Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 320 (2004) 226–232
BBRC
www.elsevier.com/locate/ybbrc

A single treatment with microcapsules containing a CXCR4 antagonist suppresses pulmonary metastasis of murine melanoma

M. Takenaga,[a,*] H. Tamamura,[b] K. Hiramatsu,[b] N. Nakamura,[a] Y. Yamaguchi,[a] A. Kitagawa,[a] S. Kawai,[a] H. Nakashima,[c] N. Fujii,[b] and R. Igarashi[a]

[a] *Institute of Medical Science, St. Marianna University School of Medicine, Kawasaki 216-8512, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*
[c] *Department of Microbiology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan*

Received 15 May 2004
Available online 10 June 2004

Abstract

Biodegradable poly D,L-lactic acid (PLA, molecular weight: ca. 5000) microcapsules containing a CXCR4 antagonist (4F-benzoyl-TE14011) were prepared (4F-benzoyl-TE14011-PLA), and their anti-metastatic activity was evaluated in mice. A single subcutaneous administration of 4F-benzoyl-TE14011-PLA significantly reduced the number of colonies formed by pulmonary metastasis of B16–BL6 melanoma cells expressing CXCR4. The same dose of 4F-benzoyl-TE14011 in a single or a series of treatments affected little. The substance 4F-benzoyl-TE14011 dose-dependently suppressed B16–BL6 cell growth. In the cells cultured with SDF-1, a more potent suppression was observed. 4F-Benzoyl-TE14011 was rapidly released from 4F-benzoyl-TE14011-PLA for an initial period, both in vitro and in vivo. A steady release was thereafter observed. Therefore, this drug release profile might contribute to prevention of melanoma metastasis at the steps involving the migration and cell growth. These results also show that a sustained drug release formulation could be a useful drug delivery system for CXCR4 antagonists.

Keywords: B16–BL6; CXCR4 antagonist; Melanoma; Metastasis; Poly D,L-lactic acids; Sustained release

CXCR4 is a member of the GPCR protein family and is the receptor for a chemokine, stromal cell-derived factor (SDF)-1 [1,2]. The interaction with SDF-1 induces chemotaxis, immunomodulation, and other regulatory functions. CXCR4 also plays an important role in human immunodeficiency virus type-1 (HIV-1) infection. An HIV-1 envelope glycoprotein, gp120, initially binds to a CD4 molecule on the host cell surface [3]. This interaction leads to conformational changes in gp120 that increases its affinity for the HIV-1 co-receptor, the chemokine receptor CXCR4 or CCR5 [4,5]. Subsequent association of gp120 with CXCR4 or CCR5 promotes conformational changes in an HIV-1 transmembrane envelope glycoprotein, gp41 [6], leading to membrane fusion. The binding of HIV to CXCR4 is therefore of paramount importance to the viral infection.

Recently, CXCR4 has been reported to be involved in cancer metastasis [7–12]. Cancer metastasis is a complex event with many factors and steps. Robledo et al. [9] reported that human melanoma cells express the chemokine receptors CXCR3 and CXCR4, which mediate agonist-dependent cell migration and activation, and that these receptors might be relevant to tumor cell invasion and growth. Müller et al. [7] demonstrated that primary tumors of breast cancer and melanoma cell lines express CXCR4 as well as CCR7 at the messenger RNA level. Moreover, exposure of breast cancer cells to a function-blocking anti-CXCR4 mAb inhibited metastasis to the lungs, suggesting that CXCR4 might be involved in the selective metastasis of cancer cells. Furthermore, based on in vitro adhesion and chemotaxis experiments, several investigators have suggested roles for SDF-1 and CXCR4 in the metastasis of

* Corresponding author. Fax: +81-44-976-3747.
E-mail address: m2take@marianna-u.ac.jp (M. Takenaga).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.05.155

neuroblastoma [8], melanoma [9], and prostate cancer cells [10]. Murakami et al. [13] showed that an excessive expression of CXCR4 dramatically enhanced the metastatic accumulation of B16 melanoma cells in mice lungs. They also showed that the CXCR4 antagonist T22, developed in our laboratory [14], blocked pulmonary metastasis in mice injected with B16 cells transduced with CXCR4.

The T22 analog 4F-benzoyl-TE14011 used in the present study is one of the most potent CXCR4 antagonists that we have synthesized [14–20]. Its EC_{50} value, evaluated as anti-HIV activity in HIV-1-infected MT-4 cells, was 2.4 nM. However, due to its hydrophilicity and small molecular size, it might be rapidly eliminated from the body after dosing.

We have recently investigated a sustained-release formulation of insulin, for therapy of diabetic patients who require multiple dosages. We have developed this formulation by encapsulating insulin with a biodegradable polymer, poly co-poly(D,L-lactic/glycolic) acid (PLGA) [21–23]. Formulations prepared using biodegradable polymers such as PLGA and PLA have attractive attributes for a controlled and sustained release of drugs. In contrast to insulin, 4F-benzoyl-TE14011 would not require a strict controlled release. Since a single treatment can be effective for a long period, the formulation would be a valuable development, not only for anti-cancer-metastatic agents but also anti-HIV agents. The present study focused on the possibility of developing a sustained release formulation of 4F-benzoyl-TE14011.

Materials and methods

Reagents. 4F-Benzoyl-TE14011 was synthesized as reported previously [20]. Anti-human CXCR4-phycoerythrin (clone 12G5) was purchased from R&D systems (Minneapolis, MN). SDF-1 was purchased from ProTech EC (London, UK).

Cells. B16–BL6 mouse melanoma cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sanko Junyaku, Tokyo, Japan) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY).

Animals. Six-week-old male C57BL/6 or ICR mice were purchased from SLC Experimental Animals (Shizuoka, Japan). Animals were housed at a constant temperature (23 ± 1 °C) and humidity (50–60%) with free access to a standard diet and water. The animal room had a 12-h light/dark cycle (lights on from 6:30 to 18:30). The study protocol was approved by the Animal Experimentation Committee of St. Marianna University.

PLA microcapsules containing a CXCR4 antagonist. The preparation method was based on the double emulsion diffusion technique. 4F-Benzoyl-TE14011 (50 mg) and fatty acid ester saccharide (50 mg, J-1216, Mitsubishi-Kagaku Food Corporation, Tokyo, Japan) were dissolved in 2 ml of distilled water. This solution was poured into 20 ml of an acetone/methylene chloride (1:1, v/v) solution containing PLA (450 mg) and fatty acid ester saccharide (50 mg, J-1205, Mitsubishi-Kagaku Food Corporation). A water-in-oil (w/o) emulsion was then formed by stirring at 4000 rpm. This w/o emulsion was further added to a 0.5% carboxymethylcellulose (CMC) aqueous solution with stirring to achieve the (water-in-oil)-in-water (w/o/w) double emulsion system. This emulsion was stirred at 1200 rpm for 24 h to reduce the organic solvent volume. Lyophilization then gave the formulated drug (4F-benzoyl-TE14011-PLA). Particle size was determined by using a particle analyzer (Multisizer IIE, Beckman Coulter, Tokyo, Japan). The CXCR4 antagonist-loaded microcapsules were examined under a SEM 4300 scanning electron microscope (Hitachi, Ibaraki, Japan) at an acceleration voltage of 1.0 kV. The 4F-benzoyl-TE14011 contents of the prepared microcapsules were determined after extraction with methylene chloride and 0.01 M HCl according to the method of Lowry et al. [24], using 4F-benzoyl-TE14011 as a standard.

Experimental pulmonary metastasis of B16–BL6 melanoma cells. Anti-melanoma-metastatic activity was assessed using an experimental metastasis assay. Mice were inoculated via the tail vein with 1×10^4 viable B16–BL6 cells and were sacrificed by decapitation 2 weeks after tumor inoculation. The lungs were isolated, fixed with 70 (v/v)% ethanol, and examined for tumor nodules. The number of metastatic nodules on the lungs was counted in each mouse under a dissecting microscope. Drugs were administered subcutaneously 30 min before tumor cell inoculation. Daily treatment with 4F-benzoyl-TE14011 was carried out at approximately 10 o'clock in the morning. 4F-Benzoyl-TE14011-PLA was dispersed (10 w/v%) with 5% mannitol solution at pH 6.5 containing 0.5% CMC and 0.1% Tween 80, just before administration. An Alzet osmotic pump (duration, 14 days, pumping rate, 0.25 μl/h, Model 1002, ALZA, Mountain View, CA, USA) containing 35.7 mg/ml of 4F-benzoyl-TE14011 (100 μl in saline) was implanted subcutaneously 30 min before tumor cell transplantation.

Anti-HIV activity of mouse sera following the subcutaneous injection of the formulation. Male ICR mice were treated by a single injection of 4F-benzoyl-TE14011-PLA using a subcutaneous route. Blood samples were obtained from the inferior ophthalmic vein before and after this treatment.

The anti-HIV activity of serum samples on 2.5×10^4 Molt-4 cells infected with HIV-1 (HIV-1 IIIB, activity: 52×10^4 $TCID_{50}$/ml) was assessed by MTT assay as previously described [25].

In vitro release. 4F-Benzoyl-TE14011-PLA (100 mg) was suspended in 1.0 ml of phosphate-buffered saline (PBS) (pH 7.4) in a test tube and kept at 37 °C. At appropriate intervals, supernatants were collected after centrifugation, 1.0 ml of the buffer was added, and the mixture was further incubated. The content of the supernatant was determined by the method of Lowry et al. [24] using 4F-benzoyl-TE14011 as a standard.

Immunohistochemistry. B16–BL6 cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 24 h and washed by PBS, followed by treatment with 0.5% Triton-X in PBS for 5 min.

Mice were anesthetized and perfused with 4% paraformaldehyde in PBS by intracardiac injection. Lungs were fixed by immersion in fresh fixative for 1 h at room temperature. Tissue was cryoprotected by immersion in 10% sucrose in PBS for 1 h at room temperature and overnight in 30% sucrose in PBS at 4 °C. After cryoprotection, lungs were frozen in embedding OTC compound (Tissue Tek, Sakura, Torrance, CA), and then cut into 5 μm sections on a cryostat and placed on poly-L-lysine-coated glass slides.

After fixation and blocking by 5% BSA in PBS, lung sections were reacted overnight with phycoerythrin-conjugated anti-CXCR4 antibody. Fluorescence images were acquired using a conventional microscope equipped with epifluorescence optics (model IX71/CoolSNAP-HQ, Olympus, Melville, NY). Hematoxylin–eosin staining was also carried out.

Cell growth. B16–BL6 cells (1×10^3) suspended in FCS-free culture medium were seeded into a 96-well tissue culture plate. 4F-Benzoyl-TE14011 was dissolved in PBS (pH 7.4) and diluted by DMEM to make the desired concentrations. Various concentrations of 4F-benzoyl-TE14011 were cultured with cells for 3 days in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cell growth was assessed by [^{3}H]thymidine (methyl[1′,2′-^{3}H]thymidine (1.40 TBq/mmol),

Amersham, UK) incorporation over the last 4 h of incubation. The radioactivity was counted with a scintillation counter (MicroBeta TRILUX, Pharmacia, Sweden). Cell growth was also examined in the presence of SDF-1.

Statistical analysis. Statistical analysis was performed by using Mann–Whitney U test and $p < 0.05$ was taken as indicating significance.

Results

PLA microcapsules containing 4F-benzoyl-TE14011

Morphological examination study using a scanning electron microphotograph showed spherical surfaces of the PLA particles, with a diameter of 14.57 ± 11.21 μm. The 4F-benzoyl-TE14011 content was 5.6%, indicating that the loading efficacy was approximately 90%.

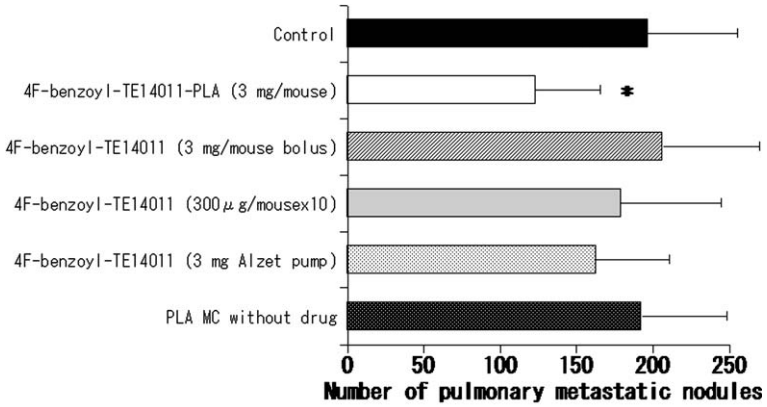
Experimental pulmonary metastasis of B16–BL6 melanoma cells in mice

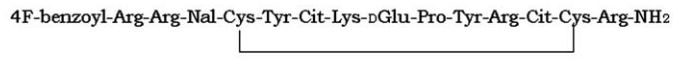
In the vehicle-treated control group, the number of pulmonary metastatic nodules was 196.4 ± 58.6 (mean ± SE) per mouse (Fig. 1). 4F-Benzoyl-TE14011-PLA reduced the number of colony formations, even though not preventing metastatic incidence. When 4F-benzoyl-TE14011-PLA (3 mg as 4F-benzoyl-TE14011) was injected to mice, the number of metastatic nodules was 122.9 ± 42.6 per mouse, which was significantly lower than that in the control group ($p < 0.05$). The PLA formulation alone showed no effect (192.3 ± 56.1). Bolus subcutaneous injection of the parent agent, 4F-benzoyl-TE14011 (3 mg), showed no significant difference compared with the control group. Daily treatment by subcutaneous injection of 4F-benzoyl-TE14011 (300 μg/mouse) for 10 days showed a partial, but not significant, reduction (178.9 ± 65.8). Subcutaneous injection of 4F-benzoyl-TE14011 using an Alzet pump implanted before tumor cell inoculation also reduced the number of colony formations (162.5 ± 47.9).

When 4F-benzoyl-TE14011-PLA was treated 30 min after tumor cell inoculation, the number of metastatic nodules was also reduced. However, there was no significant difference. (168 ± 42.5, vs. control 201.4 ± 49.1). On day 14, the formulation of 4F-benzoyl-TE14011-PLA was detected little at the injected site. The 4F-benzoyl-TE14011-treated group showed a tendency to reduce the metastatic nodule overall size, but not significance.

Anti-HIV activity of mouse sera following the subcutaneous administration of the formulation

The anti-HIV activity of serum samples is thought to reflect the plasma concentrations of 4F-benzoyl-TE14011 after subcutaneous injection of 4F-benzoyl-TE14011-PLA. 4F-Benzoyl-TE14011-PLA (3 mg as 4F-benzoyl-TE14011) showed a transient augmentation of anti-HIV activity (Fig. 2A). Thereafter, the activity again increased and then gradually decreased. A single injection of 4F-benzoyl-TE14011 showed only a transient increase of anti-HIV activity.





Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline

Fig. 1. Effects of 4F-benzoyl-TE14011-PLA on experimental pulmonary metastasis. B16–BL6 cells (1×10^4/0.2 ml) were injected into the tail veins of mice. 4F-Benzoyl-TE14011-PLA, 4F-benzoyl-TE14011 (bolus injection, 3 mg drug), 4F-benzoyl-TE14011 (300 μg/mouse/day for 10 days), 4F-benzoyl-TE14011 (Alzet pump, constant release of total 3 mg 4F-benzoyl-TE14011 over 2 weeks), or PLA microcapsules (MC) without drug was administered through a subcutaneous route 30 min prior to tumor cell inoculation. Alzet pump containing 4F-benzoyl-TE14011 was also implanted 30 min before tumor cell injection. On day 14, mice were sacrificed, and tumor nodules on the surface of the lungs were counted. The chemical structure of 4F-benzoyl-TE14011 was shown below. Data represent means ± SE. $n = 5$–8. *$p < 0.05$, vs. control.

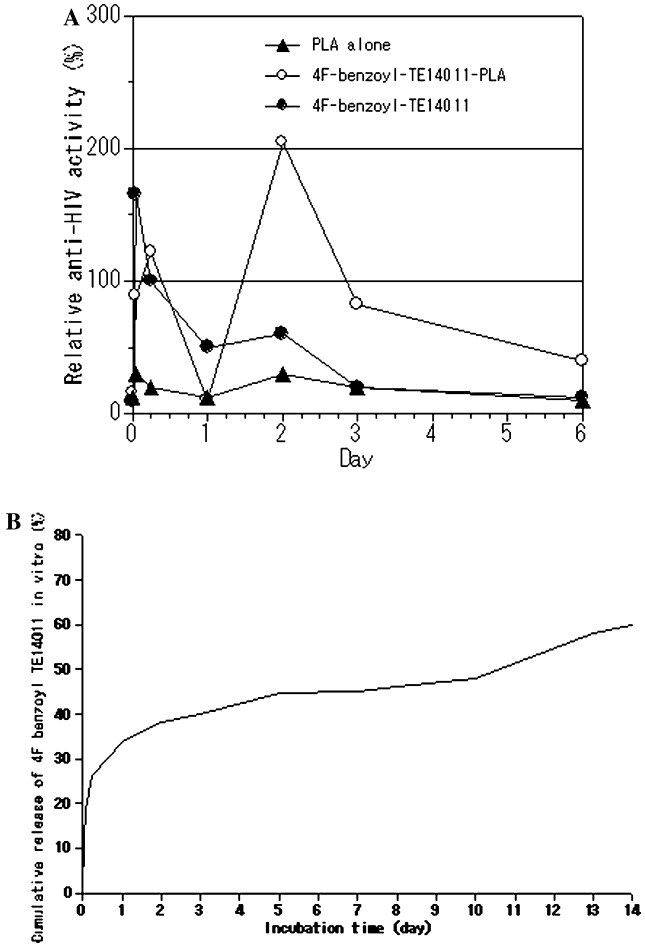


Fig. 2. (A) Serum anti-HIV activity following subcutaneous treatment of 4F-benzoyl-TE14011-PLA and (B) In vitro drug release from 4F-benzoyl-TE14011-PLA. (A) Serum anti-HIV activity following subcutaneous treatment of 4F-benzoyl-TE14011-PLA (3 mg as 4F-benzoyl-TE14011), 4F-benzoyl-TE14011 (bolus injection, 3 mg drug), or PLA alone was determined as described in Materials and methods. The relative anti-HIV activity was calculated from the concentration required for ED80. Data are means obtained from 3 mice. (B) In vitro 4F-benzoyl-TE14011 release from 4F-benzoyl-TE14011-PLA was evaluated. Data represent means obtained from 3 to 5 separate experiments.

In vitro release of 4F-benzoyl-TE14011 from 4F-benzoyl-TE14011-PLA

The in vitro release from 4F-benzoyl-TE14011-PLA was examined (Fig. 2B). The cumulative amount of 4F-benzoyl-TE14011 released from 4F-benzoyl-TE14011-PLA was 11.7% (2 h) and 33.8% (1 day). An acceleration of drug release was again seen after 10 days incubation.

Expression of CXCR4 on B16–BL6 cells and lung specimen

Immuno-fluorescence microscopic analysis demonstrated that B16–BL6 cells express CXCR4 on their surface (Figs. 3A and B). CXCR4 expression in the lung sections was also observed, especially along the epithelial cells Figs. 3C and D.

Cell growth and CXCR4 expression of lung specimens after B16–BL6 melanoma inoculation

HE staining revealed that tumor cells migrated and grew around not only the epithelial cells but also blood vessels (Fig. 3F). They also grew on the surface of lung (Figs. 3G and H). The metastatic nodule on the surface of lung showed CXCR4 expression 2 weeks after cell inoculation (Figs. 3I and J). Lung sections of mice (Figs. 3H and I) were ones treated with 4F-benzoyl-TE14011-PLA, and the sections (Figs. 3F, G, and J) were control. The intensity of the CXCR4 expression following nodule formation showed no significant difference between the two. However, the grown tumor cells were localized less around the epithelial cells in the 4F-benzoyl-TE14011-PLA-treated group.

Effect of 4F-benzoyl-TE14011 on cell growth

The growth of B16–BL6 cells was examined under conditions that included extremely low concentrations of serum. Dose-dependent suppression of the cell growth was observed by the treatment with 4F-benzoyl-TE14011 (Table 1). 4F-Benzoyl-TE14011 (300 μg/ml) reduced thymidine incorporation (dpm) by one-tenth. The inhibitory activity was less under serum conditions (data not shown).

SDF-1 enhanced the cell growth by nearly 2-fold compared to that in the absence of SDF-1. It should be noted that even 3 μg/ml of 4F-benzoyl-TE14011 significantly reduced the cell growth.

Discussion

Our present study showed that a subcutaneous single administration of 4F-benzoyl-TE14011-PLA, a biodegradable polymer microcapsule formulation containing a CXCR4 antagonist (4F-benzoyl-TE14011), significantly reduced the number of colonies formed by pulmonary metastasis of B16–BL6 melanoma cells.

CXCR4 antagonists have been identified as anti-HIV agents [26–29]. 4F-Benzoyl-TE14011 is one of the most potent CXCR4 antagonists that we have synthesized based on T22 and its smaller analog, T140 [14–20]. 4F-Benzoyl-TE14011, more potent than AZT in the MTT assay, is stable in mouse serum and rat liver homogenate [20].

4F-Benzoyl-TE14011-PLA was proven to have anti-melanoma metastatic activity following a single subcutaneous dosing, indicating that this drug delivery system is promising. Murakami et al. [13] have already shown

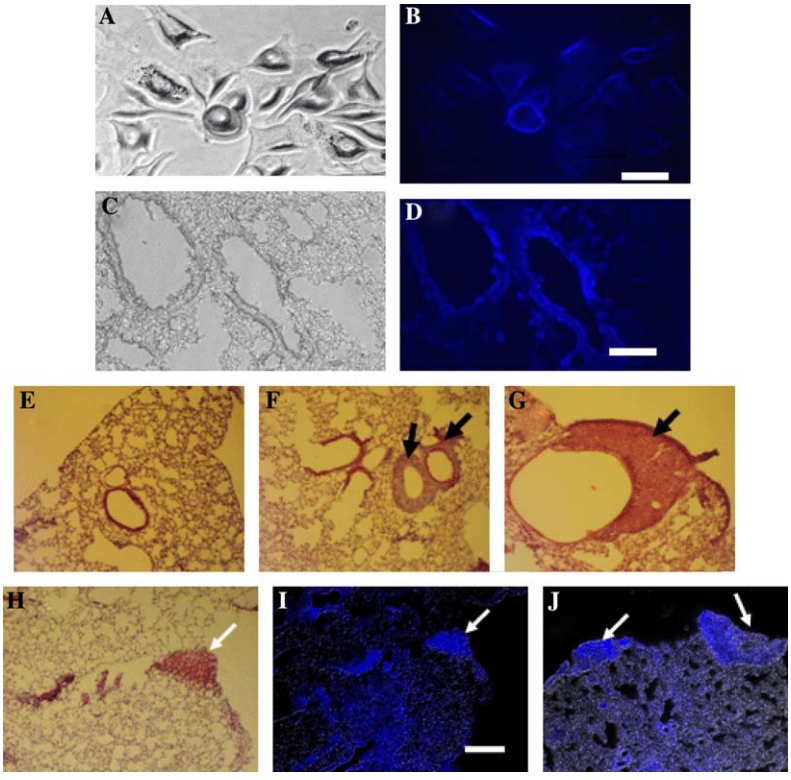


Fig. 3. B16–BL6 melanoma cells and lung specimen before or after cell inoculation. (A,B) B16–BL6 melanoma cells (scale bar is 10 μm.) (C–E) The normal lung section (scale bar is 20 μm.) (F–J) The lung section 2 weeks after tumor cell inoculation. Arrow; tumor cell growth. (B, D, I, and J) Immunostaining of CXCR4 was performed. (E–H) Hematoxylin–eosin (HE) staining.

Table 1
Effects of 4F-benzoyl-TE14011 on the SDF-1-stimulated cell growth in vitro

Addition of SDF-1 (ng/ml)	4F-benzoyl-TE14011 (μg/ml)				
	0	0.3	3	30	300
0	6698.1 ± 1962.9	8115.7 ± 1733.0	7556.0 ± 1892.0	6187. ± 1444.2	530.3 ± 516.5**
5	11128.9 ± 1417.0#	9320.5 ± 478.0	8555.1 ± 762.2*	7784.4 ± 2445.4*	845.7 ± 562.4**
50	11251.2 ± 678.7#	9622.1 ± 949.0	8916.1 ± 625.7**	7342.4 ± 573.0**	606.4 ± 224.2**

B16–BL6 cells (1×10^3/well) suspended in FCS-free culture medium were seeded to a culture plate in the presence or absence of SDF-1. Various concentrations of TE14011 were added and subsequently culture was done for 3 days. Cell growth was assessed by [^{3}H]thymidine incorporation over the last 4 h of incubation. Data are means ± SD (dpm) ($n = 8$).

#$p < 0.01$ vs. no addition of SDF-1.

*$p < 0.05$, **$p < 0.01$ vs. no addition of 4F-benzoyl-TE14011.

that daily treatment with T22 (4 μg peptide/mouse) via an i.p. route for 14 days reduced the number of nodules formed by pulmonary metastasis of melanoma cells. Even though a single treatment, 4F-benzoyl-TE14011-PLA required higher amounts of drug. This may be partly explained by the difference in the administration route. Another major reason would derive from the dependency of metastasis on the CXCR4–SDF-1 system. They demonstrated that transfection of the CXCR4 gene in B16–F10 cells dramatically enhanced the metastatic accumulation of tumor cells in the lungs of mice. B16–BL6 melanoma cell lines used in our study have a high metastatic potency, especially to the lungs. Although we confirmed the expression of CXCR4 on the surface, the metastatic potency seemed to be less dependent on the CXCR4-ligand system than that of CXCR4 gene-transfected B16–F10 cells used by Murakami et al. Therefore, their metastatic system might have required less amount of 4F-benzoyl-TE14011.

Nevertheless, it is evident that the CXCR4–SDF-1 system is involved in the growth of B16–BL6 cells. SDF-1 significantly promoted the cell growth. It was notable that the SDF-1-mediated cell growth was effectively suppressed by 4F-benzoyl-TE14011. This shows the possibility that 4F-benzoyl-TE14011 released from 4F-benzoyl-TE14011-PLA suppressed the cell growth at the metastatic destination sites in vivo. HE staining revealed that the cell growth spread not only on the surface of lungs but also around blood vessels and the epithelial cells. The grown tumor cells were associated with

CXCR4 expression. The expression was not diminished by 4F-benzoyl-TE14011-PLA, but the tumor growth was less around the epithelial cells. These evidences are support for 4F-benzoyl-TE14011 suppression of the metastatic process through CXCR4 containing cell growth.

The same dose of 4F-benzoyl-TE14011 alone (bolus injection) did not significantly reduce the number of metastatic nodules. Successive subcutaneous treatments had been expected to be potent, but no significant difference was observed. These could be partly ascribed to its easy elimination from the body, as seen in serum anti-HIV activity. Alzet pump administration causes a constant drug release without an initial large release, but it was less effective. 4F-Benzoyl-TE14011-PLA showed a transient increase of anti-HIV activity, which is consistent with an initially rapid drug release in vitro. 4F-Benzoyl-TE14011-PLA thereafter kept on releasing the drug with a lower but a relatively steady rate in vitro. This finding prompted us to advance the hypothesis that the desired pharmacological activity requires a profile with an initial large release followed by lower constant release. In vitro release study showed an acceleration of the drug release after 10 days incubation. This phenomenon was also seen in the case of insulin [21]. Plasma insulin level showed a lag phase and gradual augmentation in vivo, which was similar as in vitro release. In general, the drug release is associated with PLA polymer degradation. The serum anti-HIV activity may not completely reflect drug bio-distribution, but it might have been transiently but again higher after day 6, and have contributed to the pharmacological effect.

An advantage of formulations based on biodegradable polymers such as PLGA and PLA is an ability to maintain a relatively sustained drug release. Since these polymers are degraded by hydrolysis, followed by a drug release when exposed to aqueous media, they have been used to achieve sustained releases of various drugs [30–33]. Leuprolein acetate-loaded PLGA has already been used in a clinical setting and has made an important contribution to the remarkable improvement in the physiological condition of patients with prostate cancer, etc. A small molecular and hydrophilic drug is difficult to encapsulate, and susceptible to being released rapidly into aqueous media. PLA has an adequate entrapping efficacy for such drugs, compared to PLGA. However, PLA brings a rapid release, and there are many problems that need to be solved for achievement of a more constant release. In the present study using this system, the first rapid release of 4F-benzoyl-TE14011 might contribute to the suppression of metastasis.

4F-benzoyl-TE14011 might suppress both the migration of B16–BL6 cells into the target organs and the formation of nodules. In addition, several other papers have shown the efficacy of CXCR4 antagonists derived from T140 as anti-cancer agents: 4F-benzoyl-TN14003 showed significant suppression of pulmonary metastasis of MDA-MB-231 breast cancer cells in mice [34]. TN14003 was proven to inhibit SDF-1-induced migration and invasion of several human pancreatic cancer cells [35]. T140 was shown to block SDF-1-induced chemotaxis and attenuated the migration of pre-B acute lymphoblastic leukemia cells into bone marrow stromal layers [36], and to inhibit the interaction of small cell lung cancer cells with stromal cells [37]. Taken together, T140 analogs are promising agents for cancer chemotherapy.

The present study provides insight into the possibility of a new administration strategy for CXCR4 antagonists.

Acknowledgments

We are very grateful to Ms. Akemi Hamaguchi and Ms. Kayo Matsumoto for their excellent assistance. The authors wish to thank Prof. Scott McN. Sieburth, Department of Chemistry, Temple University, PA, USA, for proofreading the manuscript and providing useful comments.

References

[1] C.C. Bleul, M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, T.A. Springer, The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry, Nature 382 (1996) 829–833.

[2] E. Oberlin, A. Amara, F. Bachelerie, C. Bessaia, J.L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J.M. Heard, I. Clark-Lewis, D.F. Legler, M. Loetscher, M. Baggiolini, B. Moser, The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1, Nature 382 (1996) 833–835.

[3] A.G. Dalgleish, P.C. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves, R.A. Weiss, The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus, Nature 312 (1984) 763–767.

[4] L. Wu, N.P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A.A. Cardoso, E. Desjardin, W. Newman, C. Gerard, J. Sodroski, CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5, Nature 384 (1996) 179–183.

[5] A. Trkola, T. Dragic, J. Arthos, J.M. Binley, W.C. Olson, G.P. Allaway, C. Cheng-Mayer, J. Robinson, P.J. Maddon, J.P. Moore, CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5, Nature 384 (1996) 184–187.

[6] P.L. Jones, T. Korte, R. Blumenthal, Conformational changes in cell surface HIV-1 envelope glycoproteins are triggered by cooperation between cell surface CD4 and co-receptors, J. Biol. Chem. 273 (1998) 404–409.

[7] A. Müller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verastegul, A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis, Nature 410 (2001) 50–56.

[8] H. Germinder, O. Sagi-Assif, L. Goldberg, T. Meshel, G. Rechavi, I.P. Witz, A. Ben-Baruch, A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the

development of bone marrow metastasis in neuroblastoma, J. Immunol. 167 (2001) 4747–4757.
[9] M.M. Robledo, R.A. Bartolome, N. Longo, J.M. Rodriguez-Frade, M. Mellado, I. Longo, G.N.P. Muijen, P. Sanchez-Mateos, J. Teixido, Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells, J. Biol. Chem. 276 (2001) 45098–45105.
[10] R.S. Taichman, C. Cooper, E.T. Keller, K.J. Pienta, N.S. Taichman, L.K. McCauley, Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone, Cancer Res. 62 (2002) 1832–1837.
[11] S. Hatse, K. Princen, G. Bridger, E. De Clercq, D. Schols, Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4, FEBS Lett. 527 (2002) 255–262.
[12] R.J. Phillips, M.D. Burdick, M. Lutz, J.A. Belperio, M.P. Keane, R.M. Strieter, The SDF-1/CXCL12/CXCR4 biological axis in non-small cell lung cancer metastases, Am. J. Respir. Crit. Care Med. 167 (2003) 37–93.
[13] T. Murakami, W. Maki, A.R. Cardones, H. Fang, A.T. Kyi, F.O. Nestle, S.T. Hwang, Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells, Cancer Res. 62 (2002) 7328–7334.
[14] N. Fujii, H. Nakashima, H. Tamamura, The therapeutic potential of CXCR4 antagonists in the treatment of HIV, Expert. Opin. Investig. Drugs 12 (2003) 185–195.
[15] W.B. Zhang, J.M. Navenot, B. Haribabu, H. Tamamura, K. Hiramatu, A. Omagari, G. Pei, J.P. Manfredi, N. Fujii, J.R. Broach, S.C. Peiper, A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists, J. Biol. Chem. 277 (2002) 24515–24521.
[16] H. Tamamura, K. Hiramatsu, K. Miyamoto, A. Omagari, S. Oishi, H. Nakashima, N. Yamamoto, Y. Kuroda, T. Nakagawa, A. Otaka, N. Fujii, Synthesis and evaluation of pseudopeptide analogues of a specific CXCR4 inhibitor, T140: the insertion of an (E)-alkene dipeptide isostere into the betaII'-turn moiety, Bioorg. Med. Chem. Lett. 12 (2002) 923–928.
[17] H. Tamamura, A. Omagari, K. Hiramatsu, S. Oishi, H. Habashita, T. Kanamoto, K. Gotoh, N. Yamamoto, H. Nakashima, A. Otaka, N. Fujii, Certification of the critical importance of L-3-(2-naphthyl)alanine at position 3 of a specific CXCR4 inhibitor, T140, leads to an exploratory performance of its downsizing study, Bioorg. Med. Chem. 10 (2002) 1417–1426.
[18] H. Tamamura, A. Omagari, K. Hiramatsu, K. Gotoh, T. Kanamoto, Y. Xu, E. Kodama, M. Matsuoka, T. Hattori, N. Yamamoto, H. Nakashima, A. Otaka, N. Fujii, Development of specific CXCR4 inhibitors possessing high selectivity indexes as well as complete stability in serum based on an anti-HIV peptide T140, Bioorg. Med. Chem. Lett. 11 (2001) 1897–1902.
[19] H. Tamamura, K. Hiramatsu, S. Kusano, S. Terakubo, N. Yamamoto, J.O. Trent, Z. Wang, S.C. Peiper, H. Nakashima, A. Otaka, N. Fujii, Synthesis of potent CXCR4 inhibitors possessing low cytotoxicity and improved biostability based on T140 derivatives, Org. Biomol. Chem. 1 (2003) 3656–3662.
[20] H. Tamamura, K. Hiramatsu, M. Mizumoto, S. Ueda, S. Kusano, S. Terakubo, M. Akamatsu, N. Yamamoto, J.O. Trent, Z. Wang, S.C. Peiper, H. Nakashima, A. Otaka, N. Fujii, Enhancement of the T140-based pharmacophores leads to the development of more potent and bio-stable CXCR4 antagonists, Org. Biomol. Chem. 1 (2003) 3663–3669.
[21] M. Takenaga, Y. Yamaguchi, A. Kitagawa, Y. Ogawa, Y. Mizushima, R. Igarashi, A novel sustained-release formulation of insulin with dramatic reduction in initial rapid release, J. Control. Rel. 79 (2002) 81–91.
[22] M. Takenaga, Y. Yamaguchi, A. Kitagawa, Y. Ogawa, Y. Mizushima, R. Igarashi, A novel insulin formulation can keep providing steady levels of insulin for much longer periods, J. Pharm. Pharmacol. 54 (2002) 1189–1194.
[23] Y. Yamaguchi, M. Takenaga, A. Kitagawa, Y. Ogawa, Y. Mizushima, R. Igarashi, Insulin-loaded biodegradable PLGA microcapsules-initial burst release controlled by the hydrophilic additives, J. Control. Rel. 81 (2002) 235–249.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[25] M. Premanathan, H. Nakashima, R. Igarashi, Y. Mizushima, K. Yamada, Lecithinized superoxide dismutase: an inhibitor of human immunodeficiency virus replication, AIDS Res. Hum. Retroviruses 13 (1997) 283–290.
[26] J. Zhao, L.H. Ben, Y.L. Wu, W. Hu, K. Ling, S.M. Xin, H.L. Nie, L. Ma, G. Pei, Anti-HIV agent trichosanthin enhances the capabilities of chemokines to stimulate chemotaxis and G protein activation, and this is mediated through interaction of trichosanthin and chemokine receptors, J. Exp. Med. 190 (1999) 101–111.
[27] C.W. Hendrix, C. Flexner, R.T. MacFarland, C. Giandomenico, E.J. Fuchs, E. Redpath, G. Bridger, G.W. Henson, Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers, Antimicrob. Agents Chemother. 4 (2000) 1667–1673.
[28] K. Kanbara, N. Fujii, H. Nakashima, A study of anti-HIV compounds which interfere the virus entry via coreceptor CXCR4, Kansenshogaku Zasshi 74 (2000) 237–244.
[29] K. Ichiyama, S. Yokoyama-Kumakura, Y. Tanaka, R. Tanaka, K. Hirose, K. Bannai, T. Edamatsu, M. Yanaka, Y. Niitani, N. Miyano-Kurosaki, H. Takaku, Y. Koyanagi, N. Yamamoto, A duodenally absorbable CXC chemokine receptor 4 antagonist, KRH-1636, exhibits a potent and selective anti-HIV-1 activity, Proc. Natl. Acad. Sci. USA 100 (2003) 4185–4190.
[30] H. Okada, One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate, Adv. Drug Deliv. Rev. 13 (1997) 43–70.
[31] T. Chandy, G.S. Das, G.H. Rao, 5-Fluorouracil-loaded chitosan coated polylactic acid microspheres as biodegradable drug carriers for cerebral tumours, J. Microencapsul. 17 (2000) 625–638.
[32] C. Ustariz-Peyret, J. Coudane, M. Vert, V. Kaltsatos, B. Boisramene, Labile conjugation of a hydrophilic drug to PLA oligomers to modify a drug delivery system: cephradin in a PLAGA matrix, J. Microencapsul. 17 (2000) 615–624.
[33] Y.Y. Huang, T.W. Chung T, Microencapsulation of gentamicin in biodegradable PLA and/or PLA/PEG copolymer, J. Microencapsul. 18 (2001) 457–465.
[34] H. Tamamura, A. Hori, N. Kanzaki, K. Hiramatsu, M. Mizumoto, H. Nakashima, N. Yamamoto, A. Otaka, N. Fujii, T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer, FEBS Lett. 550 (2003) 79–83.
[35] T. Mori, R. Doi, M. Koizumi, E. Toyoda, D. Ito, K. Kami, T. Masui, K. Fujimoto, H. Tamamura, K. Hiramatsu, N. Fujii, M. Imamura, CXCR4 antagonist inhibits stromal cell-derived factor 1-induced migration and invasion of human pancreatic cancer, Mol. Cancer Ther. 3 (2004) 29–37.
[36] J. Juarez, K.F. Bradstock, D.J. Gottlieb, L.J. Bendall, Effects of inhibitors of the chemokine receptor CXCR4 on acute lymphoblastic leukemia cells in vitro, Leukemia 17 (2003) 1294–1300.
[37] M. Burger, A. Glodek, T. Hartmann, A. Schmitt-Graft, L.E. Seilberstein, N. Fujii, T.J. Kipps, J.A. Burger, Functional expression of CXCR4 (CD184) on small cell lung cancer cells (SCLC) mediates migration, integrin activation, and adhesion to stromal cells, Oncogene 22 (2003) 8093–8101.