polyphemusin [9, 10]. On the basis of the structure of T22, we designed and synthesized several downsized analogs, 14-residue peptides [11, 12]. Among them, T140 showed the greatest inhibitory effect on the binding of an anti-CXCR4 monoclonal antibody to CXCR4 and the strongest inhibitory activity against HIV-1 entry [12]. The aim of this study is to develop a radiolabeled T140 derivative as an imaging agent for metastatic tumors. Considering that the three residues on the restricted backbone (L-3-(2-naphthyl)alanine (Nal)^3, Tyr^5 and Arg^{14}) and the single residue in the flexible region (Arg^2) form the intrinsic pharmacophore of T140 [13–15], we designed a 14-residue peptidic inhibitor, Ac-TZ14011, as the precursor for radiolabeled peptides (Figure 1). This precursor contains the above four residues which are necessary for the inhibitory activity against CXCR4. Furthermore, for site-selective conjugation of radiolabels, Ac-TZ14011 has a single amino group (D-Lys^8), which is distant from the pharmacophore, and the carboxyl group of Arg^{14} of Ac-TZ14011 is protected *via* amidation for stability *in vivo* [16, 17].

^{111}In constitutes one of the most useful radionuclides for the radiolabeling of peptides for diagnostic applications in nuclear medicine. Diethylenetriaminepentaacetic acid (DTPA) is still an attractive chelating agent with which to prepare ^{111}In-labeled peptides since it provides ^{111}In-labeled peptides with highly specific activity. In addition, the development of a monoreactive DTPA derivative has provided an easy and efficient way to prepare DTPA-conjugated

peptides [18, 19]. In this study, DTPA-Ac-TZ14011 was prepared using a monoreactive DTPA derivative and coordinated with nonradioactive In or radioactive ^{111}In. Furthermore, the antagonistic activity of In-DTPA-Ac-TZ14011 and *in vivo* behavior of ^{111}In-DTPA-Ac-TZ14011 were investigated and the applicability of ^{111}In-DTPA-Ac-TZ14011 as a radiopharmaceutical for imaging tumors was evaluated.

2. Materials and methods

2.1. Reagents and chemicals

$^{111}InCl_3$ (74 MBq/mL in 0.02 N HCl) was kindly supplied by Nihon Medi-Physics Co. Ltd. (Nishinomiya, Japan). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and 4-(2',4'-dimethoxyphenylaminomethyl)phenoxy (SAL) resin were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) or Calbiochem-Novabiochem Japan, Ltd. (Tokyo, Japan). 1-*tert*-Butyl hydrogen 3,6,9-tris((*tert*-butoxycarbonyl)methyl)-3,6,9-triazaundecanedioic acid (mDTPA) was synthesized as reported previously [18]. All the other chemicals were purchased from either Nacalai Tesque Inc. (Kyoto, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ion spray mass spectra (IS-MS) were obtained with the API III model (PerkinElmer Sciex Instruments, Thornhill, Canada). Cellulose acetate electrophoresis (CAE) strips were run in veronal buffer (pH 8.6, I = 0.06) at a constant current of 0.8 mA for 40 min. TLC analyses were performed with silica plates (Silica gel 60, Merck

KGaA, Darmstadt, Germany) with 10 % aqueous ammonium chloride-methanol (1:1) as the developing solvent.

2.2. Synthesis of In-DTPA-Ac-TZ14011

Figure 2 shows the scheme for the synthesis of In-DTPA-Ac-TZ14011. A protected peptide was constructed using Fmoc-based solid-phase synthesis on SAL resin and its N-terminus was acetylated. After being treated with thioanisole/trifluoroacetic acid (TFA) in the presence of *m*-cresol and 1,2-ethanedithiol, the crude peptide was air-oxidized and purified by reversed-phase HPLC (RP-HPLC). RP-HPLC was carried out with a Cosmosil 5C18-AR column (20 × 250 mm, Nacarai Tesque Inc.) eluted with a linear gradient of 10-30% acetonitrile in 0.1% aqueous TFA in 30 min at a flow rate of 7 mL/min. Fractions containing the peptide were collected, and the solvent was removed by lyophilization to afford Ac-TZ14011 as a white powder. IS-MS calcd for $C_{92}H_{144}N_{35}O_{19}S_2$ [M+H$^+$]: m/z 2107.1, found: m/z 2107.4.

DTPA-Ac-TZ14011 was prepared by mDTPA conjugation. Briefly, to a solution of mDTPA (19 mg, 30.8 μmol) in acetonitrile (350 μL) were added *N*-hydroxysuccinimide (3.74 mg, 32.3 μmol) and *N,N*-dicyclohexylcarbodiimide (6.67 mg, 32.3 μmol) at 0°C, and the mixture was incubated overnight at room temperature. After cooling to 0°C again, 200 μL of Ac-TZ14011 (10.2 mg, 3.65 μmol) in a mixture of acetonitrile and phosphate-buffered saline (pH 7.4) (1:1) was added to the reaction

mixture, and incubated overnight at room temperature. After treatment with 95% TFA, the crude peptide was purified by RP-HPLC under the same conditions as above. IS-MS calcd for $C_{106}H_{165}N_{38}O_{28}S_2$ [M+H$^+$]: m/z 2482.2, found: m/z 2482.9.

Fifty micro-liters of DTPA-Ac-TZ14011 (610 μg, 0.20 μmol) in 0.1 M acetic acid was reacted with 25 μL of nonradioactive $InCl_3 \cdot 4H_2O$ (64.5 μg, 0.22 μmol) in 0.02 N HCl for 30 min at room temperature. Subsequent purification by RP-HPLC was carried out with a Hydrosphere C18 column (4.6 × 250 mm, YMC Co. Ltd., Kyoto, Japan) eluted with 17% acetonitrile in 0.1% aqueous TFA at a flow rate of 1 mL/min. Fractions containing the peptide were collected, and the solvent was removed by lyophilization to afford In-DTPA-Ac-TZ14011 as a white powder. IS-MS calcd for $InC_{106}H_{161}N_{38}O_{28}S_2$ [M+H$^+$]: m/z 2594.2, found: m/z 2594.3.

2.3. Synthesis of ^{111}In-DTPA-Ac-TZ14011

$^{111}InCl_3$ (3.7 MBq) in 0.02 N HCl (100 μL) was added to DTPA-Ac-TZ14011 (10 μg) in 0.1 M acetic acid (200 μL), and the mixture was incubated for 30 min at room temperature. Then, ^{111}In-DTPA-Ac-TZ14011 was separated from DTPA-Ac-TZ14011 by RP-HPLC under the same conditions used for the purification of In-DTPA-Ac-TZ14011. The radiochemical purity of ^{111}In-DTPA-Ac-TZ14011 was determined by TLC, CAE and RP-HPLC.

2.4. Binding assay

The binding assay was performed according to the procedure of Hesselgesser et al. [20] with a slight modification. The stable CXCR4-transfected Chinese hamster ovary (CHO) cell lines were prepared by transfection with cDNA encoding alanine scanning mutants in pcDNA3 (Invitrogen, Carlsbad, CA) using lipofectamine (GIBCO, Rockville, MD) and selection in neomycin (G418 500 mg/ml; GIBCO). The expression of CXCR4 on the surface of each transfectant was measured by flow cytometry. CXCR4-transfected CHO cell lines were suspended in the binding buffer (Ham's F-12 containing 20 mM HEPES and 0.5% BSA) and placed in siliconized tubes (5×10^5 cells/120 μL/tube). Binding reactions were performed on ice for 1 h in the presence of [^{125}I]SDF-1α (PerkinElmer Life Sciences, Boston, MA) and various concentrations of peptides. Cells were separated from the buffer by centrifugation through a dibutylphthalate/olive oil mixture. After removal of the water and oil layer, cell-associated radioactivity was measured. The 50% inhibitory concentration (IC_{50}) of peptides was determined based on inhibition of the binding of SDF-1α to CXCR4-transfected CHO cells.

2.5. Calcium fluorimetry

Calcium fluorimetry was performed as described previously [21]. CXCR4-transfected CHO cell lines were placed in wells of a microtiter tray (3×10^4

cells/100 μL/well) and incubated for 1 day at 37°C in a CO_2 incubator. The cells were loaded with 5 μM of Fura-2-AM (Dojindo Laboratories, Kumamoto, Japan), 2.5 mM probenecid (Sigma, St Louis, MO) and 20 mM HEPES (pH 7.4) in Ham's F-12 (80 μL/well) for 1 h at 37°C. After the cells were incubated with various concentrations of T140 analogs for 3 min, recombinant human SDF-1α (PeproTech EC Ltd., London, UK) was added. Changes in intracellular Ca^{2+} concentrations were measured by spectrofluorometer (96-well Fluorescence Drug Screening System, Hamamatsu Photonix, Hamamatsu, Japan) using a modified version of the Fura-2 method [22]. The IC_{50} of peptides was determined based on the inhibition of Ca^{2+} mobilization induced by SDF-1α through CXCR4.

2.6. Biodistribution study in tumor-bearing mice

Animal experiments were conducted in accordance with our institutional guidelines and were approved by the Kyoto University Animal Care Committee. Athymic nude BALB/c mice (8-week-old, female) were inoculated subcutaneously with CXCR4-expressing pancreatic carcinoma cells, AsPC-1 [23, 24]. When tumors were approximately 0.5 cm in diameter, the animals were intravenously injected with ^{111}In-DTPA-Ac-TZ14011 (25-30 kBq). The biodistribution of radioactivity was monitored at 1, 6 and 24 h postinjection. Groups of five mice were used for the experiments. Organs of interest were excised and weighed, and the radioactivity counts

were determined with a well counter (ARC380CL Aloka Co. Ltd., Tokyo, Japan). For the *in vivo* blocking experiment, mice were co-injected with Ac-TZ14011 (10 mg/kg).

2.7. Statistical analysis

Statistical analysis was performed by applying the unpaired *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

T140 and its analogs have one disulfide bond and maintain an antiparallel β-sheet structure connected by a type II′ β-turn with D-Lys^8-Pro^9 at the (i + 1) and (i + 2) positions, and the side chain of D-Lys^8 is distant from the pharmacophore for the antagonistic activity [14, 15]. Therefore, we designed Ac-TZ14011 as a mother compound that contains the residues indispensable for the antagonistic activity and has a single amino group of D-Lys^8 for site-selective conjugation of DTPA (Figure 1). In calcium fluorimetric assays, this compound showed strong inhibitory activity equal to that of T140 (Table 1). To assess the effect of the conjugation of In-DTPA with Ac-TZ14011 on the antagonistic activity toward CXCR4, nonradioactive In-DTPA-Ac-TZ14011 was synthesized (Figure 2). In binding assays with CXCR4, In-DTPA-Ac-TZ14011 maintained strong inhibitory activity although its IC_{50} value was slightly larger than that of Ac-TZ14011 (Table 1). This result indicated the validity

of the chemical design of In-DTPA-Ac-TZ14011 based on structure-activity relationships.

In RP-HPLC analyses, In-DTPA-Ac-TZ14011 and DTPA-Ac-TZ14011 showed well-separated peaks as shown in Figure 3. After purification by RP-HPLC under the same conditions, ^{111}In-DTPA-Ac-TZ14011 was obtained with high radiochemical purity (over 96%) as determined by TLC, CAE and RP-HPLC. The radioactivity pharmacokinetics of ^{111}In-DTPA-Ac-TZ14011 was evaluated in nude mice bearing the CXCR4-expressing pancreatic carcinoma AsPC-1 (Table 2). ^{111}In-DTPA-Ac-TZ14011 showed a rapid clearance from the blood and a marked accumulation and retention in the liver, kidney and spleen. The accumulation of radioactivity was greater in the tumor than in the blood or muscle (Table 2). In mice, CXCR4 mRNA is highly expressed in various lymphoid tissues and cells such as spleen, thymus, lymph node, bone marrow and leukocytes [25, 26]. Thus, since liver and spleen are concerned with the immune system, the accumulation of ^{111}In-DTPA-Ac-TZ14011 in these organs should be mediated by CXCR4-binding. In fact, co-injection of Ac-TZ14011 significantly reduced the accumulation in the liver by over one-tenth and in the spleen by over one-third. This marked reduction of radioactivity in the liver and spleen on the co-injection of Ac-TZ14011 caused the high levels of radioactivity in the blood and consequently increased the accumulation of radioactivity in organs which were small in size and/or did not take up much radioactivity (Table 2). The accumulation in the

tumor was also increased by co-injection of Ac-TZ14011, but the tumor-to-blood and tumor-to-muscle ratios were significantly reduced (Table 2). Since there is very little or no CXCR4 in the muscle [26], tumor-to-muscle ratios reflect target-to-nontarget ratios. Thus, the reduction in the tumor-to-muscle ratio caused by the co-injection of Ac-TZ14011 indicated that ^{111}In-DTPA-Ac-TZ14011 accumulated in the tumor through CXCR4. On the other hand, co-injection of Ac-TZ14011 did not alter the levels of ^{111}In-DTPA-Ac-TZ14011 in the kidney, suggesting a nonspecific accumulation. This is consistent with previous findings that CXCR4 mRNA levels expressed in the kidney were very low [25, 26]. Recent studies indicated that an electrostatic interaction between positively charged peptides and the negatively charged surface of renal proximal tubular cells plays an important role in the re-absorption of peptides into proximal tubular cells [27–29]. Since five Arg residues are contained in the peptide ^{111}In-DTPA-Ac-TZ14011, the highly positive charge would cause a greater nonspecific accumulation in the kidney even compared to other ^{111}In-DTPA-peptides [28, 30, 31]. Due to its accumulation in nontarget organs, ^{111}In-DTPA-Ac-TZ14011 may be unavailable as a radiopharmaceutical for screening small tumors, particularly in the kidneys and their surroundings.

It was reported that CXCR4 expression could be a powerful predictive factor for prognosis (recurrence, metastasis or survival rate) in colorectal cancer [32, 33], malignant melanoma [34] and osteosarcoma [35]. Therefore, a CXCR4 imaging agent

would be a new type of radiopharmaceutical for predicting the prognosis of cancer patients. CXCR4 also represents a novel target for tumor therapy, and some CXCR4 inhibitors have been investigated as anti-metastatic agents [36–39]. These agents showed positive effects in suppressing tumor metastasis, however, they would also have deleterious effects on normal physiological functions since CXCR4 plays a crucial role in numerous biological processes [2]. Therefore, *in vivo* imaging of CXCR4 expression could be a potential method for determining the dose of anti-metastatic agents and monitoring their therapeutic efficacy.

In conclusion, we designed ^{111}In-DTPA-Ac-TZ14011 based on the structure-activity relationships of peptidic CXCR4 inhibitors. In-DTPA-Ac-TZ14011 showed strong inhibitory activity against the binding of CXCR4 to an endogenous ligand. Furthermore, the accumulation of ^{111}In-DTPA-Ac-TZ14011 in the CXCR4-expressing tumor was greater than that in the blood or muscle, being mediated by this receptor. These findings suggest that ^{111}In-DTPA-Ac-TZ14011 is a potential radiopharmaceutical for the imaging of CXCR4 expression in metastatic tumors *in vivo* for predicting the prognosis of cancer patients and monitoring the therapeutic efficacy of anti-metastatic agents.

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Table 1. Antagonistic activity of T140 derivatives

	IC_{50} (nM)	
	SDF-1α binding [a]	Ca^{2+} mobilization [b]
In-DTPA-Ac-TZ14011	7.9	ND [c]
Ac-TZ14011	1.2	2.6
T140	ND [c]	2.2

[a] Values are the concentrations for 50% inhibition of the binding of [^{125}I]SDF-1α to CXCR4.

[b] Values are the concentrations for 50% inhibition of Ca^{2+} mobilization induced by SDF-1α through CXCR4.

[c] Not determined.