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Synthesis and evaluation of a ^{99m}Tc-labeled chemokine receptor antagonist peptide for imaging of chemokine receptor expressing tumors

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ABSTRACT

Objective: The chemokine receptor CXCR4 is highly expressed in tumor cells and plays an important role in tumor metastasis. In the present study, we report on the evaluation of a new radiopharmaceutical peptide for its potential to visualization for CXCR4-expressing tumors in vivo.
Methods: A CXCR4 antagonist analogue was synthesized using a standard Fmoc solid phase strategy and labeled with ^{99m}Tc via HYNIC and EDDA/tricine as coligands. In addition, stability in human serum, receptor binding internalization, in vivo tumor uptake, and tissue biodistribution were evaluated. Labeling procedure has been accomplished at 100 °C. RTLC and HPLC analysis methods have been used to confirm the procedure. The receptor binding internalization rate studied using B16-F10 melanoma tumor cells. C57BL/6 mice bearing B16-F10 tumor were used for radiopeptide biodistribution studies.
Results: Labeling yield of $>95\%$ ($n = 3$) was obtained corresponding to a specific activity of 123 ± 60 GBq/μmol. Efficient stability in the presence of human serum was observed. The radioligand showed specific internalization (of total add) into B16-F10 cells ($1.57 \pm 0.27\%$ at 2 h). In animal biodistribution study, the uptake in mouse tumor was $2.74 \pm 0.47\%$ ID/g after 15 min (percentage of injected dose per gram of tissue).
Conclusion: Results of this study show that labeled peptide conjugate could be a potential candidate for diagnosis of malignant tumors.

1. Introduction

The CXC chemokine receptor 4 (CXCR4) belongs to G-protein-coupled cell-surface receptors, which is over-expressed in many type of solid tumors and metastasis. Therefore, it is an emerging target in the field of molecular imaging and therapy [1–3]. CXCR4 is specific for chemokine ligand 12 (CXCL12), which is also called stromal-derived-factor-1 (SDF-1), as a homeostatic chemokine [4]. The CXCR4/SDF-1 pathway plays a pivotal role in stimulating the metastatic process of various tumors [5], where a plethora of phenomena such as angiogenesis, invasion, chemotaxis, and proliferation can be activated with CXCR4. These phenomena have been demonstrated with different methodological approaches in breast cancer [6,7], small cell lung cancer [8], ovarian cancer [9,10], pancreatic cancer [11], lymphoma [12,13], neuroblastoma [14], glioblastoma [15,16], renal cell carcinoma [17], thyroid cancer [18,19], prostate cancer [20], and colorectal cancer [21]. Moreover, expression of CXCR4 was observed in human melanoma cell lines and in patient samples, which is very important due to difficulties in melanoma diagnosing, even for an erudite dermatologist or physician [22].

Importance of CXCR4 roles in multiple diseases has encouraged the development of clinically viable CXCR4 antagonists which can disrupt CXCR4-mediated tumor cell adhesion to stromal cells [23]. Among several developed potent peptide antagonists for CXCR4, 14-residue cyclic peptide derivative (T140) was distinguished by optimality potency of a 18-residue cyclic peptide isolated from horseshoe crabs [24,25]. Although evaluation of T140 was demonstrated instability in serum, many stable analogs with modifications at each terminus were synthesized. One of these analogs was ^{111}In-DTPA-Ac-TZ14011 which has higher uptake in the tumor than in the muscle or blood, but it has 15 to 200-fold uptake increase in liver, kidney and spleen [26]. Another amidated analog of T140, TN14003, with N-terminal 4-fluorobenzyl protection (4-^{18}F-T140) has been labeled with ^{18}F which keeps original peptide structure during labeling, but it has low radiochemical yield and long synthesis time [27]. The same peptide was also radiolabeled with ^{64}Cu-DOTA on the lysines with short radiosynthesis time. Some of the limitations of this analog were binding to RBC and moderate unspecific accumulation of radioactivity in the liver [28].

To obtain a peptide with high CXCR4 affinity with suitable overall pharmacokinetic, the pharmacophore amino acid residues of T140 derivatives were downsized to a cyclic pentapeptide (FC131). The pharmacophore amino acid residues are necessary for the inhibitory

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activity against CXCR4. This new class of peptides contains CXCR4 antagonist activity equipotent to T140 but display improved metabolic stability toward enzymatic degradation associated with cyclic peptide as well as better pharmaceutical properties [29–32]. Further modifications of FC131, was leading to a peptide analogue which was labeled with ^{68}Ga. In the preparation process DOTA and AMBS (4-(aminomethyl) benzoic acid) were used as a chelator and a linker, respectively. This ^{68}Ga-CPCR4-2 showed high CXCR4 affinity with persistent tumor uptake and suitable overall pharmacokinetics [33].

In addition to the peptide antagonists, monoclonal antibodies and small molecule inhibitors including AMD3100 were tested as imaging agents for the detection of CXCR4 using various technics, including positron emission tomography and single photon emission computed tomography (SPECT) [34–36]. However development of an imaging agent capable of detecting CXCR4 expression still has a great value in the management of cancer patients.

For diagnostic application, ^{99m}Tc remains the radionuclide of choice in scintigraphy imaging because of its optimal nuclear properties (6.02 h half-life with 140 keV gamma photons), low cost and wide commercial availability via generators [37]. High specific activity of ^{99m}Tc labeling can be obtained by bifunctional chelating agents. The most widely used bifunctional chelating agent is 6-hydrazinonicotinic acid (HYNIC) [38]. To achieve effective peptide imaging agents based on ^{99m}Tc, an analogue of SDF-α containing a HYNIC-coupled pentapeptide with cyclic structure (*cyclo*(D-Tyr^1-D-[HYNIC] Orn^2-Arg^3-2-Nal^4-Gly^5)) was developed. Optimization of the radiolabeling condition, stability, lipophilicity, plasma protein binding, cell binding and internalization with biological evaluation were investigated in mice bearing B16-F10 melanoma tumor.

2. Materials and methods

All of the Fmoc-protected amino acids and 2-chlorotrityl [(2-Cl) Trt] resin were commercially available. The pro-chelator HYNIC-Boc was prepared according to Abrams et al. [39]. Solvents were obtained from Sigma-Aldrich (Munich, Germany). The reactive side chains of the amino acids protecting agents were masked with one of the following groups: D-Orn, 1-(4,4-Dimethyl-2,6-dioxocyclo-hexylidene)-3-methylbuty (ivdde); D-Tyr, tert-butyl (t-Bu); Arg, 2,2,4,6,7 pentamethyldihydrobenzofurane-5-sulfonyl (pbf). The cell culture medium was Roswell Park Memorial Institute (RPMI-1640) supplemented with 10% fetal bovine serum (FBS), amino acids, vitamins and penicillin/streptomycin (Gibco, Eggenstein, Germany). Sodium pertechnetate (Na^{99m}TcO$_4$) was prepared from a commercial ^{99}Mo/^{99m}Tc-generator (Pars Isotope Co). Analytical reversed- phase high performance liquid chromatography (RP-HPLC) was performed on a system (Sykam S7131, Eresing, Germany) with a variable wavelength UV detector operating at $\lambda = 280$ nm and with a flow-through gamma-detector containing a NaI crystal (Raytest-Gabi, Straubenhardt, Germany) and a C18 analytical column (CC 250/4.6 Reprosil-pur ODS-3.5 μm). The mobile phase consisted of 0.1% trifluoroacetic acid/water (Solvent A) and acetonitrile (Solvent B), and the gradient system used a flow rate of 1 ml/min in A:B ratio of: 95:5 at 0 min, 95:5 at 5 min, 0:100 at 25 min, 0:100 at 27 min, 95:5 at 30 min, 95:5 at 35 min. A mass spectrometer (1100/Bruker Daltonic; Agilent, Bremen, Germany) with a VL instrument (LC/MS) was also used. Radioactive samples were measured on a gamma counter (EG&G/ORTEC, Model 4001M, Jackson, USA) Mini Bin & Power Supply. Radio-TLC was performed on a Raytest-GITA scanner (Germany), using silica gel plates (LK6DF, 60 Å, 200 mm, Whatman) and ethyl methyl ketone, sodium citrate 0.1 N with pH = 5 and water: acetonitrile (1:1) as a developing solvents.

2.1. Synthesis

The protected peptide was constructed using standard Fmoc-based solid phase synthesis on 2-chlorotrityl [(2-Cl) Trt] resin with substitution, 1.4 mmol/g [40]. Coupling of each amino acid was performed in the presence of 3 mole excess of Fmoc-amino acid, *N*-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) in dimethylformamide (DMF) and 5 mole excess of diisopropylethylamine (DIPEA) in DMF. Fmoc groups were removed by adding 20% (v/v) piperidine in DMF (10 mL).The Kaiser Test determined the end of a coupling reaction. After synthesis of linear peptide, protected peptide was cleaved from the resin by treating with a mixture of 2.5% TFA and 5% of triisopropylsilane in dichloromethane (DCM) (10 mL). Thereafter protected peptide was cyclized by coupling the carboxylic group from Glycine with the amino group on D-Tyrosine. The cyclization reaction was achieved by an overnight reaction with peptide in DMF (3 mL) using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling agent in the presence of DIPEA. Due to attaching HYNIC, the protecting group of D-Ornithine residual chain (ivdde) was removed by 2% hydrazine hydrate in DMF. Each HYNIC-Boc (1.2 mmol) coupled with *O*-(7-azabenzotriazol-1-yl)-1, 1, 3, 3, tetramethyluronium hexafluorophosphate (HATU) (1.2 mmol) and DIPEA (3.6 mmol) in DMF (5 mL) to the amino group of D-Ornithine residual chain (1 mmol). The amino acid side chains of the peptide HYNIC conjugate were also deprotected by treatment with a cocktail of TFA, thioanisole, water and triisopropylsilane (92.5:2.5:2.5:2.5) for 30 min at 25 °C temperature. After removing the organic solvents in vacuum, the crude product was precipitated by adding a solution of cold petroleum ether and diisopropyl ether (50:50).

2.2. ^{99m}Tc-labeling of HYNIC-peptide

Labeling was executed in accordance with previous reports. A stock solution was provided by dissolving the HYNIC-peptide in distilled water. Radiolabeling of peptide was performed by adding the stock solution (5 μg of peptide; 20 μL), tricine (15 mg) and EDDA (5 mg) to water (0.5 mL). This mixture was mixed with nitrogen-purged stannous chloride dihydrate solution (in 0.1 M HCl; 2 mg/mL; 20 μL) and ^{99m}Tc-pertechnetate (370–740 MBq) in saline (0.5 mL) in an inert atmosphere. Finally the mixture was incubated for 10 min at 100 °C. After contents were cooled to ambient temperature, quality control tests were accomplished as described below.

2.3. Radiochemical analysis and quality control

Radiochemical analysis of ^{99m}Tc-HYNIC-peptide was evaluated by analytical RP-HPLC in above mentioned analytical condition. Instant thin layer chromatography (ITLC) strips (silica gel 60; Merck) were loaded with sample and developed using three different mobile phases: (i) methyl ethyl ketone for ^{99m}Tc-pertechnetate ($R_f = 1.0$); (ii) aqueous sodium citrate (0.1 M; pH = 5) to determine ^{99m}Tc-coligand and ^{99m}Tc-pertechnetate ($R_f = 1.0$); and (iii) water: acetonitrile [1:1] for ^{99m}Tc-colloid ($R_f = 0.0$). The radioactivity was quantified by TLC scanner.

2.4. Serum stability

In vitro stability of labeled compound was checked according to HPLC and ITLC method using a sample of labeled peptide stored in NaCl 0.9% (W/V) and in human serum at room temperature during 1, 4, 6 and 24 h post labeling. The level of protein-bound radioactivity versus non-bound radioactivity was assayed using a precipitation method. ^{99m}Tc-HYNIC-peptide (86 MBq; 0.1 mL) was added to a freshly prepared human serum (1 mL) and the mixture was incubated at 37 °C for 24 h. After 2 h, three samples of 100 μl aliquots were removed and treated with ethanol (100 μL). Each sample was centrifuged for 5 min at 3000 rpm to separate the precipitated serum proteins. Radioactivity in the supernatant was compared with that in sediment to determine the percentage of radiopeptide protein binding. For determination of labeled compound stability, the supernatant was analyzed with HPLC.