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T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer

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Abstract **A chemokine receptor, CXCR4, and its endogenous ligand, stromal cell-derived factor-1 (SDF-1), have been recognized to be involved in the metastasis of several types of cancers. T140 analogs are peptidic CXCR4 antagonists composed of 14 amino acid residues that were previously developed as anti-HIV agents having inhibitory activity against HIV-entry through its co-receptor, CXCR4. Herein, we report that these compounds effectively inhibited SDF-1-induced migration of human breast cancer cells (MDA-MB-231), human leukemia T cells (Sup-T1) and human umbilical vein endothelial cells at concentrations of 10–100 nM in vitro. Furthermore, slow release administration by subcutaneous injection using an Alzet osmotic pump of a potent and bio-stable T140 analog, 4F-benzoyl-TN14003, gave a partial, but statistically significant ($P \leq 0.05$ (*t*-test)) reduction in pulmonary metastasis of MDA-MB-231 in SCID mice, even though no attempt was made to inhibit other important targets such as CCR7. These results suggest that T140 analogs have potential use for cancer therapy, and that small molecular CXCR4 antagonists could potentially replace neutralizing antibodies as anti-metastatic agents for breast cancer.**

Key words: CXCR4 antagonist; Pulmonary metastasis; T140; Breast cancer

1. Introduction

Cancer metastasis represents the most serious step in the progress of this disease. There have been ample recent precedents supporting the involvement of chemokine receptors, such as CXCR4 and CCR7, in progression and metastasis of cancer cells [1–13]. CXCR4 and CCR7 belong to a 7TM GP-CR family that normally acts as receptors for chemokines, stromal cell-derived factor-1 (SDF-1/CXCL12) [14–17] and CCL21 [18–20], respectively. Chemokines belong to a chemotactic cytokine family that plays fundamental roles in the physiology of inflammatory processes by attracting and simulating leukocytes. Chemokine-induced leukocyte trafficking shares similarities with cancer cell migration and metastasis [2]. We have reported that SDF-1 mRNA is expressed in pancreatic cancer tissues, while CXCR4 mRNA is expressed both in pancreatic cancer tissues and in pancreatic cancer cell lines, and we have indicated that interactions between CXCR4 and SDF-1 are involved in pancreatic cancer progression [1]. Breast cancer and melanoma have similar metastatic patterns in terms of organ selectivity that involves lymph nodes, bone marrow, lung and liver. Müller reported that chemokine/chemokine receptor systems, such as SDF-1/CXCR4 and CCL21/CCR7 (and CCL27/CCR10 in melanoma), have been associated with metastasis of breast cancer and melanoma [2]. CXCR4 and CCR7 (and CCR10 in melanoma) are highly expressed in human breast cancer cells and malignant melanoma cells. Alternatively, SDF-1 and CCL21 (and CCL27 for melanoma) show high levels of expression in lymph nodes, bone marrow, lung and liver (and skin for melanoma), which constitute the most common metastasis sites of breast cancer and melanoma. In vitro, the interactions between CXCR4 and SDF-1 and between CCR7 and CCL21 trigger actin polymerization and pseudopodia formation and subsequently induce invasion of malignant cells in breast cancer. In vivo, metastasis of breast cancer cells can be inhibited by neutralization using anti-CXCR4 antibodies in mice. According to recent papers, the SDF/CXCR4 interaction axis is involved in cell progression and metastasis of several types of cancer, including prostate cancer [7], kidney cancer [8], neuroblastoma [4], non-Hodgkin's lymphoma [9], lung cancer [11], ovarian cancer [6,10], multiple myeloma [5], and chronic lymphocytic leukemia [12], in addition to pancreatic cancer, breast cancer and melanoma [3,13], angiogenesis [21,22]. Prior to the discovery of CXCR4 involvement in cancer progression and metastasis, CXCR4 had already been identified as a co-receptor in association with CD4 that is involved in the entry of T cell line-tropic (X4-) HIV-1 [23]. To date, we have developed several CXCR4 antagonists as HIV-entry inhibitors. These include T140 analogs [24–28]. T140 is a 14-residue peptide having a single disulfide bridge that possesses high anti-HIV and CXCR4 antagonistic activities. Here, in order to evaluate the potency of small molecule CXCR4 antagonists as anti-cancer-metastatic agents we investigated whether T140 ana-

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logs inhibit migration of breast cancer, endothelial and leukemia cells in vitro and breast cancer metastasis in vivo.

2. Materials and methods

2.1. Materials

T140 and its analogs, TC14012, TE14005 and 4F-benzoyl-TN14003, were synthesized by methodology described elsewhere [24,26–28].

2.2. Cell lines and cell culture

Jurkat T cell lymphomas (Jurkat E6-1 cells) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo (Osaka, Japan). HUVEC were maintained in Endothelial-SFM medium (Gibco BRL, Rockville, MD, USA) supplemented with 2% heat-inactivated fetal bovine serum (FBS), 2.5 ng/ml basic fibroblast growth factor (R&D systems, Minneapolis, MN, USA) and 10 ng/ml epidermal growth factor (Wako Pure Chemical Ind., Osaka, Japan). MDA-MB-231 human breast adenocarcinoma cells and Sup-T1 human T cell lymphoma were also obtained from the American Type Culture Collection. MDA-MB-231 cells were maintained in Leibovitz's L-15 medium supplemented with 10% FBS. Sup-T1 cells were maintained in RPMI1640 medium supplemented with 10% FBS.

2.3. Binding assay [29]

Jurkat cells were harvested and resuspended in the binding buffer (Dulbecco's PBS containing 20 mM HEPES and 0.5% bovine serum albumin, pH 7.0). Binding reactions were performed at room temperature for 1 h in the presence of 50 pM [^{125}I]-SDF-1α (specific activity: 2200 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) and various concentrations of peptide. Binding reactions were terminated by filtration over GF/C filters that were washed with cold PBS, and cell-associated radioactivity was counted by Top-count™ scintillation counter (Perkin Elmer) ($n = 3$).

2.4. RT-PCR

Poly A RNA extraction from cultured MDA-MB-231 cells was performed with QuickPrep mRNA purification kit (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's procedure. Sense and antisense primers were prepared including: CXCR4, 5′-ATCTGGAGAACCAGCGGTTA and 3′-ATGAGGACACTGCTGTAGAG (459 bp); CCR7, 5′-TGGTGGCTCTCCTTGTCATT and 3′-GATGGCCACGTAGCGGTCAA (440 bp); β-actin, 5′-ATCGAGCACGGCATCGTCACCA and 3′-GAGGAGCTGGAAGCAGCCGT (495 bp). Samples underwent thermal cycling at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min for 30 cycles. Fragment detection was performed using 2% agarose gel electrophoresis.

2.5. Cell migration assays [30]

Migration assays were performed in 24-well cell culture chambers using inserts with 8-μm pore membranes (Transwell, model 3422, Corning Inc., Corning, NY, USA). Membranes were pre-coated with bovine fibronectin (10 μg/ml) (Yanagi Corp., Yamagata, Japan). The lower chamber was filled with migration buffer (DMEM/0.1% BSA/12 mM HEPES) containing various concentrations of peptides and SDF-1 (30 nM for Sup-T1 cells, 100 nM for MDA-MB-231 cells and HUVEC) (R&D systems). Peptide solutions and cell suspension (2×10^5 cells/well) were added to the upper chamber. When Sup-T1 cells were assayed, cell numbers in the lower chamber were counted using a Coulter particle counter after incubation at 37°C for 4 h. When MDA-MB-231 cells and HUVEC were assayed, after incubation at 37°C for 15 h, non-migrating cells on the upper surface were fixed and stained with 0.5% crystal violet in 25% methanol. The filters were washed in distilled water, and the crystal violet was eluted by 0.1 M sodium citrate in 50% ethanol. Migrating cells were estimated by measuring OD_{550nm} with a microplate reader (Corona Electric, Ibaraki, Japan).

2.6. In vivo metastasis studies

CB-17 severe combined immunodeficient (SCID) mice (5 week old, female, Crea Japan Inc., Tokyo, Japan) 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 μl/h, Model 1002, ALZA Corp., Mountain View, CA, USA) 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 lungs green. The lungs were extracted and fixed with Bouin's fixative (picric acid: folmalin: acetic acid = 15:5:1), and the tumor area was also stained yellow. Ratios of tumor area to total area on the lung surface were calculated from difference of color between tumor and normal lung area using image analyzing techniques (MAC scope, Mitani Corp., Fukui, Japan).

2.7. Statistical analysis

Statistical significance of results in the migration assays was analyzed using Williams' test (depending on homogeneity of variance). A value of $P \leq 0.025$ was considered significant. Results of in vivo metastasis assays were assessed with Student's t-test. The level of significance was defined as $P \leq 0.05$.

3. Results

3.1. Evaluation of CXCR4-binding potency of T140 analogs

Among several T140 analogs, TC14012, TE14005 and 4F-benzoyl-TN14003 were utilized for this study (Table 1). CXCR4-binding potencies of these compounds were examined based on inhibition of [^{125}I]-SDF-1 binding to CXCR4-expressing Jurkat cells. We have previously reported the strong anti-HIV and CXCR4 antagonistic activities of these agents [26–28]. TC14012 and TE14005 showed approximately the same CXCR4-binding potency as that of T140, whereas 4F-benzoyl-TN14003 exhibited slightly higher potency than T140. These results are compatible with our previous reports.

3.2. CXCR4 and CCR7 mRNA expression

RT-PCR was performed using specific primers to examine CXCR4 and CCR7 mRNA expression in human breast carcinoma cell lines, MDA-MB-231. CXCR4 mRNA expression was clearly detected in MDA-MB-231 whereas the presence of CCR7 mRNA was minimal (Fig. 1).

3.3. Inhibition of migration of breast cancer, leukemia and endothelial cells in vitro

As reported by Müller [2], SDF-1/CXCL12 (100 nM) doubled the migration of the CXCR4-positive, human breast carcinoma cell line MDA-MB-231, as compared to control (Fig. 2A,C,E). T140 analogs inhibited SDF-1-induced migra-

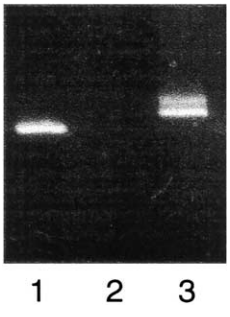

Fig. 1. CXCR4 and CCR7 mRNA expressions in human breast carcinoma cell lines, MDA-MB-231. Lane 1, CXCR4 mRNA expression; lane 2, CCR7 mRNA expression; lane 3, β-actin mRNA expression.

Table 1
Sequences and CXCR4-binding activity of T140 analogs

Compound	Sequence[a]	IC_{50} (nM)[b]
T140	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-OH	2.4
TC14012	H-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Cit-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2	2.9
TE14005	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-D-Glu-Pro-Tyr-Arg-Cit-Cys-Arg-OH	2.2
4F-benzoyl-TN14003	4-fluorobenzoyl-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2	0.99

[a]Each peptide has a single disulfide bridge between Cys^4 and Cys^{13}.
[b]IC_{50} values are the concentrations for 50% inhibition of binding of [^{125}I]-SDF-1 to CXCR4-expressing Jurkat cells. Data are mean values for three experiments.

tion of MDA-MB-231 in dose-dependent manners. At a concentration of 100 nM, TC14012, TE14005, and 4F-benzoyl-TN14003 caused 68%, 64%, and 78% reductions, respectively, of MDA-MB-231 chemotaxis induced by SDF-1 (100 nM). These compounds also reduced SDF-1-induced migration of Sup-T1 leukemia cells and HUVEC endothelial cells at a concentration of 10 nM (data shown, Fig. 2B: TC14012-HUVEC, Fig. 2D: 4F-benzoyl-TN14003-Sup-T1).

3.4. *Reduction of pulmonary metastasis in SCID mice with inoculation of MDA-MB-231 by slow release administration of 4F-benzoyl-TN14003*

Effect of the CXCR4 antagonist, 4F-benzoyl-TN14003, was investigated by using experimental metastasis models of breast cancer MDA-MB-231 cells. In this model, cells were injected intravenously (i.v.) into the tail vein of SCID mice and trapped in the lung through heart and pulmonary artery. Four mice were administered 4F-benzoyl-TN14003 by s.c. injection using an Alzet pump beginning from the day preceding transplantation of MDA-MB-231. Seven mice were not administered the agent as control models. By histological detection on day 28, agent-treated mice showed a relative suppression of tumor accumulation on lung surface based on the metastasis of MDA-MB-231 cells, as compared to control mice. Quantitative analyses revealed that ratios of tumor area to total lung surface area in treated mice were statistically smaller than those of control mice (Table 2). 4F-benzoyl-TN14003 significantly reduced pulmonary metastasis of MDA-MB-231 cells in SCID mice.

4. Discussion

CXCR4 represents an important therapeutic target for several diseases, including AIDS, cancer and rheumatoid arthritis. We have previously developed the 18- and 14-mer peptides, T22 [31] and T140 [24], respectively, as specific CXCR4 antagonists that prevent X4-HIV-1 entry mediated by this coreceptor. T140 possesses the highest level of anti-HIV ac-

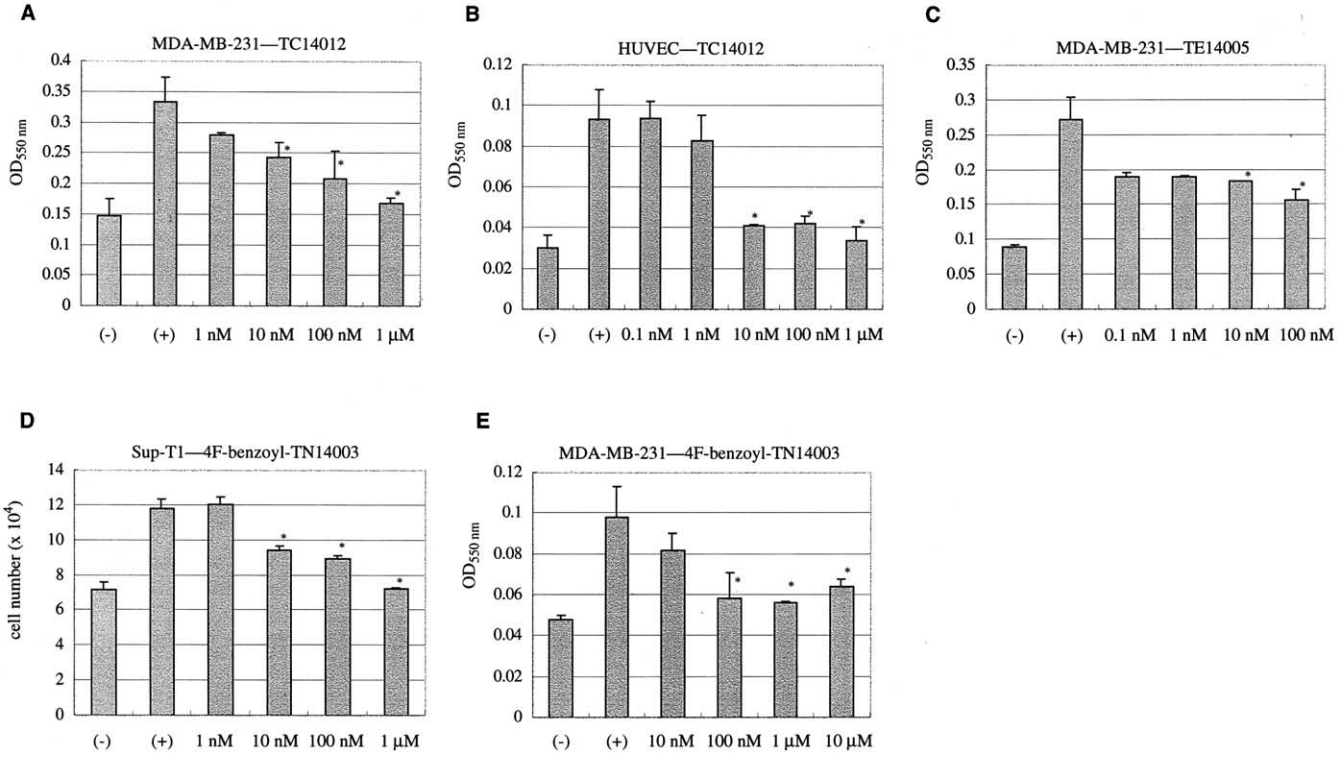

Fig. 2. Effects of T140 analogs on SDF-1-induced migration of MDA-MB-231, HUVE and Sup-T1 cells. MDA-MB-231 cells were treated by SDF-1 (100 nM) and various concentrations of TC14012 (A), TE14005 (C) and 4F-benzoyl-TN14003 (E). HUVEC were treated by SDF-1 (100 nM) and various concentrations of TC14012 (B). Sup-T1 cells were treated by SDF-1 (30 nM) and various concentrations of 4F-benzoyl-TN14003 (D). Control migrating cells in the absence and presence of SDF-1 are shown as (-) and (+), respectively. Data are expressed as means ± S.D. ($n = 2$). *$P \leq 0.025$ (Williams' test).

Table 2
Effects of 4F-benzoyl-TN14003 against pulmonary metastasis of breast cancer MDA-MB-231 cells in SCID mice

	Tumor area/total area of lung surface (%)[a]	S.D. (%)	Statistical significance
Control	39.35	8.20	
Treated[b]	24.80	4.66	*

*$P \leq 0.05$ (t-test).
[a]Mean values of four treated and seven control mice.
[b]Subcutaneous injection of 4F-benzoyl-TN14003 (80 mg/ml) using an Alzet osmotic pump.

tivity among all the CXCR4 antagonists that have been reported up to 1998. The Cit/Glu-substitution study based on T140 has generated several effective compounds, such as TC14012 and TE14005, where the total positive charge, which is related to cytotoxicity and non-specific binding, has been reduced from that of T140 [26,27]. TC14012 is completely stable in mouse serum due to its C-terminally amidated form. Furthermore, N-terminal acylation of T140 derivatives by a 4-fluorobenzoyl group to yield 4F-benzoyl-TN14003 increases anti-HIV activity and biostability in rat liver homogenate [28]. Since neutralization of SDF-1/CXCR4 interactions by anti-CXCR4 antibodies significantly impairs metastasis of breast cancer cells to lung and regional lymph nodes in mice [2], development of specific CXCR4 antagonists has been considered as a potential approach toward anti-metastatic agents. In the present study, we investigated whether CXCR4 antagonists derived from HIV-coreceptor inhibitors show anti-cancer-metastatic activity in vitro and in vivo. TC14012 and TE14005, which have CXCR4-binding ability comparable to that of T140 (Table 1), were used for cell migration assays in vitro. 4F-benzoyl-TN14003, which possesses relatively higher binding ability and increased biostability, was used for cell migration assays in vitro and metastasis studies in vivo.

Tumor cell invasion, including adhesion to matrix components (integrin, laminin, fibronectin, vitronectin, selectin, etc.), and migration through basement membranes, are significant parts of the process of cancer metastasis [32]. Cell migration, which is recognized as a critical step in metastasis, is partly induced by chemokines, such as SDF-1 and CCL21. In MDA-MB-231 human breast carcinoma cells, CXCR4 mRNA expression was confirmed (Fig. 1). Thus, SDF-1 induces migration of MDA-MB-231 cells through its interaction with CXCR4 (Fig. 2A,C,E) [2]. T140 analogs inhibit SDF-1-induced migration of MDA-MB-231 cells and Sup-T1 leukemia cells in dose-dependent manners, suggesting that these compounds might suppress tumor spread. Since TC14012 also inhibits SDF-1-induced migration of HUVEC endothelial cells, these compounds might also suppress tumor angiogenesis. These results prompted us to investigate whether T140 analogs suppress breast cancer metastasis in vivo.

Murakami reported that daily i.p. treatment with T22 [31] reduces pulmonary metastasis in mice following inoculation of CXCR4-transduced B16 melanoma cells, whereas T22 did not block metastasis in mice following inoculation of wild type B16 cells [13]. T22 inhibits CXCR4-mediated increase in melanoma metastasis. In our present study, the s.c. administration of 4F-benzoyl-TN14003 using an Alzet pump-mediated controlled slow release starting before transplantation of MDA-MB-231 cells, significantly reduced the pulmonary growth in the experimental metastasis model. This metastasis model mainly reflects the process of reattachment to distant organ, migration, invasion and proliferation, not detachment from primary organ and circulation. SDF-1 is strong chemoattractant factor on not only leukocytes but also various types of tumor cells and did not show any effects on the growth of MDA-MB-231 cells (data not shown), indicating that the metastasis suppression of 4F-benzoyl-TN14003 is due to the inhibition of the SDF-1-induced migration of MDA-MB-231 cells into lung tissue. It is reasonable that the T140 analog did not completely suppress metastasis (the decrease of tumor area: 39.4% to 24.8%), since the T140 analog can only block CXCR4 action but it cannot affect CCR7 action, and cancer metastasis is a process mediated by orchestration of several factors as described above. However, this result strongly suggests that small molecule CXCR4 antagonists, such as T140 analogs, could replace neutralizing anti-CXCR4 antibodies as anti-metastatic agents for the treatment of breast cancer.

In conclusion, CXCR4 antagonists, T140 analogs, inhibit SDF-1-induced migration of human breast cancer, leukemia and endothelial cells in vitro, in a fashion relevant to tumor spread and angiogenesis. Furthermore, a potent and bio-stable T140 analog, 4F-benzoyl-TN14003, reduced pulmonary metastasis of breast cancer in SCID mice. The suppression of SDF-1/CXCR4 interactions may represent a novel therapeutic strategy against cancer metastasis which involves this ligand-receptor system. These CXCR4 antagonists as well as other antagonists, including AMD3100 [33], ALX40-4C [34] and KRH-1636 [35], have the potential of becoming promising anti-cancer agents. Since T140 is an inverse agonist for a constitutively active mutant of CXCR4 and wild type CXCR4 and lacks partial agonistic activity (SDF-1-like activity) [36], it and its analogs might have some advantage in clinical development.

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