reduced the antiapoptotic effect caused by CXCL12, as well as stromal cell-mediated protection of CLL cells from spontaneous apoptosis. Co-culture of CLL cells and marrow stromal cells protected CLL cells from drug-induced apoptosis, causing stromal CAM-DR. Treatment with T140 analogues resensitised the above CLL cells to fludarabine-induced apoptosis. It suggests that T140 analogues might overcome CAM-DR, which is one of serious problems in clinical chemotherapy, and that use of a CXCR4 antagonist alone or in combination with CLL cell-directed drug (i.e., fludarabine) would be promising chemotherapy in CLL.

5.8 Effect of T140 analogues against acute lymphoblastic leukaemia

The growth and survival of precursor-B (pre-B) ALL cells might be caused by their intimate contact with bone marrow stromal layers using the β_1 integrins. The migration of these cells through stromal layers requires these integrins and is regulated by CXCL12, because CXCR4 is uniformly and highly expressed on pre-B ALL cells. T140 0.1 μM completely blocked CXCL-12-induced chemotaxis and attenuated the migration of pre-B ALL cells into bone marrow stromal layers. Furthermore, a T140 analogue enhanced the cytotoxic and antiproliferative effects of the cytotoxic agents, vincristine and dexamethasone, suggesting that T140 analogues might overcome CAM-DR in ALL chemotherapy [23].

6. T140 analogues and rheumatoid arthritis

6.1 Anti-RA activity of a biostable T140 analogue (reduction of the delayed-type hypersensitivity reaction)

Inflammatory cytokines, such as IL-1, IL-6, IFN-γ and TNF-α, and activation markers play a critical role in the chronic RA synovium [27]. Development of biological drugs targeting these cytokines, such as humanised monoclonal antibodies, has produced useful results in clinical therapy of RA patients. However, this therapy has not yet reached a perfect stage, and development of other drugs, which are not associated with the above cytokine's functions, is required for the improvement of RA

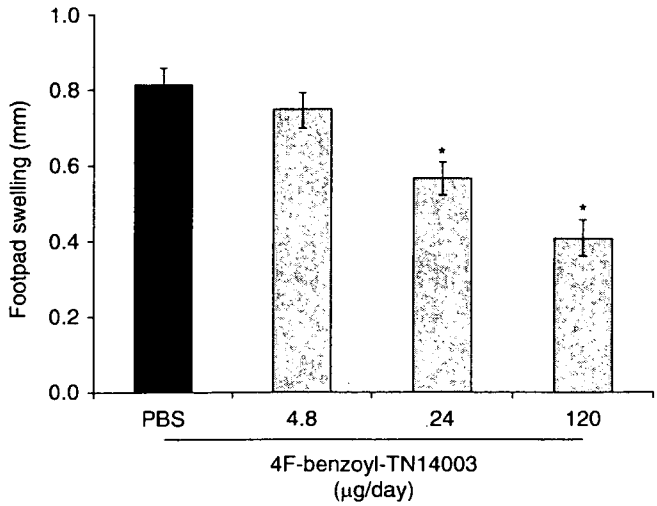

Figure 9. Inhibition of the mouse DTH response by 4F-benzoyl-TN14003. The gain of thickness of right footpad by swelling 24 h after challenge was measured by a micrometer. PBS (control models) or 4F-benzoyl-TN14003 (4.8, 24 or 120 μg/day) was administered by subcutaneous injection using an Alzet pump from the day before immunisation. Data are expressed as means ± SE (n = 7). *$p \leq 0.025$ (Williams' test)
PBS: Phosphate-buffered saline; SE: Standard error.
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chemotherapy. As an *in vivo* experimental model of the cellular immune response, mouse delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells was adopted for evaluation of the activity of 4F-benzoyl-TN14003 [56]. The gain of thickness of right footpad by swelling 24 h after challenge, was measured. As a result, s.c. injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump significantly suppressed the DTH response in a dose-dependent manner. The 24 and 120 μg daily injections showed inhibitory percentages of 31 and 51%, respectively (**Figure 9**).

6.2 Anti-RA activity of a bio-stable T140 analogue (suppression of collagen-induced arthritis)

As the second *in vivo* experimental model of RA, collagen-induced arthritis in mice was adopted. Several symptoms of arthritis (score increase, body weight loss, ankle swelling and weight gain in the limbs) were observed in mice that were treated with 4F-benzoyl-TN14003 using an Alzet osmotic pump (s.c.) after the bovine Type II collagen (CII) emulsion booster, as compared to those in the control mice that developed arthritis [56]. 4F-benzoyl-TN14003-treated mice showed significant suppression of these symptoms and apparent suppression of the increase in levels of serum antibovine CII IgG2a antibody observed in the control mice (**Figure 10**). 4F-benzoyl-TN14003, therefore, interferes with the humoral immune response to CII.

7. Identification of T140 as an inverse agonist

Antagonists are generally classified into two categories: inverse agonists that show no agonistic activity at all, and partial agonists that show weak agonistic activity. Because partial agonists of CXCR4 have CXCL12-like agonistic activity through CXCR4, which is weaker than that of CXCL12, these compounds might migrate and activate various cancer cells and memory T cells that highly express CXCR4. Thus, inverse agonists have a great clinical advantage due to no positive effect on CXCR4, especially in terms of cancer and RA chemotherapy. The authors' collaborators, Peiper *et al.*, prepared a constitutively active mutant of CXCR4 by coupling CXCR4 to the pheromone response pathway in yeast [57]. Conversion of Asn119 to Ser or Ala in CXCR4 conferred autonomous signalling in yeast and mammalian cells. Exposure to AMD3100 or ALX40-4C (see Section 11) induced G-protein activation through CXCR4 wild type and this mutant, whereas T140 decreased autonomous signalling, disclosing that T140 is an inverse agonist whereas AMD3100 and ALX40-4C are partial agonists. The amino acid residues in CXCR4 used for binding to T140 and AMD3100 were comparatively studied using Ala-scanning mutagenesis and computation docking simulation analysed by Trent *et al.* [58]. Critical residues for both T140 and AMD3100 bindings are mainly located in the second extracellular loop of CXCR4, but these are distinctly different, suggesting that the different mechanisms of these antagonists are due to the subtle difference of their binding sites on CXCR4. Development of a new generation of agents, such as T140 analogues, which lack partial agonistic activity, may reduce toxicities and side effects on the usage of clinical applications.

8. CXCL12-mediated CXCR4 signalling in neural progenitor cells

Zheng *et al.* found that CXCR4 is expressed in abundance on rat and human neural progenitor cells, and that CXCL12 induced human neural progenitor cell chemotaxis *in vitro*, which was abrogated by T140 [59]. Knockout studies also suggested that the CXCL12–CXCR4 axis plays essential roles in cerebellar, hippocampal and neocortical neural cell migration during embryogenesis.

9. CXCR4-mediated germinal centre organisation

Germinal centre (GC) dark and light zones segregate cells undergoing somatic hypermutation and antigen-driven selection. Cyster *et al.* reported that GC organisation was absent from mice deficient in CXCR4, and that GC B cells, which have high expression of CXCR4, migrated towards the dark zone where CXCL12 was more abundant than that in the light zone [60]. Genetic ablation of CXCR4 and its pharmacological inhibition by a T140 analogue disrupted GC

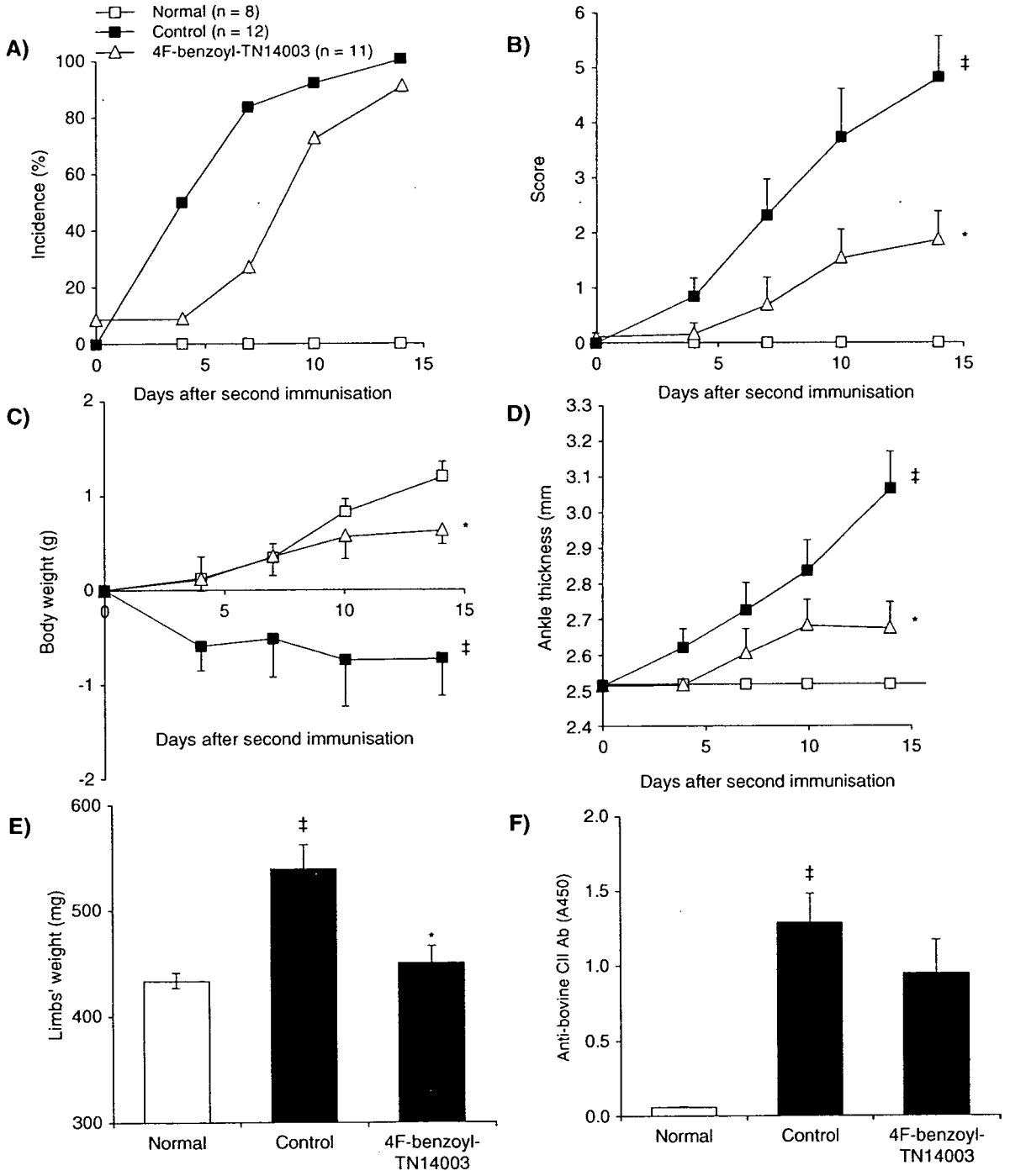

Figure 10. Suppression of CIA in mice by 4F-benzoyl-TN14003. The incidence (**A**) and the score expressing the clinical severity (**B**) of arthritis were evaluated, and body weight (**C**) and the thickness of the hind ankles (**D**) were measured after the second immunisation twice a week. The weights of 4 limbs were measured 2 weeks after the second immunisation (**E**). Levels of antibovine CII IgG2a antibody in serum, which was obtained 2 weeks after the second immunisation, were measured by ELISA (**F**). PBS (control models, n = 12) or 4F-benzoyl-TN14003 (120 μg/day, n = 11) was administered by subcutaneous injection using an Alzet pump from the day before the second immunisation. In normal models (n = 8), mice were not immunised. Data are expressed as means ± SE. *$p \leq 0.01$ (t test): comparison with control models (scores were compared by nonparametric Steel test). ‡ $p \leq 0.01$ (t test): comparison with normal models.
CIA: Collagen-induced arthritis; ELISA: Enzyme-linked immunosorbent assay; SE: Standard error.
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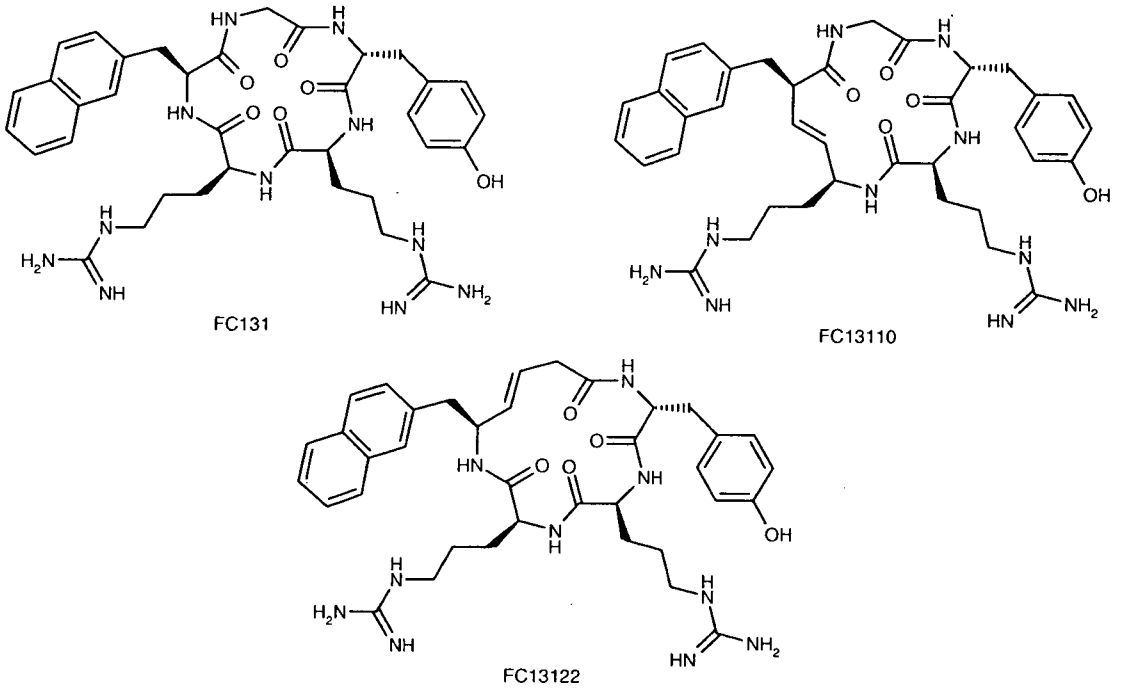

Figure 11. Structures of a cyclic pentapeptide, FC131, and its pseudopeptides, FC13110 and FC13122.

compartmentalisation into the dark zone, suggesting that CXCR4 is essential for proper GC organisation. On the other hand, CXCR5 helps direct cells to the light zone.

10. Low molecular weight CXCR4 antagonists based on cyclic penta- and tetrapeptides

As described in Section 2, the four residues of T140, Arg2, Nal3, Tyr5 and Arg14, are indispensable to express high potency [47]. These residues are located in close proximity to each other in the spatial structure, as shown in conformational analysis by NMR [46]. Thus, the T140 pharmacophore-guided approach based on these four residues was attempted for the development of low molecular weight CXCR4 antagonists. Initially, cyclic pentapeptide libraries containing two Arg, Nal and Tyr were constructed. Cyclic pentapeptides have been used as conformational-constrained templates disposing functional groups by medicinal chemists [61-66]. Practically, the authors devised a library using two L/D-Arg, L/D-Nal and L/D-Tyr in addition to Gly as a spacer, and succeeded in efficient discovery of a hit compound, FC131, which has strong CXCR4-antagonistic activity comparable to that of T140 [67] (Figure 11). NMR and simulated annealing molecular dynamics (SA-MD) analysis of FC131 showed convergent backbone structures involving an almost symmetrical pentagonal shape.

Next, for reduction of the peptide character of FC131, contributions of each amide bond to the biological activity were investigated by introduction of (*E*)-alkene dipeptide isosteres (EADIs) [68-73] and reduced amide-type dipeptide isosteres (RADIs), which have been intensively utilised in SAR studies of biologically active peptides. Several FC131 analogues, in which the above isosteres were substituted for the backbone amide bonds between Arg and Nal and between Nal and Gly, were prepared by the synthetic strategy as reported in previous papers [74-76] (Figure 11). These synthetic pseudopeptides showed weaker CXCR4-antagonistic activity than FC131, demonstrating that the amide bonds are necessary for high potency. It suggests that either a deletion of the hydrogen bonding interaction with CXCR4 by the EADI introduction or a change in hydrophobicity might not be appropriate. As NMR and SA-MD analysis showed that FC131 and these pseudopeptides have nearly equal distances between any two β-carbons in all of the side chains, and that these compounds maintain similar dispositions of pharmacophores, biological differences are caused by the amide bond replacement [77]. SAR studies by substitution of these isosteres provided useful information for the future design of CXCR4 antagonists.

The cyclic pentapeptide, FC131, and its pseudopeptides have a Gly residue as a spacer for cyclisation. To reduce the ring size, several cyclic tetrapeptide-scaffolds have been prepared and investigated for structural tuning of FC131. A γ-amino acid-containing peptide, FC151, where the Nal-Gly sequence was replaced by 4-amino-5-naphthalen-2-yl-pentanoic acid (γ-Nal), disulfide-bridged cyclic peptides, 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], showed significant CXCR4-antagonistic

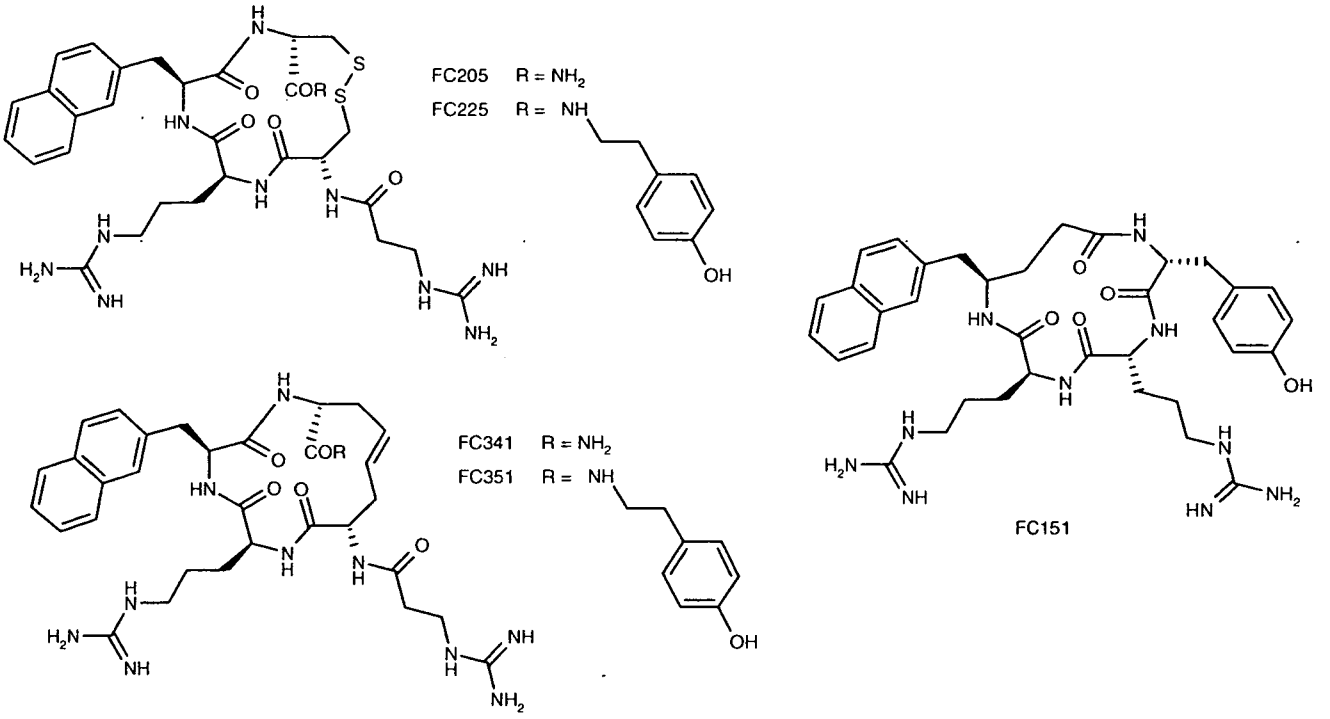

Figure 12. Structures of a γ-amino acid-containing cyclic peptide, FC151, disulfide-bridged cyclic peptides, FC205 and FC225, and olefin-bridged cyclic peptides, FC341 and FC351.

activity (IC_{50} = 54, 690 and 530 nM, respectively) (Figure 12). Furthermore, cyclic compounds that were bridged by an olefin using ring-closing metathesis, FC341 and FC351, exhibited moderate CXCR4-antagonistic activity (IC_{50} = 1 – 10 μM) [78]. Examination on further downsizing and reduction of peptide character is now in progress.

11. Other CXCR4 antagonists

To date, several CXCR4 antagonists, other than T140-related compounds, have been reported [79,80]. In 1997, the bicyclam AMD3100 (AnorMED, Inc.) [81] (Figure 13) and ALX40-4C (Ac-[D-Arg]9-NH_2; NPS Allelix) [82] were reported at the same time as T22 [43]. These compounds have a high basicity as a common property. In association with AMD3100, an *N*-pyridinylmethylene cyclam (monocyclam) AMD3465 (AnorMED, Inc.) [83], a non-cyclam AMD8665 (AnorMED, Inc.) [84] and AMD070 (AnorMED, Inc.) [85] were found as new antagonists (Figure 13). Bifunctional drugs based on AMD3100 and galactosylceramide analogue conjugates were reported [86]. Intensive modification of the *N*-terminal tripeptide of T140, Arg-Arg-Nal, might produce KRH-1636 (Kureha Chemical & Sankyo), which is an orally bioavailable agent [87]. The 4-[[[pyridin-2-ylmethyl]amino]methyl]phenyl group constitutes a common substructure unit of AMD3465, AMD8665 and KRH-1636, which might be a critical pharmacophore. Arg-mimetic conjugates, CGP-64222, R3G and NeoR, were also developed as cationic CXCR4 antagonists [88-90]. A distamycin analogue, NSC651016 [91], and a flavonoid compound, ampelopsin [92], were found to be CXCR4 antagonists that have different structures. These low molecular weight antagonists might be useful agents for chemotherapy of AIDS, cancer, RA and so on.

12. Expert opinion and the future of the therapeutic potential of CXCR4 antagonists

The authors found strong anti-HIV agents, T22 and its downsized analogue T140, which inhibit entry to T cells by X4-HIV-1 through their specific binding to the coreceptor CXCR4. T140 and its derivatives were also identified to have anticancer-metastasis, antileukaemia and anti-RA activities. Downsizing and reduction of peptide character based on T140 were explored to develop new low-molecular weight CXCR4 antagonists. Furthermore, several other CXCR4 antagonists, which are not associated with T140, have been developed. However, CXCR4 is constitutively expressed in several organs and tissues, and plays a critical role in embryogenesis, homeostasis and inflammation in fetus, especially in haematopoietic, cardiovascular and nervous systems. Although one has to carefully consider risky reactions toward living bodies based on blocking the CXCL12-CXCR4 axis, these CXCR4 antagonists might be promising agents for clinical chemotherapy of HIV infection, cancer metastasis, leukaemia progression and RA. 1) CXCR4 antagonists play a

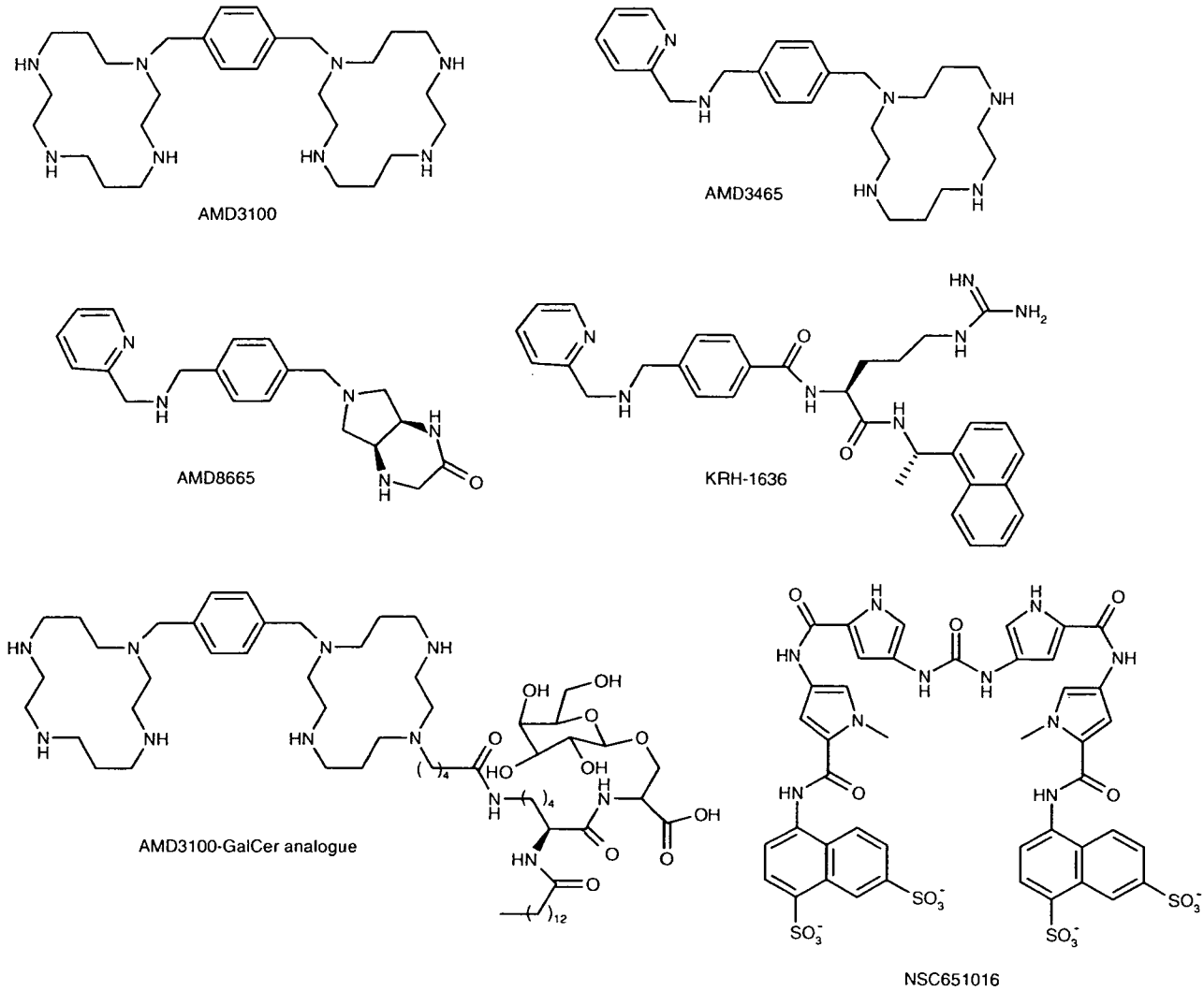

Figure 13. Structures of other CXCR4 antagonists.

critical role in HIV-infected patients who have X4 HIV-1 strains that emerge late in this disease. Furthermore, CXCR4 antagonists might inhibit the appearance of X4 or dual-tropic strains in patients who have R5 strains that constitute majority in the early stage of HIV infection. Thus, combinational use of CXCR4 antagonists with CCR5 antagonists and fusion inhibitors might improve clinical chemotherapy of HIV infection and AIDS. 2) Blocking the CXCL12/CXCR4 interactions might represent a novel and useful chemotherapy of cancer metastasis and leukaemia. Furthermore, CXCR4 antagonists would overcome CXCL12-mediated CAM-DR. 3) RA chemotherapy based on CXCR4 antagonists is promising, because the CXCL12–CXCR4 axis is not associated to the inflammatory cytokines, such as TNF-α. Because T140-related compounds are inverse agonists, which have no CXCL12-like activity, these compounds do not migrate or activate various cancer cells and rheumatoid T cells that highly express CXCR4. Downsizing, reduction of the peptide character and investigation on administration routes of these CXCR4 antagonists are thought to become important in the chemotherapy of multiple diseases relevant to CXCR4.

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Kisspeptin-10-Induced Signaling of GPR54 Negatively Regulates Chemotactic Responses Mediated by CXCR4: a Potential Mechanism for the Metastasis Suppressor Activity of Kisspeptins

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Abstract

The product of the *KiSS-1* gene is absent or expressed at low level in metastatic melanoma and breast cancer compared with their nonmetastatic counterparts. A polypeptide derived from the KiSS-1 product, designated kisspeptin-10 (Kp-10), activates a receptor coupled to Gαq subunits (GPR54 or KiSS-1R). To study the mechanism by which Kp-10 antagonizes metastatic spread, the effect on CXCR4-mediated signaling, which has been shown to direct organ-specific migration of tumor cells, was determined. Kp-10 blocked chemotaxis of tumor cells expressing CXCR4 in response to low and high concentrations of SDF-1/CXCL12 and inhibited mobilization of calcium ions induced by this ligand. Pretreatment with Kp-10 did not induce down-modulation of cell surface CXCR4 expression, reduce affinity for SDF-1/CXCL12, or alter Gαi subunit activation stimulated by this ligand. Although Kp-10 stimulated prolonged phosphorylation of extracellular signal-regulated kinase 1/2, it inhibited the phosphorylation of Akt induced by SDF-1. The ability of Kp-10 to inhibit signaling and chemotaxis induced by SDF-1 indicates that activation of GPR54 signaling may negatively regulate the role of CXCR4 in programming tumor metastasis. (Cancer Res 2005; 65(22): 10450-6)

Introduction

The capacity for metastatic spread is a critical aspect of tumor cell biology that has a profound effect on clinical behavior. There is significant evidence that the metastatic phenotype is a composite effect of multiple mechanisms, including breach of normal architectural boundaries, angiogenesis, directed migration, and target site modification. Expression microarray analysis of high bone metastatic variants biologically selected from a nonmetastatic cell line identified *CXCR4* among a cadre of genes that confer the metastatic phenotype (1).

CXCR4, the receptor for the CXC chemokine stromal cell-derived factor 1 (SDF-1/CXCL12), is a G-protein-coupled receptor (GPCR) expressed by a wide spectrum of cells and its physiologic importance in hematopoiesis, development of the vasculature and of the central nervous system has been emphasized by the lethal phenotype of its knockout in mice. Among the candidate genes that were implicated in the metastatic phenotype, expression of CXCR4 alone was found to significantly increase the metastatic behavior, and bone metastasis was further increased by the coordinated expression of other prometastatic genes (1). Common target organs for the metastatic spread of breast cancer secrete SDF-1/CXCL12, including lung, lymph node, liver, and bone marrow. Blockade of this receptor with a monoclonal antibody has been shown to inhibit spread of human breast cancer cells to lungs and regional lymph nodes in a mouse xenograft model (2). Although CXCR4 has been implicated in the pathogenesis of metastatic spread of multiple malignant tumors, the regulation of this mechanism has not yet been elucidated (3). It is unclear whether expression of CXCR4 is sufficient to program migration of tumor cells to target organs that secrete SDF-1/CXCL12, or the sensitivity of this receptor to the chemotactic gradient of ligand may be positively and/or negatively regulated by independent factors.

Genes having a metastasis suppressor function are candidates for the negative regulation of prometastatic mechanisms (4). The *KiSS-1* gene was originally identified by its altered expression in metastatic melanoma but not in localized tumors (5). Programming of KiSS-1 expression in human breast carcinoma cell lines decreased metastatic spread in mouse xenograft models (6). The KiSS-1 protein (also known as metastin or kisspeptin) contains 145 residues and multiple shorter products resulting from naturally occurring proteolytic cleavage have been identified. Metastin (45-54) [also known as metastin (112-121) or KiSS-1 (112-121), kisspeptin-10 (Kp-10)] is a 10-residue peptide derived from the product of the KiSS-1 (7–11). Kisspeptins bind to the same GPCR (KiSS1-R: hOT7T175, AXOR12, and GPR54; refs. 7–9). Qualitatively, all the different forms of the polypeptide (natural or synthetic) have a similar activity but different affinities for their receptor, the decapeptide being the most active. Exposure of cancer cell lines (melanoma, pancreatic carcinoma, or Chinese hamster ovary, CHO) with endogenous or programmed expression of GPR54 to metastin decreased expression of metalloproteinase 9, motility, and proliferation *in vitro* and prevented metastasis *in vivo* (6–8, 12, 13). Because metastin decreased the motility and migration of cell lines exposed to fetal bovine serum (FBS), it was hypothesized that the antimetastatic action of this ligand may involve negative regulation of prometastatic mechanisms, including directed chemoattraction to target organs. In this study, the effect of Kp-10 on the function of CXCR4, including chemotaxis and intracellular signaling, was determined in CHO and HeLa cell transfectants.

Materials and Methods

Materials. The COOH-terminally amidated decapeptide Kp-10 (YNWNSFGLRF-NH_2) was synthesized at the University of Kyoto, Japan and was used in all the experiments. CHO and HeLa were selected because of their total absence of response to Kp-10 as assessed by calcium mobilization and activation of extracellular signal-regulated kinase 1/2 (ERK1/2)/mitogen-activated protein kinase (MAPK). CXCR4 is endogenous.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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in HeLa and expressed by transfection in CHO. Human GPR54 was subcloned in pcDNA3.1 (Invitrogen, Carlsbad, CA) with a Myc-tag at the NH_2 terminus and transfected in both cell lines. Transfectants were selected by magnetic sorting (Miltenyi Biotec, Auburn, CA).

Chemotaxis assay. Cells were resuspended in MEM-α/0.5% bovine serum albumin (BSA) at a density of 2×10^6/mL for CHO or 5×10^5 for HeLa, and 100 μL were added to the top chamber of 24-well transwell apparatus (6.5-mm diameter, 8.0-μm pore size; Corning, New York, NY) coated with collagen (human, type IV, Sigma, St Louis, MO). SDF-1 (Leinco Technologies, St Louis, MO) was added to the lower chamber, and Kp-10 was added either to the bottom chamber or both to the bottom and to the cells in the top chamber. The plates were incubated for 4 hours at 37°C. Cells were fixed with 20% ethanol/0.5% crystal violet, and the cells at the top of the membrane were wiped off. The cells on the bottom face of the membrane were stained with 4′,6-diamidino-2-phenylindole and counted with a fluorescence microscope. Alternatively, the cells migrating in the bottom chamber were resuspended in the medium and counted for 1 minute by flow cytometry (LSRII, Becton Dickinson, San Jose, CA) after appropriate gating.

Calcium mobilization. CHO cells were loaded with 2 μg/mL Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR). Agonist-dependent increases in cytoplasmic calcium were determined as described (14). Calcium mobilization was also studied by flow cytometry. The cells were then prepared in a similar way except that Indo-1 (Molecular Probes) was used instead of Fura-2. The cells were analyzed on a LSRII flow cytometer equipped with a solid-state UV laser (Xcyte, Lightwave Electronics, Mountain View, CA). The fluorescence of Indo-1 was split by a 450-nm LP dichroic mirror and measured by two separate detectors after passage through a 530/30 band pass filter (calcium-free form) and a 405/20 band pass filter (calcium-bound form). Acquisition and analysis of the ratio of fluorescence over time was done with the Diva software (Becton Dickinson). About 300 cells per second were analyzed over the entire experiments.

Flow cytometry. Internalization of CXCR4 was measured by indirect immunofluorescence and flow cytometry. Cells were detached with citrate buffer, resuspended in MEM-α/0.5% BSA at 1×10^7 cells/mL. The cells (100 μL) were then exposed to 100 nmol/L of SDF-1 or 100 nmol/L of Kp-10 for 30 minutes at 37°C (or medium alone for the positive control). After an acid wash (pH 3) to elute the ligand from the receptor, cells were incubated with a saturating concentration of a monoclonal antibody to CXCR4 (A145, 10 μg/mL; ref. 15) followed by a phycoerythrin-labeled antibody to mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Ligand binding and displacement. Membrane preparations of CHO cells stably expressing the GPR54 were incubated with 0.1 nmol/L [^{125}I] metastin (45-54) (Amersham Biosciences, Piscataway, NJ) in the presence of incremental concentrations of Kp-10 as described previously (8). The affinity was calculated using Prism (GraphPad Software, San Diego, CA) and is expressed as the EC_{50} ± SD based on duplicate samples of each concentration.

γ[^{35}S]GTP binding assay. The γ[^{35}S]GTP binding assay was carried out as described previously (14). The effect of Kp-10 on the capacity of CXCR4 to activate G-proteins was measured either by incubating the membrane fraction with Kp-10 immediately before adding SDF-1 or following preincubation of the cells with Kp-10 for 5 minutes at 37°C to allow cross-desensitization before preparation of the membranes.

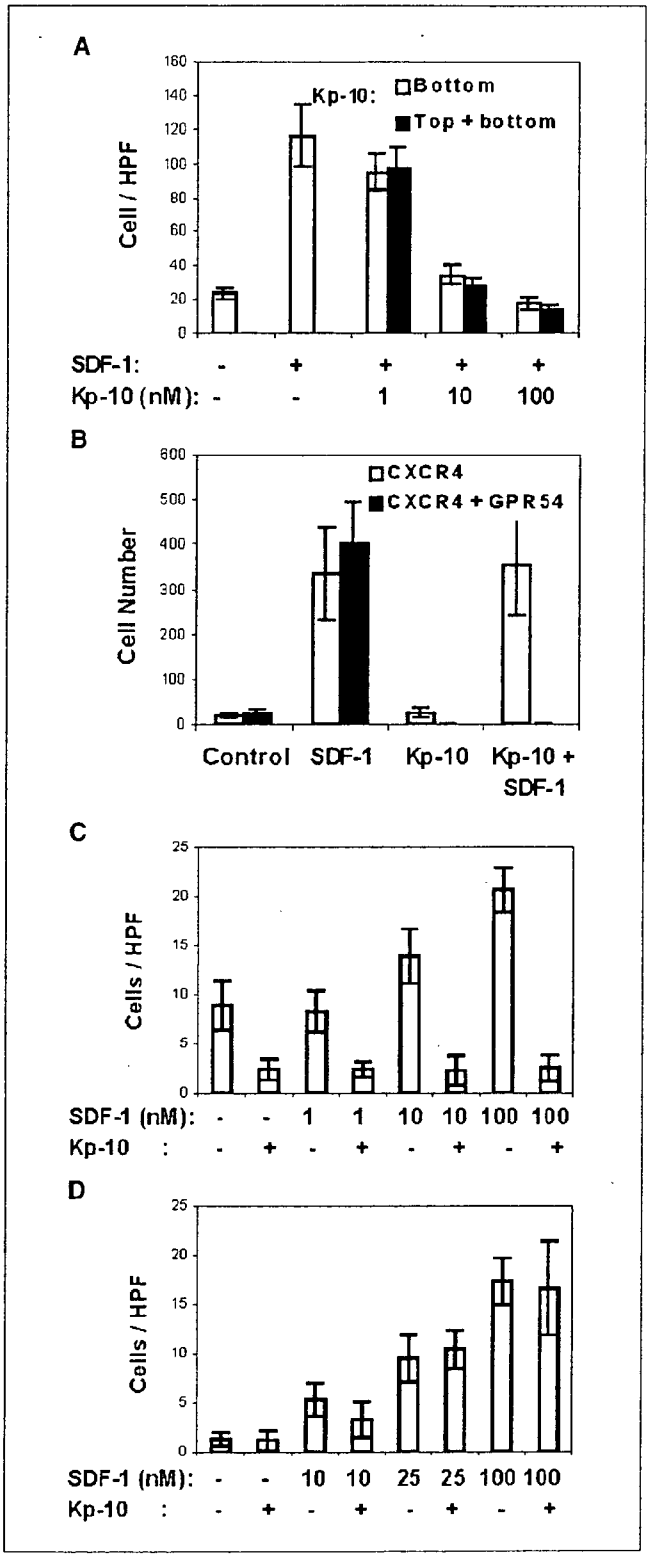
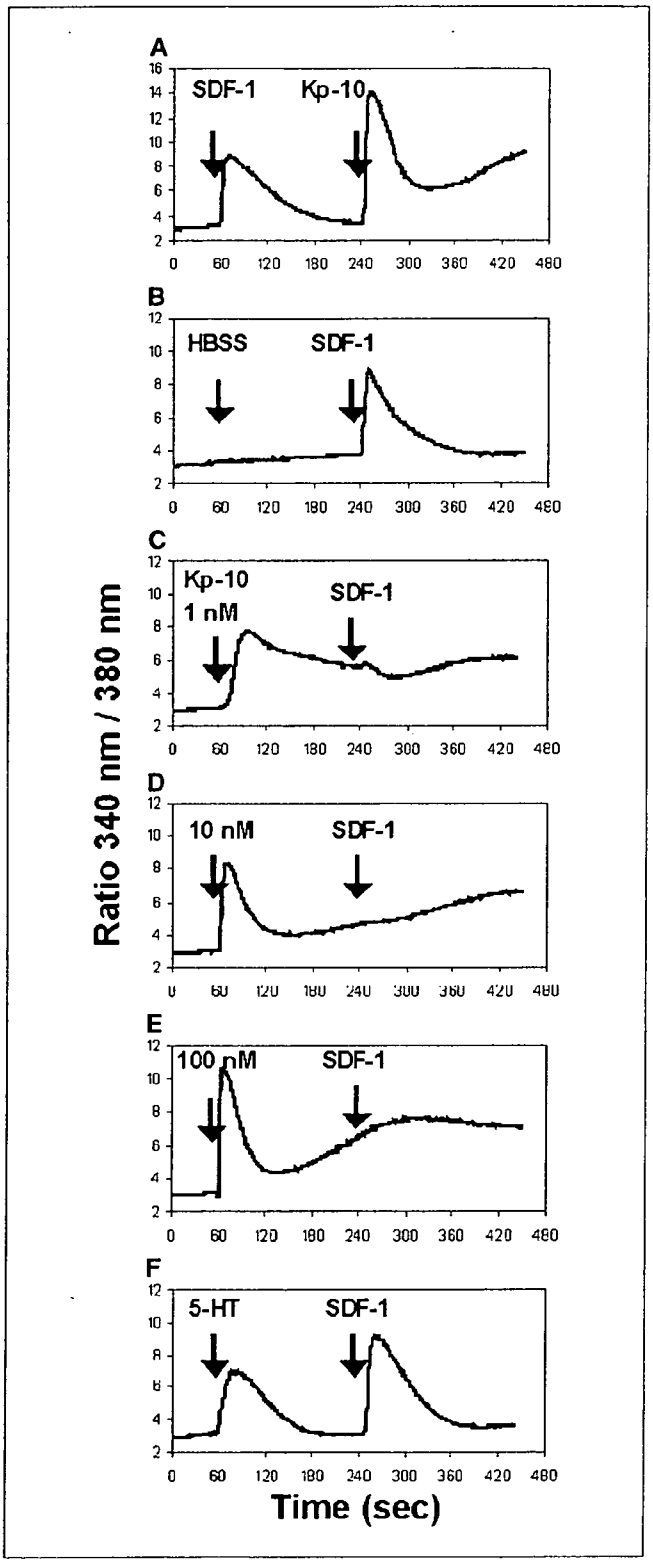


Figure 1. Kp-10 inhibits chemotaxis mediated by SDF-1 in CHO and HeLa cells expressing CXCR4 and the GPR54. *A*, Kp-10 inhibits chemotaxis toward 50 nmol/L of SDF-1 in a dose-dependent manner. The reduction of the number of migrating CHO cells depends on the concentration of Kp-10 ($P < 0.05$ at 1 nmol/L, $P < 0.001$ at 10 and 100 nmol/L), and the effect is independent of location in the top or the bottom chamber. Results are based on the number of cells counted in 8 high power fields (*HPF*, ×40) for each condition. *B*, the inhibitory effect of Kp-10 on CXCR4 requires the presence of GPR54 in CHO cells. Parallel experiments were done in triplicate in CXCR4 transfectants or CXCR4/GPR54 double transfectants. The cells in the bottom chamber of the transwell were counted for 1 minutes by flow cytometry. Almost no cells could be counted when GPR54 transfectants were exposed to Kp-10. *C*, inhibition of chemotaxis toward increasing doses of SDF-1 as well as chemokinesis is also observed in HeLa cells transfected with GPR54. Results are based on the number of cells counted in 8 high power fields and are representative of three independent experiments. *D*, Kp-10 does not inhibit chemotaxis or chemokinesis of HeLa cells that do not express the GPR54.

Western blot. CHO cells were seeded in 60-mm dishes (5×10^5), grown for 24 hours in MEM-α /10% FBS, and starved overnight in DMEM/0.5% BSA. Stimulation of the cells with 100 nmol/L of SDF-1 or Kp-10 or both was done at 37°C. The cells were then washed with ice-cold PBS and resuspended in lysis buffer (50 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, protease, and phosphatase inhibitors) for 1 hour. After centrifugation (15 minutes at 13,000 rpm), the soluble fraction was diluted in SDS sample buffer. SDS-PAGE and transfer to polyvinylidene difluoride were done according to standard protocols. Antibodies to p44/42 MAPK (ERK1/2), phospho-p44/42, Akt, phospho-Akt Ser^{473}, and phospho-Akt Thr^{308} (Cell Signaling Technology, Beverly, MA) were detected using horseradish peroxidase–labeled secondary antibodies (Jackson ImmunoResearch Laboratories) and Enhanced Chemiluminescence-Plus (Amersham Biosciences).

Statistics. When relevant, quantitative data were analyzed using the Student's *t* test.



Results

KiSS-1 inhibits the chemotactic response to SDF-1. The findings related to the relative roles of CXCR4 and Kp-10 in cancer cell metastasis suggest that signaling induced by KiSS-1 may antagonize the effects of SDF-1, thereby suppressing the metastatic spread of breast cancer. The ability of Kp-10 to inhibit the directed migration induced by SDF-1 was tested in CHO transfectants expressing GPR54 and CXCR4. As shown in Fig. 1*A*, exposure of target cells to a gradient of Kp-10 parallel to a chemotactic gradient of SDF-1 inhibited chemotaxis induced by 50 nmol/L SDF-1 in a dose-dependent fashion. A similar suppression of chemotaxis induced by SDF-1 was noted when Kp-10 was placed in the upper chamber of the transwell and mixed with the cells immediately prior the initiation of chemotaxis (Fig. 1*A*). The presence of high concentration of Kp-10 (100 nmol/L) had no effect on SDF-1-induced chemotaxis of cells expressing CXCR4 but not GPR54 (Fig. 1*B*). Parallel experiments were done with HeLa cells, which have endogenous CXCR4 expression and were transfected with GPR54. As shown in Fig. 1*C*, exposure of HeLa-GPR54 transfectants to 100 nmol/L Kp-10 completely inhibited chemotaxis stimulated by SDF-1 at concentrations of 10 and 100 nmol/L. The magnitude of transmigration of cells exposed to Kp-10 was less than basal levels, consistent with the previous finding that kisspeptins decrease basal cytokinesis (8). Like in the case of CHO cells, no effect of Kp-10 on the chemotaxis induced by SDF-1 and CXCR4 was observed in HeLa cells that did not express the GPR54 (Fig. 1*D*). The transient nature of the suppressive effect on chemotaxis induced by SDF-1 was evident from the significant decrease in inhibition detected when chemotaxis to SDF-1 was initiated 4 hours following addition of Kp-10 (Supplementary Data 1).

Unilateral desensitization of CXCR4 signaling by GPR54 activation. The effect of GPR54 activation on CXCR4 signaling induced by SDF-1 was determined to elucidate potential mechanisms responsible for the suppression of chemotaxis stimulated by the latter ligand. Both Gi-coupled CXCR4 (and other chemotactic GPCRs) and Gq-coupled GPR54 can mobilize intracellular calcium by activating phospholipase C-β, either directly through the α subunit of Gq (GPR54) or through the Gβγ heterodimer (CXCR4). Crosstalk between GPCR can occur

Figure 2. Kp-10 inhibits the cytosolic calcium signaling response induced by the stimulation of CXCR4 by SDF-1 in CHO cells. Transfectants of CHO cells expressing CXCR4 and GPR54 were stimulated sequentially by SDF-1 or Kp-10. The addition of the first and the second ligand (*arrow*) are 3 minutes apart. *A*, cells stimulated with 100 nmol/L of SDF-1 first then 100 nmol/L of Kp-10 can fully respond to each ligand. *B-E*, the cells were exposed to either HBSS (*B*) or 1 nmol/L Kp-10 (*C*), 10 nmol/L Kp-10 (*D*), or 100 nmol/L Kp-10 (*E*; *first arrow*) followed by 100 nmol/L of SDF-1 (*second arrow*). The response to SDF-1 was almost completely abolished after exposure to 1 nmol/L of Kp-10 and was undetectable with higher concentrations. Representative of at least three independent experiments. Similar results were obtained in HeLa-GPR54 cells.

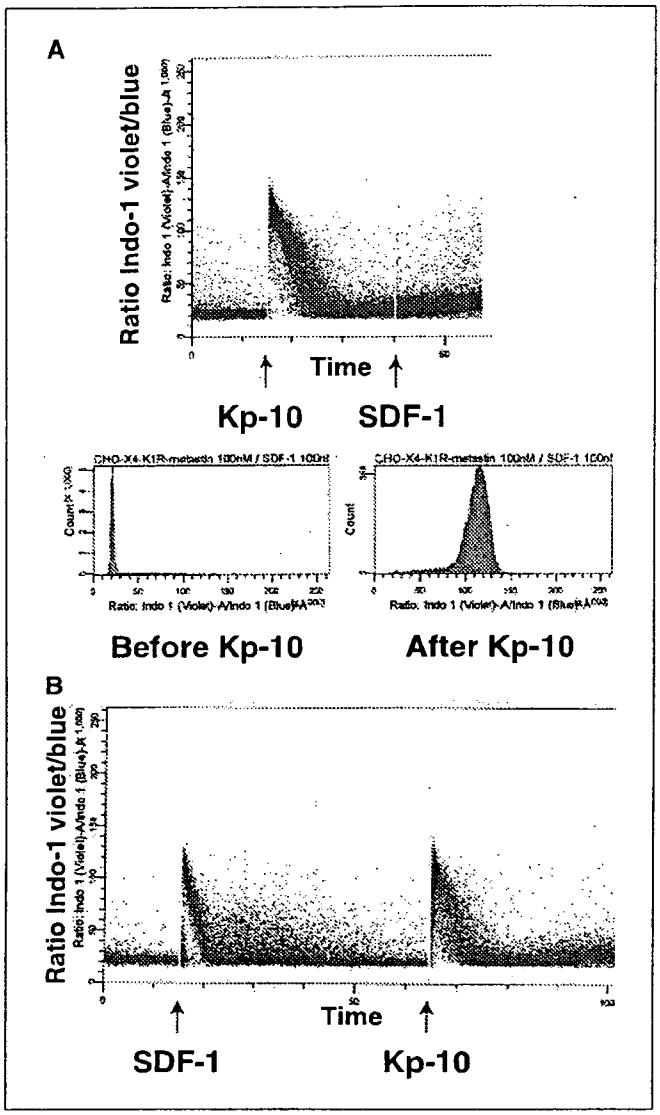


Figure 3. Calcium mobilization induced by SDF-1 and Kp-10 in CHO cells expressing CXCR4 and GPR54. *A*, the cells were sequentially exposed to 100 nmol/L of Kp-10 than 100 nmol/L of SDF-1. Time gating before and immediately after addition of Kp-10 (*bottom*) shows that over 99% of the cells responded 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 γ[^{35}S]GTP. Exposure of membrane fractions from CHO-CXCR4/GPR54 transfectants to SDF-1 resulted in a significant increase in γ[^{35}S]GTP over basal levels (Fig. 4*C*). In contrast, incubation with Kp-10 did not increase γ[^{35}S]GTP binding. Preincubation of membrane fractions with Kp-10 before exposure to SDF-1 did not alter γ[^{35}S]GTP binding

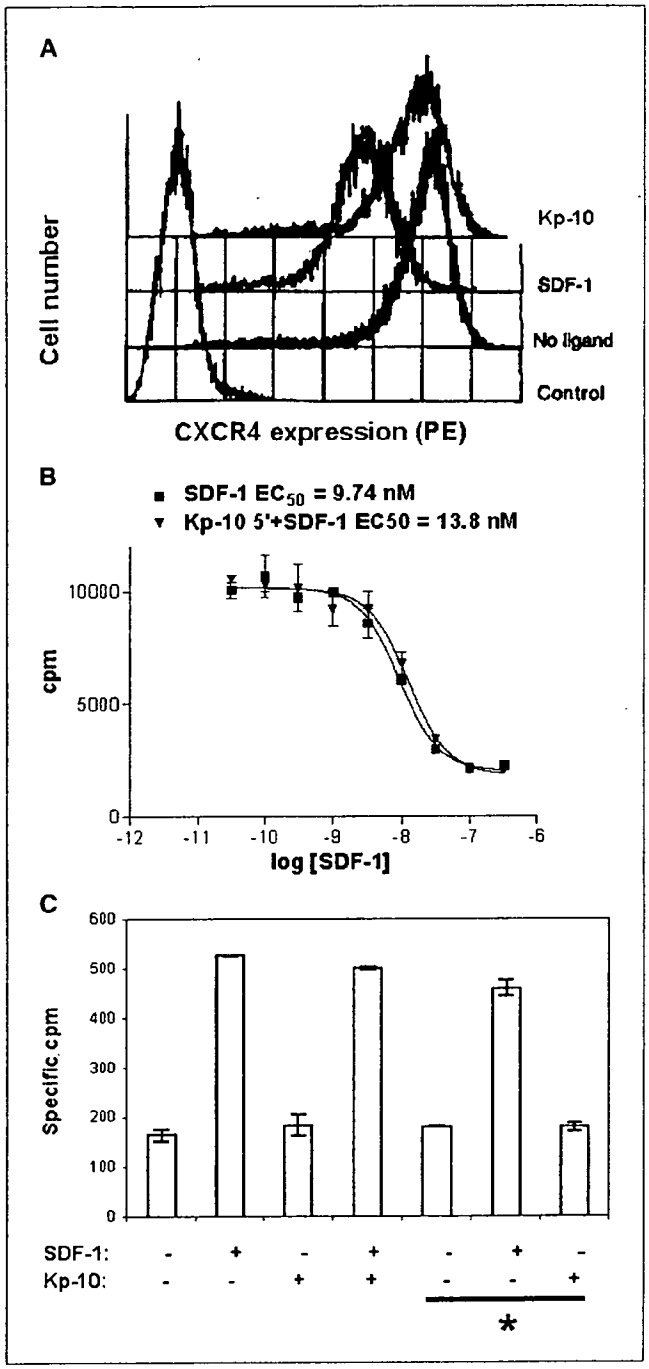


Figure 4. Stimulation of GPR54 by Kp-10 does not directly affect CXCR4. *A*, Kp-10 does not induce cross-internalization of CXCR4. CHO transfectants were either left unstimulated (no ligand) or exposed for 30 minutes to 100 nmol/L of SDF-1 or Kp-10 to allow internalization to occur. The cells were then 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

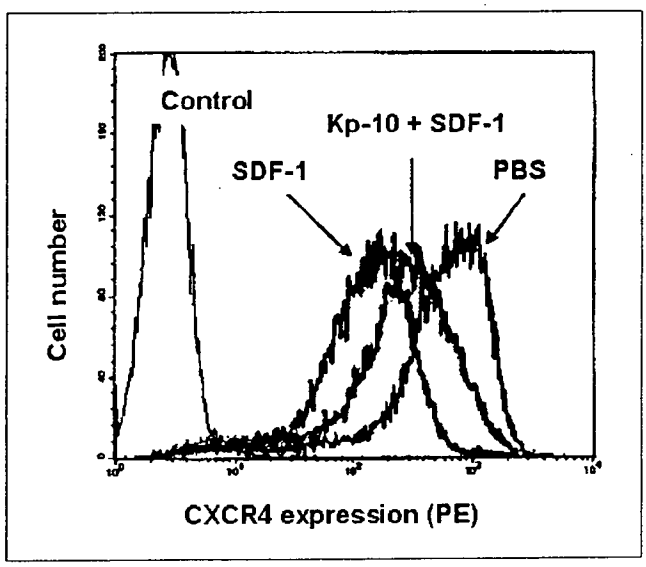


Figure 5. Internalization of CXCR4 in CHO cells after activation of the GPR54 with Kp-10. Cells were exposed to 100 nmol/L Kp-10 for 5 minutes at 37°C before addition of 100 nmol/L SDF-1 and incubation for 30 minutes at 37°C to allow internalization of CXCR4. After an acid wash, the cells were stained 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.

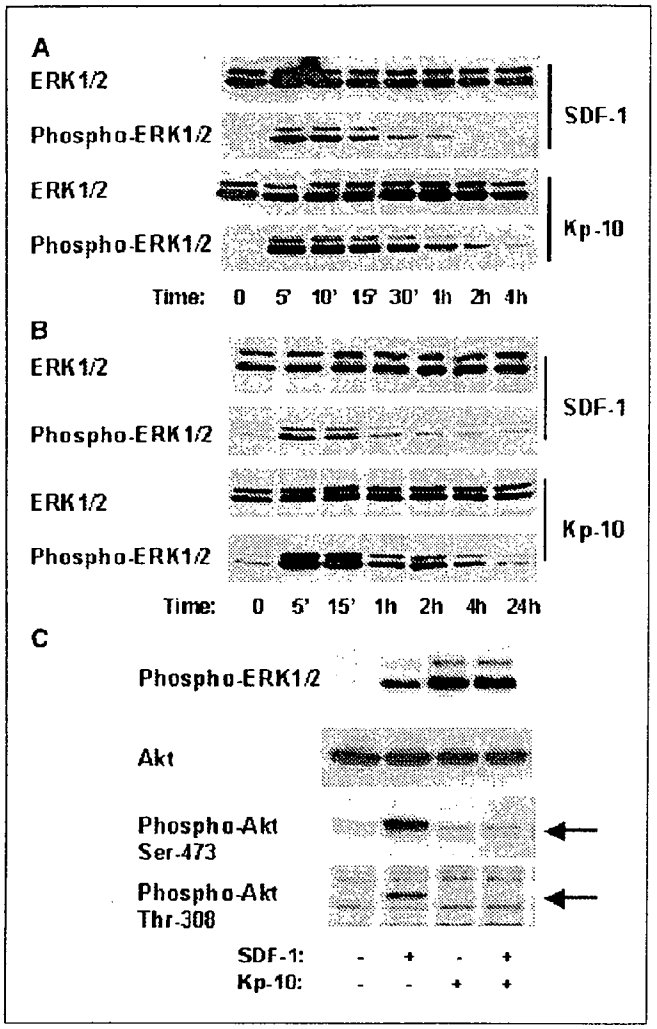


Figure 6. Signaling mechanism of GPR54 by Western blot. *A*, CHO cells expressing CXCR4 and GPR54 were exposed to 100 nmol/L of SDF-1 or Kp-10, and lysates were prepared at the indicated times from 5 minutes to 4 hours. *A*, long-lasting activation of ERK1/2 MAPK by GPR54 that is clearly detectable at 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