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Synthesis and in vitro evaluation of ^{68}Ga-DOTA-4-FBn-TN14003, a novel tracer for the imaging of CXCR4 expression

Ute Hennrich [a,*], Lisa Seyler [a], Martin Schäfer [b], Ulrike Bauder-Wüst [b], Michael Eisenhut [b], Wolfhard Semmler [a], Tobias Bäuerle [a]

[a] Department of Medical Physics in Radiology, DKFZ, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[b] Department of Radiopharmaceutical Chemistry, DKFZ, Heidelberg, Germany

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ABSTRACT

The expression of the chemokine receptor CXCR4 in tumors is associated with tumor aggressiveness and poor prognosis for the patient and contributes to metastatic seeding. Therefore it is of high interest to find a specific PET tracer for the imaging of CXCR4 expression in tumors. The aim of this study was the synthesis, ^{68}Ga labeling and first evaluation of DOTA-4-FBn-TN14003 as a potential PET tracer for this purpose. DOTA-4-FBn-TN14003 was synthesized using solid phase peptide synthesis and radiolabeling of this versatile precursor was performed with ^{68}Ga, which was obtained from a ^{68}Ge/^{68}Ga generator. ^{68}Ga-DOTA-4-FBn-TN14003 was reproducibly obtained in isolated radiochemical yields of 72.5 ± 4.9% with an excellent radiochemical purity of >99.5%. Specific activities of up to 29.8 ± 3.1 GBq/μmol were achieved. In competition binding assays with SDF-1α, human T cell lymphoma Jurkat cells expressed high levels of CXCR4 whereas human breast cancer MDA-MB-231 cells expressed significantly lower levels of this chemokine receptor. The inhibition constants (IC_{50}) of Ga-DOTA-4-FBn-TN14003 and 4-FBn-TN14003 to CXCR4 were determined in a competition assay against ^{125}I-SDF-1α using Jurkat as well as MDA-MB-231 cells. The IC_{50} values of Ga-DOTA-4-FBn-TN14003 (1.99 ± 0.31 nM) and 4-FBn-TN14003 (4.07 ± 1.00 nM) proved to be comparable, indicating negligible influence of the metal complex. These results suggest ^{68}Ga-DOTA-4-FBn-TN14003 as a promising agent for the imaging of CXCR4 expression in tumors and metastases.

1. Introduction

Chemokines play a critical role in tumor progression and metastasis. In this regard, the chemokine receptor CXCR4 is expressed by tumor cells including breast, prostate and lung cancer that are metastasizing to the respective target organs.[1–3] In breast cancer, overexpression of CXCR4 predicts poor clinical outcome and is closely associated with lymph node metastasis.[4,5] During the development of metastasis, CXCR4 interacts with its ligand CXCL12 (stromal cell-derived factor-1, SDF-1α) which is expressed in liver, lung, lymph nodes, bone and other tissues.[6] Binding of SDF-1α to CXCR4 results in a variety of responses such as chemotaxis as well as cell survival, migration and proliferation. The expression of SDF-1α by these organs is supposed to be crucial for homing and colonization of disseminated tumor cells that express CXCR4. Inhibition of the CXCR4-SDF-1α axis was reported to reduce orthotopic and metastatic tumor growth in experimental models of breast cancer.[7,8] Consequently, CXCR4 is a potential imaging target for the diagnosis, staging and therapeutic monitoring of metastases.

Imaging of tumors and in particular metastases is currently limited to assessing morphology and metabolic activity by magnetic resonance imaging (MRI), computed tomography (CT), scintigraphy, single photon emission computed tomography (SPECT) or positron emission tomography (PET). Use of these techniques, however, results in a relatively late detection of disseminated lesions, that is, after tumors or macrometastases in the range of several millimeters have developed. For this reason, specific imaging of molecular targets expressed by disseminated tumor cells and micrometastases is attractive for early diagnosis. In this context, we recently presented the integrins αvβ3 and αvβ5 as molecular targets for the imaging of bone metastases.[9]

Several approaches have been performed to image CXCR4 expression in tumor models using ^{111}In-labeled peptides and ^{125}I-labeled antibodies by SPECT.[10–12] Recently imaging of CXCR4 by PET was described, for example, Nimmagadda et al. reported the feasibility of imaging CXCR4 by two different ^{64}Cu-labeled PET tracers in orthotopic and metastatic breast cancer in mice[13] as well as a subcutaneous brain tumor model.[14] Here, we describe

* Corresponding author. Tel.: +49 6221 422686; fax: +49 6221 422585.
E-mail address: u.hennrich@dkfz.de (U. Hennrich).

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the synthesis, radiolabeling and evaluation of ^{68}Ga-DOTA-4-FBn-TN14003 as a novel tracer for the imaging of CXCR4 expression in MDA-MB-231 human breast cancer and Jurkat human T lymphocyte cell lines as well as the expression levels of the receptor on both cell lines.

2. Materials and methods

2.1. Materials

All commercially available chemicals were of analytical grade or better and used without further purification unless otherwise noted. The main suppliers were Sigma–Aldrich (Taufkirchen, Germany) and Merck (Darmstadt, Germany). Tris-*t*Bu-DOTA was synthesized modifying a three step procedure previously described by Wängler et al.[15] The last reaction step, the debenzylation, was not performed using hydrogen gas as described but by generating hydrogen in situ from ammonium formate in the presence of palladium black in absolute methanol at 60 °C.[16] ^{68}Ga was obtained from a self-assembled ^{68}Ge/^{68}Ga generator based on a pyrogallol resin support.[17] Typically, about 500 MBq of ^{68}Ga ($t_{1/2}$ = 68 min) were eluted with 5.5 M HCl and trapped on a small anion-exchanger column (AG1X8, Biorad, Richmond, CA, USA). The activity was eluted from this cartridge using ultrapure water (Merck, Darmstadt, Germany) in a final volume of 300 μL. For the cell assays, the radioactive samples were either counted in the γ-counter Cobra II (Packard Canberra, Minnesota, USA) or the Wizard 2 (2480 automatic gamma counter; Perkin–Elmer, Rodgau, Germany).

Analysis of the radiolabeled compound was performed using analytical reversed-phase high performance liquid chromatography (RP-HPLC). The HPLC system (system I) contained a L6200 pump from Merck-Hitachi (Darmstadt, Germany), a variable wavelength UV detector (254 nm) from Latek (Heidelberg, Germany) and a NaI(Tl) gamma detector from Bioscan (Washington, USA). Analytical HPLC was performed using a Chromolith Performance RP-18e column (100 × 4.6 mm; Merck, Darmstadt, Germany). The solvent gradient was raised from 0% to 100% methanol in 5 min at a flow rate of 4.0 mL/min. As aqueous phase water containing 0.1% TFA was used. Purification of the non-radioactive peptides was performed by semi-preparative reversed-phase HPLC using the following system (system II) from Dionex (Idstein, Germany): an Ultimate 3000 LPG-3400A pump, a variable four wavelength Ultimate 3000 VWD-3400RS UV/VIS detector (222, 254, 280 nm), analysis of HPLC data was performed with Chromeleon 6.80 software. As column a Chromolith Performance RP-18e column (100 × 10 mm; Merck, Darmstadt, Germany) was used. The solvent gradient was raised from 5% to 100% acetonitrile (a) or methanol (b) in 5 min at a flow rate of 6 mL/min. The aqueous phase consisted of water containing 0.1% TFA. MALDI-TOF-MS was performed on a microflex LT (Bruker Daltonics, Bremen, Germany).

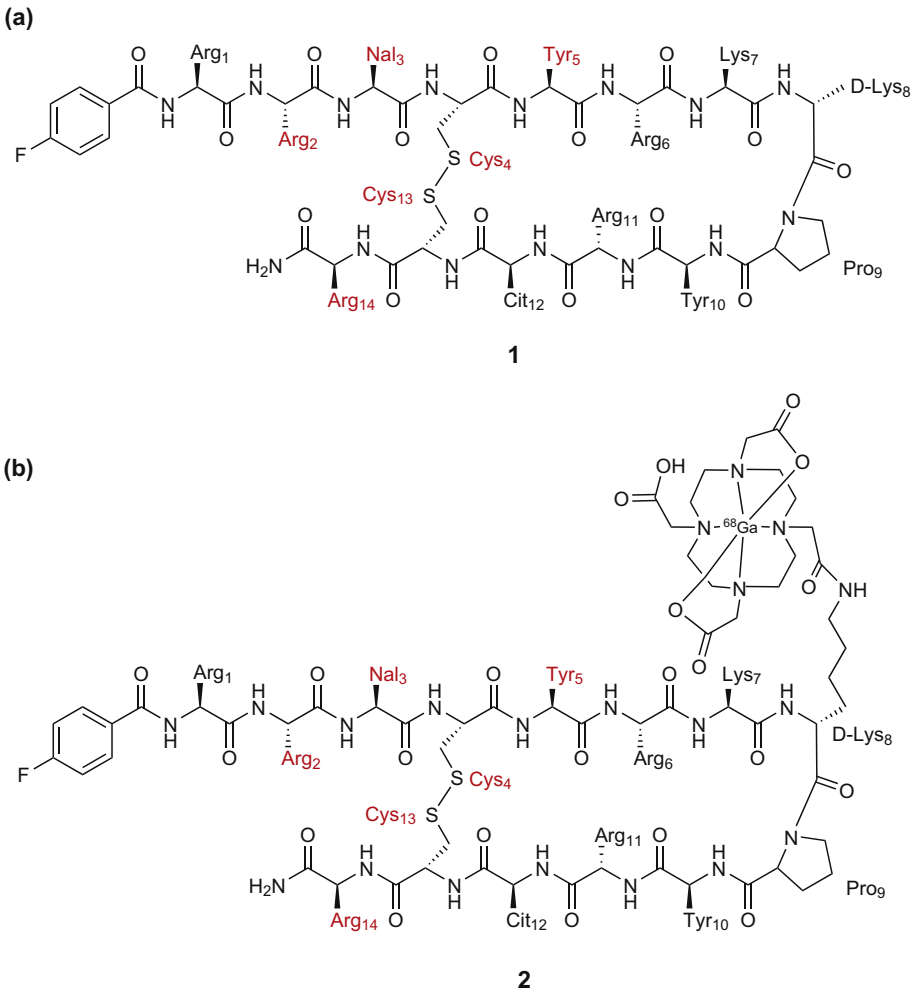

Figure 1. Structures of 4-F-Bn-TN14003 (**1**) and ^{68}Ga-DOTA-4-FBn-TN14003 (**2**).

2.2. Synthesis of DOTA-4-FBn-TN14003 and 4-FBn-TN14003

The synthesis scheme of DOTA-4-FBn-TN14003 is shown in Figure 2. The protected peptidyl resin was manually constructed using Fmoc-based solid phase synthesis on Rink Amide resin (0.71 mmol/g, 0.1 mmol scale). Fmoc-protected amino acid derivatives (4 equiv) were successively condensed, using HBTU (3.92 equiv) in the presence of DIPEA (4 equiv) for activation, in DMF, allowing a reaction time of 60–90 min for each amino acid. For side-chain protection the following groups were used: Pbf for Arg, Acm for Cys, *t*Bu for Tyr, Boc for Lys, and Mtt for D-Lys. Fmoc-deprotection was performed by treatment of the resin with 50% (v/v) piperidine-DMF for 2 and 5 min. For acylation of the N-terminal amino group the resin was reacted for 90 min with 4-fluorobenzoic acid (4 equiv) under HBTU activation as described above. The formation of the disulfide bridge between Cys_4 and Cys_{13} was achieved by reacting the resin with thallium(III) trifluoroacetate (4 equiv, technical grade) in the presence of anisole (5%) in DMF for 5 h. For the conjugation of tris-*t*Bu-DOTA, the ε-amino function of D-Lys_8 was selectively deprotected by treatment of the resin with 1.5% TFA and 3% TIS (triisopropylsilane) in DCM for 3 × 30 min, followed by washing of the resin with 10% DIPEA in DMF (5 × 5 min). Then the reaction with tris-*t*Bu-DOTA (4 equiv), activated as described above, was performed for 3 h. Cleavage from the resin as well as side-chain deprotection of the amino acid derivatives was achieved by treatment with 95% TFA, 2.5% TIS and 2.5% water for 4 h. The crude peptide was precipitated by dropwise addition of the reaction mixture to ice cold diethyl ether and collected by filtration followed by washing with ice cold diethyl ether. Purification of DOTA-4-FBn-TN14003 was performed by semi-preparative HPLC (system IIa) and it was obtained as a fluffy white powder after freeze drying the collected product fractions (t_R = 2.43 min). The identity of the peptide was confirmed by MALDI-TOF-MS (*m*/*z* calcd for $C_{113}H_{171}FN_{37}O_{26}S_2$ ($[M+H]^+$): 2546.9 found 2546.8) and NMR spectroscopy (see Supplementary data). The parent peptide 4-FBn-TN14003 was synthesized accordingly, omitting the step of DOTA-conjugation. It was also purified by semi-preparative HPLC (t_R = 2.41 min, system IIa). Because it was not pure after the first HPLC purification, the collected fractions were again purified by semi-preparative HPLC, now using system IIb (t_R = 3.31 min). The identity of 4-FBn-TN14003 was confirmed by MALDI-TOF-MS (*m*/*z* calcd for $C_{97}H_{145}FN_{33}O_{19}S_2$ ($[M+H]^+$): 2160.5 found 2160.5).

2.3. ^{68}Ga-Labeling of DOTA-4-FBn-TN14003

To the $[^{68}Ga]Ga^{3+}$ eluate (200–500 MBq) in a polypropylene screwcap vial HEPES solution (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5.5 M in ultrapure water, 110 μL) was added as well as sodium hydroxide solution (30%, 15 μL) and DOTA-4-FBn-TN14003 (5 or 10 nmol in 5 or 10 μL of ultrapure water, respectively). The vial was heated for 10 min at 80 °C. Depending on the application, the compound was either diluted to the desired concentration (cell assays) or further purified by solid phase extraction (stability studies). For purification, the reaction mixture was diluted with water (1 mL) and passed over an Oasis HLB 1 cc cartridge (Waters, Eschborn, Germany). Elution of the product was achieved with absolute ethanol (0.8 mL). In order to perform stability studies with the radiotracer, the solvent was evaporated at 80 °C under a gentle stream of air and the residue was taken up in phosphate buffered saline (PBS). Depending on the synthesis conditions for ^{68}Ga-DOTA-4-FBn-TN14003, specific activities of up to 29.3 ± 3.2 GBq/μmol were achieved at the end of synthesis. The radiochemical purity was analyzed by RP-HPLC (t_R = 3.29 min; system I) and the identity of the labeled peptide verified by co-elution with the non-radioactive reference compound.

2.4. Synthesis of Ga-DOTA-4-FBn-TN14003

The non-radioactive standard compound Ga-DOTA-4-FBn-TN14003 was obtained by reacting the peptide precursor (5 nmol in 5 μL ultrapure water and 35 μL 0.1 M HEPES buffer) with 4 equivalents of a 3 mM solution of $Ga(NO_3)_3$ hydrate (in 0.1 M hydrochloric acid) in the presence of 2.1 M HEPES buffer (10 μL) and 1 M hydrochloric acid (2 μL). The pH value of the solution was between 4.1 and 4.4 and the mixture was heated at 80 °C for 10 min. After cooling, the pH value was adjusted to 7.4 by the

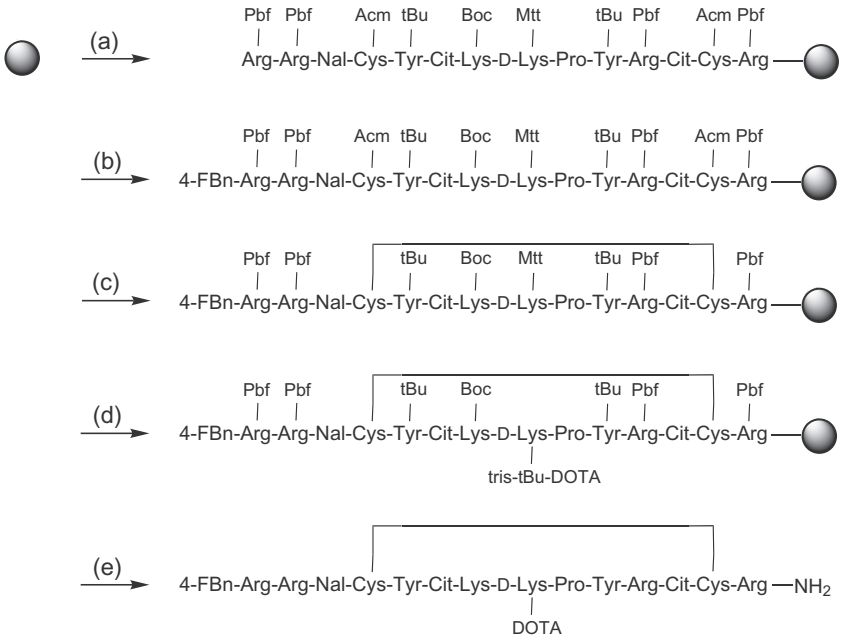

Figure 2. Synthesis of DOTA-4-FBn-TN14003. Reagents: (a) stepwise elongation on Rink Amide resin; (b) 4-fluorobenzoic acid, HBTU, DIPEA; (c) Thallium(III) triflouroacetate, anisole; (d) (1) 1.5% TFA and 3% TIS in dichloromethane, (2) tris-*t*Bu-DOTA, HBTU, DIPEA; (e) TFA, TIS, water.

addition of 1 M sodium hydroxide solution and the compound was used without further purification. The identity of the reference compound was confirmed by MALDI-TOF-MS (m/z calcd for $C_{113}H_{169}FGaN_{37}O_{26}S_2$ ($[M+H]^+$): 2613.6 found 2613.3).

2.5. Stability of ^{68}Ga-DOTA-4-FBn-TN14003

The stability of ^{68}Ga-DOTA-4-FBn-TN14003 was evaluated in PBS as well as human plasma. For the stability in PBS, the radiotracer was incubated at 37 °C and its stability repeatedly tested by analytical HPLC (system I). Human plasma (Sigma–Aldrich) was incubated with the radiotracer at 37 °C and the stability of the compound was tested after 30 and 90 min ($n = 2$, respectively). Plasma samples (100 μL) were vortexed with an equal amount of acetonitrile and centrifuged at 4 °C for 2 min at 13,000 rpm and an aliquot of the supernatant solution was analyzed by HPLC (system I). An aliquot of the plasma itself was analyzed by size exclusion chromatography (SEC; Superdex 75, 0.4 mL/min, 0.1 M phosphate buffer (pH 7.4) containing 500 mM arginine).

2.6. Cell lines and cell culture

The human T lymphocyte cell line Jurkat was purchased from European Collection of Cell Cultures (ECACC). Jurkat cells were cultured routinely in RPMI-1640 (Gibco, Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS (Sigma, Taufkirchen, Germany). All cultures were kept under controlled conditions (humidified atmosphere, 5% CO_2, 37 °C) and passaged 2–3 times a week to keep them in logarithmic growth. Sub-confluent Jurkat cells were harvested, counted on a Neubauer's chamber and resuspended in binding buffer[18] (Dulbecco's PBS containing 20 mM HEPES and 0.5% BSA, pH 7.0) to a concentration of 1.1×10^7 in 400 μL. The human breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC). MDA-MB-231 cells were cultured routinely in RPMI-1640, supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco, Invitrogen, Karlsruhe, Germany). All cultures were kept under controlled conditions (humidified atmosphere, 5% CO_2, 37 °C) and passaged 2–3 times a week to keep them in logarithmic growth. Sub-confluent MDA-MB-231 cells were harvested using 0.05% Trypsin–EDTA (Gibco, Invitrogen, Karlsruhe, Germany) and prepared as described above.

2.7. Cell binding assays using ^{125}I-SDF-1α

In order to determine the binding affinity of Ga-DOTA-4-FBn-TN14003 and 4-FBn-TN14003 (for comparison) to CXCR4 on living cells, a competitive cell binding assay was performed using Jurkat as well as MDA-MB-231 cells. The assay was modified from a published procedure.[18] For the binding assay with Jurkat cells, the cells were harvested and resuspended in binding buffer (Dulbecco's PBS containing 20 mM HEPES and 0.5% BSA, pH 7.0). A suspension of 10^5 cells, 0.055 nM human ^{125}I-SDF-1α (specific activity: 2200 Ci/mmol, Perkin–Elmer Life Sciences, Boston, MA, USA) and 12 different concentrations of Ga-DOTA-4-FBn-TN14003 (0–5000 nM) were incubated for 1 h at 37 °C. It was essential that for the binding reaction as well as the previous dilution step Protein LoBind vials (Eppendorf) were used because otherwise ^{125}I-SDF-1α could not be recovered sufficiently. The incubation was terminated by removing aliquots of the mixture and separating cells from buffer by centrifugation (12,000 rpm) through a silicone/mineral oil mixture. The cell-bound radioactivity as well as the non-bound radioactivity was measured using a γ-counter. Experiments were performed at least 4 times including quadruplicate sample measurements. Binding results were expressed as % cell bound activity to total activity and the IC_{50} values were calculated using Sigma Plot 11.0 and a sigmoidal dose-response fit. For comparison, the experiments were also performed with 4-FBn-TN14003 instead of Ga-DOTA-4-FBn-TN14003 as described above. For the respective binding assays using MDA-MB-231 cells, the cells were trypsinized, resuspended in the binding buffer and the assay was conducted as described above.

For the determination of the number of receptors per cell on the different cell lines (Jurkat and MDA-MB-231) the assay was performed using human SDF-1α (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as inhibitor under the same assay conditions as described above ($n = 3$–5). The B_{max} calculation was conducted using a competitive binding fit from GraphPad Prism 5.0 Software (San Diego, California, USA).

2.8. Cell binding assays using ^{68}Ga-DOTA-4-FBn-TN14003

For the determination of uptake kinetics as well as internalization of ^{68}Ga-DOTA-4-FBn-TN14003 both Jurkat and MDA-MB-231 cells were used. The radiotracer was added in a concentration of 250 nM and the cells were prepared as described above. For tracer uptake kinetics the cells were incubated at 37 °C for 5, 10, 15, 20, 30, 60, 90, and 120 min, respectively, and the probe workup was conducted as described for the competition binding assays. Experiments were performed 3 times including quadruplicate sample measurement and binding results were expressed as % cell bound activity to total activity.

For the internalization assays cell lines were incubated for 1 h at 37 °C. After incubation, cells were centrifuged for 2 min at 2000 rpm and the supernatant was removed. Subsequently, cells were washed 3 times with cold PBS and then, for removal of surface-bound radioactivity, 2 times for 5 min with 50 mM Glycine buffer (pH 2.8) at 4 °C. After washing with cold PBS once, the cell pellet was resuspended in 0.5 M NaOH. The radioactivity of the samples was measured in a γ-counter. Experiments were performed 3 times including triplicate sample measurements. The values are expressed as the percentage of internalized radioactivity (cell pellet) to total activity (glycine washes, last PBS wash and cell bound activity).

2.9. Immunocytochemical staining

The cytochemical staining for CXCR4 was performed with Jurkat and MDA-MB-231 cells in a concentration of 1×10^6 cells per milliliter, respectively. As primary antibody human CXCR4 (monoclonal mouse IgG_{2A}, clone 12G5; dilution 1:100) from R&D Systems (Minneapolis, USA) and as secondary antibody Texas-Red-conjugated goat anti-mouse (dilution 1:100; Dianova, Hamburg, Germany) were used. Counterstaining of cell nuclei was conducted with DAPI (4′,6-diamidino-2-phenylindole, dilution 1:500, Invitrogen, Karlsruhe, Germany). For staining the cells were either fixated on glass plates (for images) or unfixated in PBS (for analysis). With the primary antibody cells were incubated for 60 min and with the secondary antibody together with DAPI for 30–45 min at room temperature. The stainings were evaluated using a fluorescence microscope BX50 with an adapted digital camera (F-View) from Olympus Soft Imaging Solutions (Muenster, Germany). Positive area fractions (10 fields of view per staining) were analyzed with cell^F software (Olympus Soft Imaging Solutions, Muenster, Germany) using unfixated cells (magnification 20-fold, exposure time 1 s).

3. Results and discussion

The peptide 4-FBn-TN14003 (**1**, Fig. 1a) was first described by Tamamura et al. as an antagonist for CXCR4 and was developed

by improving the structure of T140 analogs.[18] It was shown that this peptide has nanomolar affinity for the chemokine receptor, that it is stable in vivo and that it reduces breast cancer lung metastasis in SCID mice. Like the parent T140 this peptide consists of 14 amino acid residues and differs from T140 by the C-terminal amide as well as the N-terminal 4-fluorobenzoyl group. These changes resulted in an improved bio-stability and affinity for CXCR4.[18] In order to make this peptide available as a PET or SPECT tracer, different radiolabeling approaches are feasible. One possibility is the direct electrophilic radioiodination of tyrosine residues in 4-FBn-TN14003 which has already been done successfully (unpublished results). However, this approach has several disadvantages and has therefore not longer been pursued. As two different tyrosine residues are present in the amino acid sequence of the peptide (Tyr_5 and Tyr_{10}), both residues could be radioiodinated. Therefore two different labeled compounds would be formed which were expected not to be easily separated chromatographically and to also exhibit different pharmacokinetics. Another cause of concern is that Tyr_5 is part of the intrinsic pharmacophore (red amino acid residues in Fig. 1) necessary for binding to CXCR4[19–21] and a radioiodination might potentially lead to a decreased binding efficiency. Another labeling approach represents the ^{18}F-fluorination of TN14003 using ^{18}F-SFB as a secondary labeling precursor which has been reported recently.[22] ^{18}F-4-FBn-TN14003 was evaluated in mice bearing CHO-CXCR4 tumors in biodistribution as well as PET studies and showed promising results. However, this labeling approach has also disadvantages which will be discussed below. A completely different radiolabeling approach is the site-specific conjugation of the peptide 4-FBn-TN14003 at the ε-amino function of a lysine residue with a suitable chelator for the complexation of radiometals. Appropriate chelators are for example DTPA (diethylenetriaminepentaacetic acid; for labeling with the SPECT isotope ^{111}In) or DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; for labeling with either ^{111}In, the PET isotopes ^{64}Cu and ^{68}Ga or the therapy isotopes ^{90}Y and ^{177}Lu). A similar ligand for CXCR4, namely Ac-TZ14011, has already been conjugated with DTPA and labeled with ^{111}In showing promising results in first evaluation studies.[10] In this peptide the Lys_7 residue of T140 was changed to an Arg residue in order to achieve site-selective conjugation of the chelator moiety to D-Lys_8. The DTPA conjugation did not interfere with the binding efficiency of the peptide to CXCR4 because the D-Lys_8 residue is not part of the pharmacophoric structure. Another recently published approach was the conjugation of two DOTA moieties to both Lys_7 and D-Lys_8 and labeling of the peptide with PET radiometals.[23] Since we are also interested in a radiometallated PET and not a SPECT tracer for CXCR4, ^{68}Ga was the isotope of our choice. ^{68}Ga combines well-suited decay properties as a PET isotope with its easy availability via a generator.[24] A suitable chelator for ^{68}Ga is DOTA which was site-selectively conjugated to the D-Lys_8 residue of 4-FBn-TN14003 (**2**, Fig. 1b). Without the need to exchange Lys_7 and still obtaining one specific product and one specific side for labeling, we received this peptide for radiolabeling with ^{68}Ga and other potentially suited radiometals such as ^{90}Y or ^{177}Lu.

3.1. Synthesis of DOTA-4-FBn-TN14003 and 4-FBn-TN14003

DOTA-4-FBn-TN14003 as well as 4-FBn-TN14003 were received by Fmoc-based solid phase synthesis on Rink Amide resin using amino acid derivatives with suitable protection groups, for example, the highly acid-labile 4-methyltrityl (Mtt) group for D-Lys and the acetamidomethyl (Acm) group for Cys. The synthesis route is shown in Figure 2. All reaction steps, including DOTA conjugation and formation of the disulfide bond, could be conducted on the solid phase support. After stepwise condensation of the 14 amino acid residues, the N-terminus was acylated with 4-fluorobenzoic acid followed by the formation of the disulfide bridge between Cys_4 and Cys_{13}. For this purpose the Acm-groups were selectively deprotected on the resin using thallium(III) trifluoroacetate and the disulfide bridge was formed.[25] The formation of the disulfide bridge was performed before DOTA conjugation to D-Lys_8 because otherwise disulfide ring formation might be impeded due to steric hindrance. Selective DOTA conjugation was achieved at D-Lys_8 using Mtt protection which was easily cleaved with 1.5% TFA while Lys_7 was protected by a Boc group which is stable under these mild conditions. The tris-*t*Bu-DOTA derivative needed for conjugation to the peptide was synthesized via a three step reaction procedure starting from commercially available cyclene [similar to[15]]. Tris-*t*Bu-DOTA was activated by HBTU and coupled to D-Lys_8. The final cleavage and deprotection yielded DOTA-4-FBn-TN14003. The reference peptide 4-FBn-TN14003 was synthesized accordingly omitting the conjugation of tris-*t*Bu-DOTA. This synthesis strategy allowed the execution of all reaction steps on the solid phase support including the site-specific conjugation of DOTA to D-Lys_8 of 4-FBn-TN14003 and the disulfide bridge formation. With DOTA-4-FBn-TN14003 a versatile labeling precursor for (radio)metal complexation was obtained. Depending on the radiometal used for complexation, the compound enables the synthesis of either a PET or SPECT tracer for the imaging of CXCR4 in tumors and metastases or a radiopharmaceutical for use as a therapeutic agent.

3.2. ^{68}Ga-Labeling of DOTA-4-FBn-TN14003

In order to synthesize a PET tracer for the imaging of CXCR4 expression in tumors and metastases, the CXCR4 inhibitor DOTA-4-FBn-TN14003 was labeled with ^{68}Ga. The radioactive labeling was optimized concerning various reaction parameters such as the amount of labeling precursor, pH value, reaction temperature, and reaction time using analytical reversed-phase HPLC. The radiolabeled product eluted in a single peak with a retention time of 3.31 min and its identity was confirmed by co-elution with the non-radioactive reference compound Ga-DOTA-4FBn-TN14003. Concerning the amount of labeling precursor, no significant difference in RCY could be observed when using 5, 10 or 15 nmol of the precursor peptide. Depending on the application of the radiotracer, the labeling reactions were therefore performed with either 5 or 10 nmol precursor. Varying the pH value of the reaction solution between 4 and 5 did not result in significant differences in RCY as well and the same was observed for the reaction time (8 and 18 min, respectively) and the reaction temperature (80 and 90 °C, respectively). For subsequent experiments a reaction temperature of 80 °C and a reaction time of 10 min were chosen because a further increase in reaction time would only lead to a loss of radioactivity due to the radioactive decay of ^{68}Ga. Routinely RCYs of 95–100% (n >15) were achieved determined by analytical HPLC.

For the evaluation of ^{68}Ga-DOTA-4-FBn-TN14003 in cell assays, no further purification of the compound was necessary. When necessary, however, purification of the radiotracer was accomplished by solid phase extraction (SPE) using a C18 cartridge. The radiochemical purity of purified ^{68}Ga-DOTA-4-FBn-TN14003 was determined by HPLC to be >99.5% and the isolated RCY was 72.5 ± 4.9%. At the end of synthesis specific activities of ^{68}Ga-DOTA-4-FBn-TN14003 of up to 29.8 ± 2.3 GBq/μmol were achieved depending on the amount of labeling precursor as well as the starting radioactivity and the RCY of the labeling reaction. The overall time for synthesis and isolation of ^{68}Ga-DOTA-4-FBn-TN14003 was 35 min, which is compatible with the half-life of ^{68}Ga (68 min). Due to the simplicity of the labeling and purification procedures, the labeling protocol may also be adoptable for automation.

As mentioned above, the CXCR4 inhibitor 4-FBn-TN14003 has already been described as a ^{18}F-labeled tracer. However, the

following advantages of labeling a peptide with ^{68}Ga as compared to ^{18}F exist: first, the availability of ^{68}Ga via a ^{68}Ge/^{68}Ga-generator whereas for the production of ^{18}F a cyclotron is needed. Secondly, for ^{18}F-fluorinations a larger amount of labeling precursor is needed which requires a HPLC separation step, removal of HPLC solvent and formulation of the final product. Thirdly, the ^{18}F-fluorination of a peptide usually necessitates a secondary labeling precursor such as ^{18}F-SFB.[26,27] This secondary labeling precursor was also used for the preparation of ^{18}F-4-FBn-TN14003 and resulted in a 5 step reaction procedure, 3 steps for the synthesis of ^{18}F-SFB and another 2 steps for the conjugation to the peptide precursor.[22] The elaborate labeling procedure resulted in an overall reaction time of more than 165 min. As long as the derivatization of peptides with a chelator and the ^{68}Ga complexation does not lead to a considerable decrease in affinity the radiometal complexation is preferable.

3.3. Stability of ^{68}Ga-DOTA-4-FBn-TN14003

The stability of ^{68}Ga-DOTA-4-FBn-TN14003 in PBS and human plasma was evaluated at 37 °C. The radiochemical stability of the compound in PBS was examined by repeated HPLC analysis, proving stability over a period of at least 4 h. In human plasma, the stability was evaluated using HPLC as well as size exclusion chromatography (SEC) after 30 and 90 min incubation time. After 30 min the radiochemical purity of the radiotracer was >99% and after 90 min >97% indicating 3% free ^{68}Ga. The recovery of the radioactive sample from the HPLC was 100%. Using SEC the binding of the radiotracer to plasma can be evaluated. After 90 min >90% of the injected sample corresponded to non-bound radiotracer and the recovery from SEC was also 100%. Taken together, these results suggest a high stability of ^{68}Ga-DOTA-4-FBn-TN14003 which is an important property of a radiotracer.

3.4. In vitro evaluation

3.4.1. Determination of the binding affinity to CXCR4

An essential property for the suitability of a compound as a PET or SPECT tracer is its affinity to the receptor of choice. While it is known that 4-FBn-TN14003 has a high affinity for CXCR4, conjugation of a chelator like DOTA including complexation with gallium might change the receptor affinity significantly. For the determination of the affinity of Ga-DOTA-4-FBn-TN14003 to CXCR4 the non-radioactive complex was synthesized and a competitive cell binding assay was performed using ^{125}I-SDF-1α, the natural ligand for CXCR4, as the specific radioligand. To evaluate the influence of the Ga-DOTA complex, the affinity of the parent peptide 4-FBn-TN14003 was determined accordingly. For this purpose Jurkat cells (derived from a human T cell lymphoma), which are known to highly express CXCR4 receptors,[28] were used as well as MDA-MB-231 human breast cancer cells which also express the receptor.[18] For the evaluations vital cells were used which provide realistic conditions of receptor expression on the cell surface. Initially some difficulties were encountered concerning the recovery of ^{125}I-SDF-1α during the assay. Because of the strong adherence of ^{125}I-SDF-1α to safe lock plastic vials (Eppendorf) when using cell medium supplemented with 10% FCS as assay buffer, different conditions were evaluated. The use of screw cap vials as well as the use of cell medium supplemented either with 0.25% BSA or without serum did not improve the results. It was essential to perform the assay in protein LoBind vials (Eppendorf) which are special vials with reduced binding of proteins to the surface. With these vials recoveries of 97.9 ± 1.9% of the radioligand ^{125}I-SDF-1α were achieved.

First, the cell binding assays were performed using Jurkat cells and evaluating the affinity of both Ga-DOTA-4-FBn-TN14003 as well as 4-FBn-TN14003. The competition curves for both compounds are depicted in Figure 3, showing that the plateau phases for low as well as high concentrations of the peptides are reached, respectively. Table 1 summarizes the IC_{50} values (inhibitory concentrations of 50%) for both compounds and both cell lines. For 4-FBn-TN14003 the IC_{50} value was determined to be 4.07 ± 1.00 nM (n = 5) indicating a high affinity of the peptide to CXCR4. This result was in the same range as previously published values for this peptide (2.5 nM using CHO-CXCR4 cells[22] and 0.99 nM using Jurkat cells[18]). The IC_{50} value of Ga-DOTA-4-FBn-TN14003 was 1.99 ± 0.31 nM (n = 4) under the same assay conditions demonstrating similar CXCR4 affinity. Obviously, the Ga-DOTA substituent had no influence on the affinity of the original 4-FBn-TN14003. This result was expected because for a similar inhibitor for CXCR4, namely Ac-TZ14011, the affinity of In-DTPA-Ac-TZ14011 was only slightly lower than that of the parent peptide (IC_{50} = 7.9 nM vs 1.2 nM, respectively[10]).

When using MDA-MB-231 cells instead of Jurkat cells downscaled competition curves were observed (Fig. 4), because the maximal cell binding of ^{125}I-SDF-1α was significantly lower. Looking at the parent peptide 4-FBn-TN14003, the maximal binding was reduced from 19.8 ± 1.0% (Jurkat, n = 5) to 10.4 ± 0.3% (MDA-MB-231, n = 4), respectively. Accordingly, for Ga-DOTA-4-FBn-TN14003 the maximal binding was reduced from 18.5 ± 0.8% (Jurkat, n = 4) to 8.4 ± 0.3% (MDA-MB-231, n = 5). These results suggest that MDA-MB-231 human breast cancer cells express less CXCR4 than human T lymphocyte Jurkat cells. To verify this observation, the expression of CXCR4 receptors on Jurkat and MDA-MB-231 cells was studied using immunocytochemical staining as well as a competition binding assay (see Section 3.4.3).

Besides the lower maximal binding, another difference between MDA-MB-231 and Jurkat cells was observed in the lower IC_{50} values for both peptides (Table 1). Due to the higher error bars as well as the downscaled competition curves the fits of the respective curves for the determination of the IC_{50} values were not as good as for the Jurkat cell experiments which might explain the discrepancy between the IC_{50} values for both cell lines. Even though the expression of CXCR4 on MDA-MB-231 cells is lower, the higher affinity of both peptides might on the other hand be caused by a slight variance of the receptor on this cell line. The conformation of the receptor might be better suited to the peptides. Slight differences in the affinity of 4-FBn-TN14003 to CXCR4 when using different cell lines were also described in previously published evaluations (see above).[18,22] To verify these considerations, further experiments should be performed using a panel of cell lines with different CXCR4 expression levels.

The competitive binding assays for both cell lines showed the high affinity of the parent peptide 4-FBn-TN14003 as well as the novel compound Ga-DOTA-4-FBn-TN14003. It could be demonstrated that the modification of the parent peptide did not result in a decrease of the affinity to CXCR4 making this peptide a promising candidate as a new PET tracer.

3.4.2. Uptake kinetics and internalization properties of ^{68}Ga-DOTA-4-FBn-TN14003

For estimating the optimal imaging time point in regard to in vivo studies using ^{68}Ga-DOTA-4-FBn-TN14003, the uptake kinetics of the tracer were evaluated in Jurkat as well as MDA-MB-231 cells. Fast uptake of the ligand was observed in both cell lines (Fig. 5), and again lower uptake for MDA-MB-231 cells could be observed as compared to Jurkat cells. While the uptake in MDA-MB-231 cells remained stable over time, at 90 and 120 min a decrease in uptake was observed for Jurkat cells. These results suggest that the kinetics of ^{68}Ga-DOTA-4-FBn-TN14003 are fast, which is expected for a small peptide, and that the half-life of ^{68}Ga is suitable for PET imaging using this radiotracer.

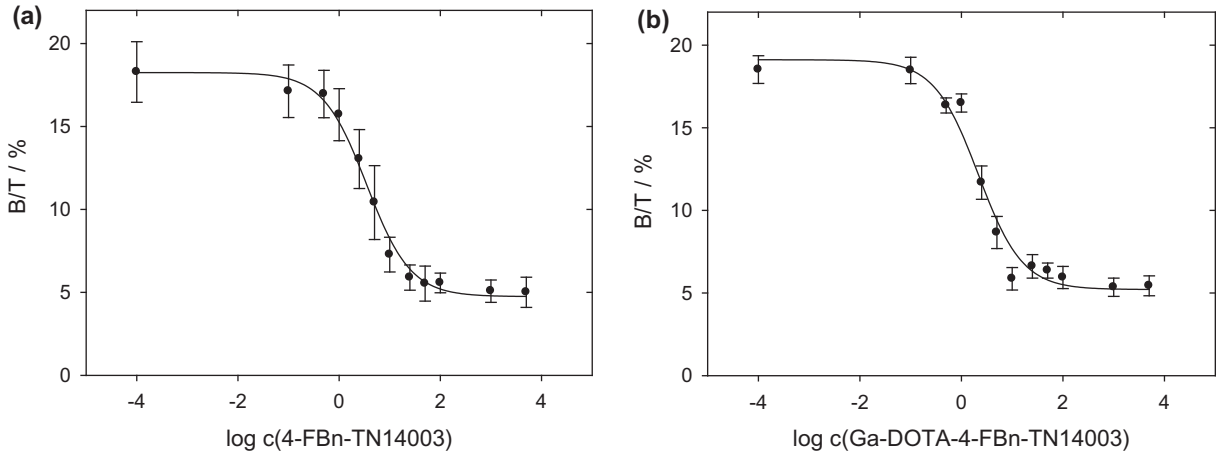

Figure 3. Competitive binding assay of 4-FBn-TN14003 (a) and Ga-DOTA-4-FBn-TN14003 (b), respectively, against ^{125}I-SDF-1α on Jurkat cells. Concentrations of the inhibitors were varied between 0–5000 nM. Values are mean ± standard deviation (n = 5 (a) and 4 (b), including quadruplicate sample measurements).

Table 1
IC_{50} values of 4-FBn-TN14003 and Ga-DOTA-4-FBn-TN14003 using Jurkat as well as MDA-MB-231 cells in a competitive binding assay with ^{125}I-SDF-1α

	IC_{50} (nM)	
	Jurkat	MDA-MB 231
4-FBn-TN14003	4.07 ± 1.00	1.75 ± 0.54
Ga-DOTA-4-FBn-TN14003	1.99 ± 0.31	1.04 ± 0.33

Values are mean ± standard deviation (n = 4 or 5).

In order to determine how much of the radiotracer that bound to the cells was actually internalized, the cells were washed with an acidic buffer to cleave off surface-bound radioactivity. At 37 °C, the internalization was 29.5 ± 1.8% for Jurkat cells and 40.0 ± 0.5% for MDA-MB-231 cells, respectively. These data are in line with recently published results for a similar peptide (^{64}Cu-T140-2D), which has two DOTA moieties conjugated to both lysine residues in the 14 amino acid peptide 4-FBn-TN14003.[23] Using this peptide, internalization of one third to one half of the radioactivity was observed in CHO-CXCR4 cells at 37 °C.

3.4.3. Expression of CXCR4

During the binding assays with ^{125}I-SDF-1α and ^{68}Ga-DOTA-4-FBn-TN14003 using Jurkat and MDA-MB-231 cells, respectively, a considerable difference in the maximal binding to both cell lines could be observed. These results suggest that MDA-MB-231 breast cancer cells express significantly less CXCR4 receptors than Jurkat T lymphocyte cells. For verification, the expression levels on both cell lines were determined by immunocytochemical staining for CXCR4 as well as a competition radioligand binding assay. The immunocytochemical staining for CXCR4 is shown in Figure 6 together with an analysis of the mean positive area fractions. In comparison, Jurkat cells expressed high amounts of CXCR4, whereas MDA-MB-231 cells expressed considerably less CXCR4.

Using a competitive binding assay by inhibiting the binding of human ^{125}I-SDF-1α by various concentrations of human SDF-1α, the determination of B_{max}, the number of binding sites per cell, was possible. In comparison to Jurkat cells, which expressed $(3.40 \pm 0.64) \times 10^5$ receptors per cell, MDA-MB-231 cells showed only $(1.13 \pm 0.44) \times 10^4$ sites per cell. This explains the results obtained above. As reported previously, Jurkat cells express CXCR4 on a high level,[28] which could be verified here in both assays. For MDA-MB-231 cells the expression of CXCR4 was also reported by Tamamura et al. as verified by RT-PCR.[18] In a recent publication by Nimmagadda et al. MDA-MB-231 cells were characterized as low expressing CXCR4 cells by flow cytometric analysis.[13] In a tumor model of MDA-MB-231-derived lung metastases in mice the same authors showed that the visualization of these metastases with ^{64}Cu-AMD3100 was feasible and that the expression of CXCR4

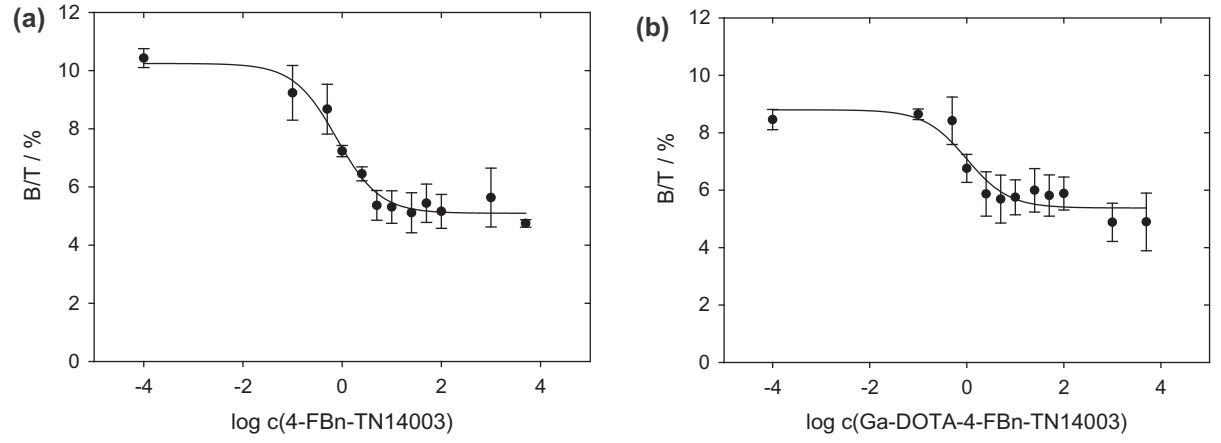

Figure 4. Competitive binding assay of 4-FBn-TN14003 (a) and Ga-DOTA-4-FBn-TN14003 (b), respectively, against ^{125}I-SDF-1α on MDA-MB-231 cells. Concentrations of the inhibitors were varied between 0–5000 nM. Values are mean ± standard deviation (n = 4 (a) and 5 (b), including quadruplicate sample measurements).

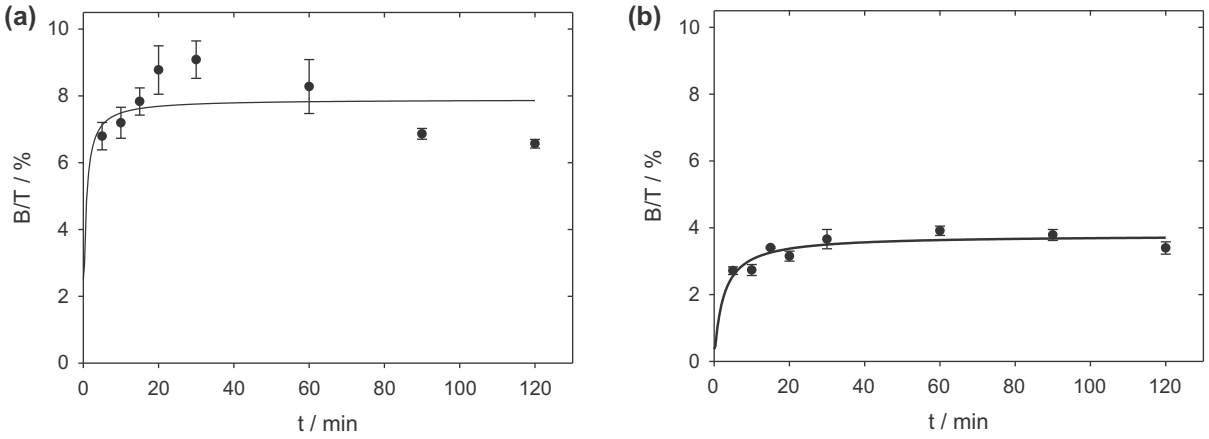

Figure 5. Uptake kinetics of ^{68}Ga-DOTA-4-FBn-TN14003 on Jurkat (a) and MDA-MB-231 cells (b), respectively. Values are mean ± standard deviation (n = 3, including quadruplicate sample measurements).

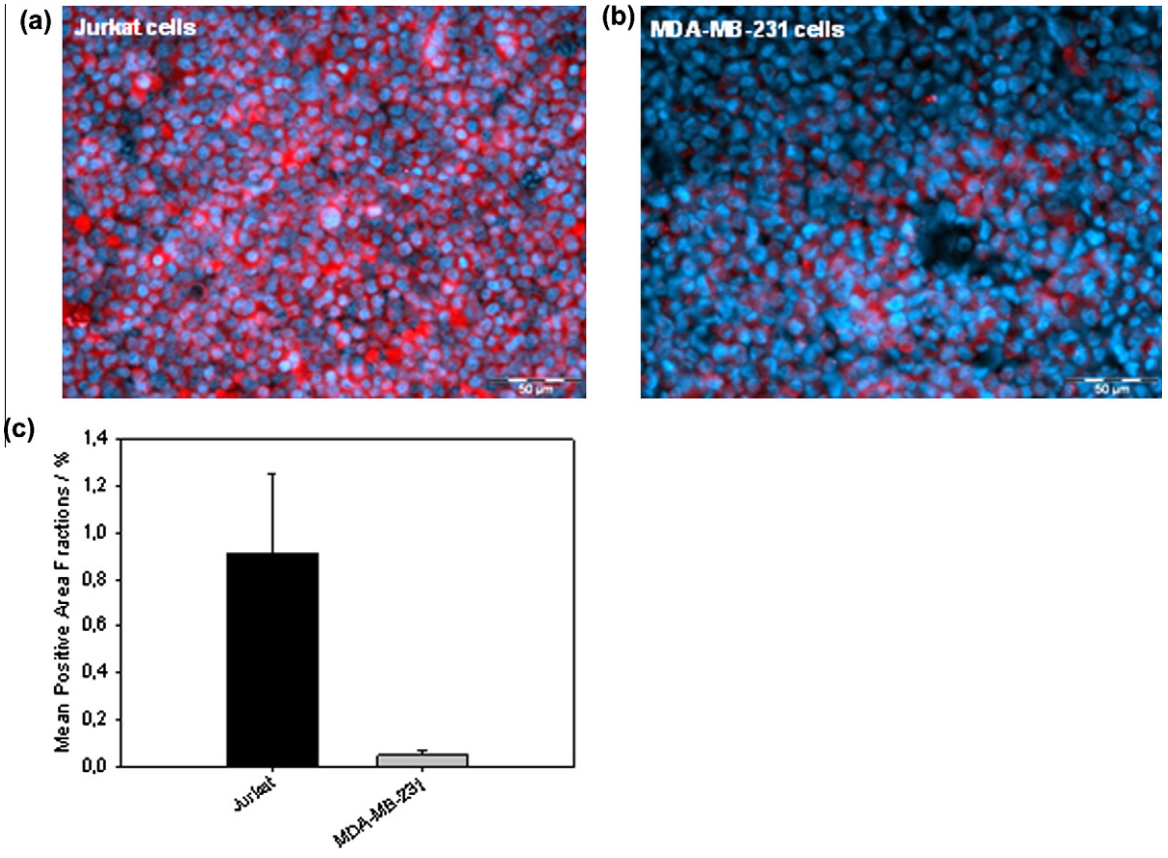

Figure 6. Immunocytochemical analysis of CXCR4: (a) Jurkat and (b) MDA-MB-231. Colored images show merged signal channels for CXCR4 (red) and DAPI (blue). Magnification 40-fold. (d) Mean positive area fractions for CXCR4 staining. Values are mean ± standard deviation (n = 10).

in the metastatic tissue was increased by 15–30%. These results indicate that the visualization of metastases by specific imaging of CXCR4 should be possible with ^{68}Ga-DOTA-4-FBn-TN14003, which we introduced here, due to its significantly higher affinity for CXCR4 than the bis(cyclam) complex ^{64}Cu-AMD3100 (IC_{50} = 1.99 ± 0.31 nM vs 62.7 μM,[29] respectively) and due to the favorable properties of ^{68}Ga as a PET isotope in comparison to ^{64}Cu (β^+ decay is only 17.6%[24]). The potency of ^{68}Ga-DOTA-4-FBn-TN14003 for the imaging of disseminated tumor cells is planned to be further evaluated in suited animal models of metastases.[30]

4. Conclusions

The favorable binding characteristics of ^{68}Ga-DOTA-4-FBn-TN14003 to CXCR4, as well as its site-specific solid phase peptide synthesis and easily adoptable radiosynthesis, gives rise to the significant potential of this compound as a novel PET tracer. Future

experiments are currently underway to evaluate its usefulness for the detection of tumors and especially metastases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.052.

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