Novel Bifunctional Cyclic Chelator for $^{89}$Zr Labeling—Radiolabeling and Targeting Properties of RGD Conjugates

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INTRODUCTION

Over the last years the positron-emitter $^{89}$Zr has attracted considerable interest for molecular imaging applications using positron emission tomography (PET). In comparison to the commonly used Radionuclides for PET ($^{18}$F, $^{68}$Ga, $^{11}$C, etc.), $^{89}$Zr with its relatively long half-life of 78.4 h is particularly suited for in vivo imaging at late time points with a mean positron energy of 0.395 MeV, which is between the positron energies of $^{18}$F (0.250 MeV) and $^{68}$Ga (0.836 MeV), it allows for high resolution PET images for small animal imaging applications.

For imaging applications at late time points $^{89}$Zr has to be stably bound to a chelator to minimize dissociation in vivo, as free $^{89}$Zr can accumulate in the bone and associate with plasma proteins. Over the years, several chelators, such as diethylene-triaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), and 1,4,7,10-tetraazacyclododecane $N,N',N'',N'''$-tetraacid (DOTA), have been evaluated with limited success. Today desferrioxamine B (DFO) is the most prominent chelator for $^{89}$Zr labeled biomolecules (Figure 1).

$^{89}$Zr DFO mAb-conjugates have been used in a number of clinical trials for in vivo tracking and quantification of monoclonal antibodies (mAbs) by PET with good imaging quality. However, there are concerns about the stability of the complexes in vivo not only in relation to image quality but in particular to radiation dose. Several preclinical studies reported bone accumulation of dissociated $^{89}$Zr ranging from 3 to 15% after 3 to 7 days. Alternative chelators for $^{89}$Zr that could eliminate the release of osteophilic $^{89}$Zr and lead to safer PET procedures with reduced radiation dose and optimal image quality are required for the development of novel targeted PET-tracers.

Several studies have focused on the conjugation moiety and recently also on the ligand itself. Considering that DFO is a linear ligand and exposed to endogenous competitive cations and natural chelators in vivo that may challenge the
stability of the metal chelate, macrocyclic structures may favor a higher kinetic inertness of their metallic complexes. Recently we demonstrated that fusarinine C (FSC), a representative of the class of hydroxamate siderophores, is a promising bifunctional chelator for $^{68}$Ga-radiolabeling (Figure 1). FSC has three primary amines, which can be derivatized in a number of ways also applying the concept of multivalency. This is the combination of several targeting units in one single molecule. By attaching a cyclic RGD peptide, binding to $\alpha_v\beta_3$ integrins that are expressed during angiogenesis, via a succinic acid linker, stable $[^{68}\text{Ga}]\text{FSC(succ-RGD)}_3$ with excellent receptor binding properties and \textit{in vivo} targeting was prepared. $[^{68}\text{Ga}]\text{FSC(succ-RGD)}_3$ showed superiority to monomeric cyclic RGD peptides, such as $[^{18}\text{F}]\text{Galacto-RGD}$ and $[^{68}\text{Ga}]\text{-NODAGA-RGD}$. FSC has a similar structure like DFO with three hydroxamate groups providing six oxygen donors for metal binding. We postulated that FSC could be an alternative for $^{89}\text{Zr}$ labeling with complex formation properties comparable to DFO but potentially higher stability due to its cyclic structure. Thus, increased image contrasts at delayed time points are possible. In an initial study we could demonstrate that cyclic siderophores can be labeled with $^{89}\text{Zr}$ displaying comparable properties to its $^{68}\text{Ga}$ counterparts. In this study triacetylfusarinine C (TAFC), the triacetylated form of FSC, was investigated, and the labeling procedure and \textit{in vitro} stability were compared with $[^{89}\text{Zr}]\text{DFO}$. Additionally, the feasibility of different FSC conjugation strategies, where the RGD peptides have been bound either directly or through a linker, has been evaluated. Moreover, radiolabeling properties, stability, and \textit{in vivo} behavior of $[^{89}\text{Zr}]\text{FSC(succ-RGD)}_3$ in comparison to its $^{68}\text{Ga}$ labeled counterpart were compared. The study also included microPET/CT imaging in mice bearing $\alpha_v\beta_3$ integrin expressing tumor xenografts.

### EXPERIMENTAL SECTION

**General.** All substances described were of reagent grade and were used without further purification. Human melanoma M21 and M21-L cells were a kind gift from D. A. Cheresh, Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, CA, USA. $^{89}\text{Zr}$ was purchased from PerkinElmer, Inc. (USA). Analytical reversed-phase high performance liquid chromatography (RP-HPLC) analysis was performed using a Vydac 218 TP5215, 150 $\times$ 3.0 mm column (SRD, Vienna, Austria) at a flow rate of 1.0 mL/min with the following acetonitrile (CH$_3$CN)/H$_2$O/0.1% trifluoroacetic acid (TFA) gradients: 0$\text{−}$0.5 min 0% CH$_3$CN, 0.5$\text{−}$7.0 min 0$\text{−}$55% CH$_3$CN. Radiolabeling efficiency and radiochemical purity were determined by instant thin-layer chromatography (ITLC) on ITLC silica gel strips (Agilent Technologies) with 50 mM EDTA (pH 7, adjust pH using 10 M NaOH) as mobile phase. The strips were scanned using a mini-scan radio TLC scanner with flow-count detector (LabLogic, Sheffield, UK).

FSC and TAFC were prepared as described previously. Briefly, the Aspergillus strains (A. fumigatus strain for FSC, A. nidulans strain for TAFC) were cultured in iron free minimal medium with 1% glucose for 36 h at 37 °C and 200 rpm. Biomass was removed by filtration, and the media-containing...
siderophores were collected. TACF was directly isolated by preparative RP-HPLC.

[Fe]Fusaringine C ([Fe]FSC). [Fe]FSC was prepared by saturating FSC solution with iron followed by purification using Amberlite XAD18 beads (Dow Chemical Company, Philadelphia, PA, USA) as column matrix. [Fe]FSC was further purified via preparative RP-HPLC and dried by lyophilization. Approximately 500 mg of a red colored solid was obtained. MALDI TOF-MS \( m/z \) [M + H] = 780.4 [C\(_{31}\)H\(_{52}\)FeN\(_{10}\)O\(_{12}\); exact mass, 779.3 (calculated)].

**Peptide Synthesis.** The cyclic pentapeptides cyclo(-Arg(Pbf)-Gly-Asp(OtBu)-DPhe-Glu-) (c(RGDfK) (Pbf, OtBu)) was synthesized via phase peptide synthesis (SPPS) as previously reported. The butyl) were prepared by cyclization of the side chain protected pentamethyl-3-hydrobenzofuran-6-sulfonyl; OtBu = (RGDfE) (Pbf, OtBu)) and cyclo(-Arg(Pbf)-Gly-Asp(OtBu)-DPhe-Glu-) (c(RGDfE)) (protecting groups: Pbf = 2,2,4,5,7-pentamethyl-3-hydrobenzofuran-6-sulfonyl; OtBu = O-tert-butyl) were prepared by cyclization of the side chain protected linear precursors, which were synthesized by Fmoc-based solid phase peptide synthesis (SPPS) as previously reported. The Z-protecting group of lysine and ODmab of glutamic acid were cleaved by hydrogenation with the Pd/C catalyst or 2% hydrazine in DMF, respectively. Pbf/OtBu was cleaved with a TFA/H\(_2\)O solution (v/v 95:5). A succinyl-derivative of c(RGDfK) (Pbf, OtBu) (succ-c(RGDfK) (Pbf, OtBu)) was synthesized using c(RGDfK) (Pbf, OtBu) reacting with molar excess of succinic anhydride. The products were purified by preparative RP-HPLC, and their identities were confirmed by ESI-MS or MALDI TOF-MS [M + H] = 1012.6 [C\(_{48}\)H\(_{69}\)N\(_{9}\)O\(_{13}\)S; exact mass, 1011.5 (calculated)].

**Demetalation.** [Fe]FSC(RGDfE)\(_3\) (10 mg, 3.9 \( \mu \)mol) or [Fe]FSC(succ-RGD)\(_3\) (10 mg, 3.5 \( \mu \)mol) or [Fe]FSC(Mal-RGD)\(_3\) (10 mg, 2.6 \( \mu \)mol) was dissolved in 40-fold of a disodium EDTA solution (25 mM, pH 4). After 1 h stirring at RT the solution was directly isolated via preparative RP-HPLC and dried by lyophilization. FSC(RGDfE)\(_3\) yield 6.2 mg (2.5 \( \mu \)mol), 64\%, MALDI-TOF-MS [M + H]\(^+\) = 2487.8, [C\(_{111}\)H\(_{183}\)N\(_{30}\)O\(_{36}\); exact mass, 2485.1 (calculated)]; FSC(succ-RGD)\(_3\) yield 6.4 mg (2.3 \( \mu \)mol), 66\%, MALDI-TOF-MS [M + H]\(^+\) = 2785.0, [C\(_{126}\)H\(_{189}\)N\(_{30}\)O\(_{36}\); exact mass, 2782.3 (calculated)]; FSC(Mal-RGD)\(_3\) yield 6.8 mg (1.8 \( \mu \)mol), 69\%, MALDI-TOF-MS [M + H]\(^+\) = 3785.2, [C\(_{168}\)H\(_{230}\)N\(_{42}\)O\(_{42}\); exact mass, 3781.7 (calculated)].

**\( ^{89}\)Zr Radiolabeling.** Approximately 30 MBq (30 \( \mu \)L) of \( ^{89}\)Zr-oxalate was transferred into a low-protein binding Eppendorf tube, followed by addition of 27 \( \mu \)L of 1 M sodium carbonate. The reaction solution was incubated for 3 min at RT. Thereafter, 100 \( \mu \)L of HEPES buffer (0.5 M, pH 7.0) was added to the reaction vial. DFO (32.8 \( \mu \)g), TACF (42.6 \( \mu \)g), or the FSC-RGD conjugates ([FSC(RGDfE)]\(_3\), FSC(succ-RGD)\(_3\), or FSC(Mal-RGD)\(_3\)) was added to the reaction vial. The pH of the reaction vial was measured to be sure that it was between 6.8 and 7.2. The solution was allowed to react at RT for 90 min. The reaction was monitored via ITLC (\( ^{89}\)Zr-complexes remained at the origin and free \( ^{89}\)Zr migrates with the solvent front, see Supporting Information, Figure S1) and confirmed by HPLC. For animal experiments, after radio-labeling 60 \( \mu \)L of CaCl\(_2\) (0.5 M) was added to the reaction vial, which resulted in a precipitate of Ca-oxide. The solution was passed through a 0.2 \( \mu \)m filter to remove Ca-oxide. The filtrate was diluted to an appropriate volume using saline for further evaluation.

**Distribution Coefficient (logD).** Aliquots of 50 \( \mu \)L of radioligand ([\( ^{89}\)Zr]DFO, [\( ^{89}\)Zr]TACF, or [\( ^{89}\)Zr]FSC-RGD conjugates) were diluted in 450 \( \mu \)L of PBS. Then 500 \( \mu \)L of octanol was added, and the mixture was vortexed for 15 min at 1400 rpm and centrifuged for 2 min at 2000 rcf. Subsequently, 50 \( \mu \)L aliquots of the aqueous and the octanol layer were collected, measured in the gamma counter, and logD values were calculated using Excel (n = 5).
Stability Assay. Determination of the stability of $^{89}$Zr-DFO, $^{89}$Zr-TAFC, and $^{89}$Zr-FSC-RGD conjugates was carried out by incubating the radioligands in PBS, 1000-fold molar excess of EDTA solution with different pH (pH 7 and pH 6; radioligand vs EDTA: 25 μM vs 25 mM) as well as in human serum for 7 days at 37 °C, respectively. At selected time points three aliquots of PBS and EDTA solution were analyzed directly via ITLC, while the three serum aliquots were mixed with 500 μL of CH₃CN, vortexed, and centrifuged at 20,000 rcf for 2 min prior to analysis.

Transchelation Study. $^{89}$Zr-DFO and $^{89}$Zr-TAFC (50 μL each) were mixed with a 1000-fold molar excess of either TAFc or DFO for 7 days at RT. At selected time points aliquots of the solutions were analyzed directly via RP-HPLC. ($^{89}$Zr)DFO $t_{1/2}$ = 4.8 min; $^{89}$Zr]TAFC $t_{1/2}$ = 5.9 min). The transchelation was determined by the ratio of $^{89}$Zr]DFO and $^{89}$Zr]TAFC. Three replicates were analyzed for each radioligand.

Protein Binding Assay. Protein binding abilities were evaluated by incubating $^{89}$Zr]DFO and $^{89}$Zr]TAFC for 7 days and $^{89}$Zr]FSC-RGD conjugates for 4 h at 37 °C in fresh human serum. Subsequently, 30 μL of the solution was passed through a size exclusion spin column (MicroSpin G-50 column, GE healthcare, Buckinghamshire, UK) via centrifugation at 2000 rcf for 2 min. Protein binding of the conjugates in triplicates was determined by measuring the activity on the column (nonprotein bound) and the activity in the eluate (protein bound) in a gamma counter.

Internalization Assay. M21 cells ($\alpha$, $\beta$, integrin positive) were incubated with RPMI 1640 (Gibco, Invitrogen Corporation, Paisley, UK) containing 1% glutamine (m/v), 1% bovine serum albumin (BSA) (m/v), CaCl₂ (1 mM), MgCl₂ (1 mM), and MnCl₂ (10 mM) to a concentration of 2.0 × 10⁶ cells/mL. Aliquots of 1 mL cell solution were transferred to Eppendorf tubes for incubation at 37 °C for 1 h. After addition of $^{89}$Zr]FSC(RGDfK)₃, $^{89}$Zr]FSC(succ-RGD)₃ or $^{89}$Zr]FSC-(Mal-RGD)₃ (approximately 1.5 × 10⁶ cpm), the cells were incubated in triplicates with either PBS with 0.5% BSA (150 μL, total series) or with 10 μM c(RGDyV) in PBS/0.5% BSA (150 μL, non-specific) at 37 °C for 90 min. After the incubation time the tubes were centrifuged and incubation was stopped by removal of the medium. Then the cells were washed twice with ice-cold TRIS-buffered saline. Subsequently, the cells were incubated twice in acid wash buffer (20 mM acetate buffer, pH 4.5) at 37 °C for 5 min. Then the tubes were centrifuged, and the supernatant was collected in plastic vials (membrane bound activity). In a last step the cells were lysed by addition of 2 M NaOH, and the radioactivity associated with cells was collected in plastic vials (internalized radioligand fraction). Protein content in the NaOH fraction was determined using a spectrometric method (Bradford assay). The internalized activity was calculated and expressed as percentage of total activity per milligram protein.

Biodistribution Studies. All animal experiments were conducted in compliance with the Austrian animal protection laws and with approval of the Austrian Ministry of Science (BMWF-66.011/000604-II/3b/2012 and BMWF-66.011/0049-WF/II/3b/2014). For the evaluation of biodistribution, four healthy female BALB/c mice (Charles River Laboratories, Sulzfeld, GER) were intravenously injected with $^{89}$Zr]TAFC (∼1.5 MBq/mouse, ∼4 μg of precursor) into the tail vein and sacrificed at 6 h postinjection (p.i.). For the evaluation of tumor uptake of $^{89}$Zr]FSC(succ-RGD)₃ female, athymic BALB/c nude mice (Charles River Laboratories) were used. For the induction of tumor xenografts mice were injected subcutaneously with 5 × 10⁶ $\alpha$, $\beta$, integrin positive M21 cells into the right hind limb and with 5 × 10⁶ $\alpha$, $\beta$, integrin negative M21-L cells (negative control) into the left hind limb of the same mouse (n = 4). The tumors were allowed to grow until they had reached a volume of 0.3 to 0.6 cm³. On the day of the experiment $^{89}$Zr]FSC-(succ-RGD)₃ ($\sim$0.5 MBq/mouse, ∼0.5 μg of peptide) was intravenously injected in the lateral tail vein. Mice were sacrificed by cervical dislocation at 1, 2, and 4 h after injection. Organs (spleen, pancreas, stomach, intestine, kidney, liver, heart, and lung), blood, muscle tissue, bone, and tumors were dissected and weighted. As a comparison, four tumor-bearing mice were intravenously injected in the tail vein with $^{68}$Ga]FSC(succ-RGD)₃ ($\sim$0.5 MBq/mouse, ∼0.5 μg of precursor) and sacrificed at 1 h p.i. Activity of the different samples was measured in the gamma counter. Results were expressed as percentage of injected dose per gram tissue (% ID/g).

MicroPET/CT Imaging. MicroPET/CT imaging experiments were conducted on an Inveon microPET/CT scanner (Siemens Preclinical Solutions, Knoxville, USA). A group of four M21-tumor xenograft bearing BALB/c nude mice were administered with $^{89}$Zr]FSC(succ-RGD)₃ (∼5 MBq/mouse, ∼5 μg of peptide) via intravenous tail vein injection. MicroPET images were acquired under general anesthesia (isoflurane/O₂) for 20 min. PET data for each mouse were recorded via static scans at 1, 4, and 24 h p.i. The microPET scans were reconstructed with Inveon Acquisition Workplace software (version 1.5; Siemens Preclinical Solutions), using a 3-dimensional fast maximum a posteriori algorithm with the following parameters: matrix, 256 × 256 × 161; pixel size, 0.4 × 0.4 × 0.8 mm; β-value of 1.5 mm resolution with uniform variance.

Statistical Analysis. Statistical analysis was performed using SPSS 17.0 software. The biodistribution data was analyzed using Student’s t test. The level of significance was set at P = 0.05.

RESULTS

Precursor Synthesis. RGD peptides could be synthesized in good yields following standardized protocols.21 Assemblies of the linear RGD peptides were accomplished on solid phase using Fmoc-protocols. Cyclization was carried out under high dilution conditions and subsequent Z-deprotection under hydrogen atmosphere for c(RGDfK)(Pbf, OtBu) or ODmab-deprotection with 2% hydrazine in DMF for c(RGDfE)(Pbf, OtBu). The lysine side chain was modified via addition of succinic anhydride enabling the coupling of the RGD peptide to the primary amino functions of [Fe]FSC. Amidation of succ-(c(RGDfK))(Pbf, OtBu) or c(RGDfE)(Pbf, OtBu) with [Fe]FSC was accomplished via in situ activation using HATU/HOAt and DIPEA. Complete deprotection and subsequent demetalation of the organometallic complexes resulted in an average yield of approximately 40% and 30% for FSC(succ-RGD)₃ and FSC(RGDfE)₃, respectively. [Fe]-FSC(Mal)₃ was synthesized by [Fe]FSC reaction with 3-maleimidopropionic acid N-hydroxysuccinimide ester. The deprotected c(RGDfK) was modified with SAT(PEG)₄ and then treated with hydroxylamine for 20 min to expose the sulfhydryl group for coupling [Fe]FSC(Mal), at neutral pH, resulting in an average yield of 55%. The removal of [Fe],
which was used to protect the complexing moiety, from [Fe]FSC-RGD conjugates was carried out using 40-fold molar excess of EDTA solution at pH 4 within 25 to 60 min with high yield (>90%). The rapid disappearance of the intense red color of the solution indicated the successful removal of [Fe]. After HPLC purification the chemical purity of the final compounds was >95%.


**89Zr Radiolabeling.** Labeling of DFO, TAFC, and the FSC-RGD conjugates with 89Zr-oxalate was performed with slight modifications as described recently.22 Radiolabeling was carried out in HEPES buffer at RT at pH 6.8 to 7.2. FSC could acquire 89Zr quantitatively from 89Zr-oxalate at RT with incubation times between 30 and 90 min depending on the concentration of precursor. Moreover, 1 μg of TACF could be quantitatively labeled with 30 MBq of 89Zr-oxalate leading to a specific activity of 25 GBq/μmol (without optimization). The time course of 89Zr-complexation of FSC(succ-RGD)3 (30 μg, 58 μM) is shown in Figure 2. Oxalate, which could be toxic due to the production of solid calcium oxalate causing kidney failure, was readily removed by preprecipitation with CaCl₂ and filtration using a 0.2 μm filter for in vivo experiments.

**In Vitro Characterization.** 89ZrDFO, 89ZrTAFC, and 89ZrFSC-RGD conjugates showed high stability in PBS and human serum over a period of 7 days, and no demetalation was observed. LogD and protein binding data are summarized in Table 1. High hydrophilic character was observed for 89ZrFSC-RGD conjugates (logD was between −2.9 and −3.0). The lower hydrophilicity of 89ZrTAFC (logD−2.0) is in accordance with the data found for 68GaTAFC.23 The low protein-bound activity (<10%) over the whole monitoring period further confirmed the stability of FSC in human serum.

**Biodistribution Studies.** 89Zr TAFC showed rapid pharmacokinetics in normal mice at 6 h p.i. with fast blood clearance (0.05 ± 0.01% ID/g), low uptake of bone (0.04 ± 0.02% ID/g), and predominant renal excretion with some retention at this late time point (0.86 ± 0.48% ID/g). Biodistribution and specific tumor uptake of 89ZrFSC(succ-RGD)3 were investigated at 1, 2, and 4 h postinjection in BALB/c nude mice bearing M21 (α,β3 integrin positive) as well as M21-L (α,β3 integrin negative) tumor xenografts (n = 4) (Figure 4). The uptake of 89ZrFSC(succ-RGD)3 in the α,β3 integrin positive M21 tumor was 2.83 ± 0.60% ID/g at 1 h p.i. At 2 h a slight increase to the maximum uptake of 3.48 ± 0.45% ID/g was seen and a slow clearance to 2.86 ± 0.30% ID/g at 4 h p.i. In contrast, a constant but low uptake (0.5% ID/g) was observed at all time points in the contralateral α,β3 integrin negative M21-L tumors. This highly significant difference in tumor uptake between receptor-positive and receptor-negative tumors confirmed the receptor-selective uptake found in vitro.

The fast blood clearance (0.16 ± 0.04% ID/g, 0.15 ± 0.0% ID/g, and 0.10 ± 0.01% ID/g at 1, 2, and 4 h p.i., respectively) led to an increased tumor/blood ratio. The comparably high activities in kidneys (6.42 ± 0.72% ID/g, 5.77 ± 0.20% ID/g, and 5.26 ± 0.90% ID/g at 1, 2, and 4 h p.i., respectively) could be related to renal excretion and partial retention. Notably, the low amount of activity in the bone remained stable (0.71 ± 0.05% ID/g, 0.72 ± 0.28% ID/g, and 0.73 ± 0.27% ID/g at 1, 2, and 4 h p.i., respectively) indicating the high in vivo stability of 89ZrFSC(succ-RGD)3. Compared with the biodistribution data of 68GaFSC(succ-RGD)3 at 1 h, significant differences of uptake in blood, spleen, liver, and M21-L tumors were observed. The higher uptakes of 68GaFSC(succ-RGD)3 in the spleen (3.27 ± 0.64% ID/g vs 1.78 ± 0.22% ID/g) and in M21-L tumors (1.10 ± 0.30% ID/g vs 0.50 ± 0.12% ID/g) are related to the prolonged presence of the 68Ga-tracer in the blood (0.63 ± 0.26% ID/g vs 0.16 ± 0.04% ID/g).

A microPET/CT imaging study with tumor-bearing nude mice was performed in order to investigate the in vivo pharmacokinetics of 89ZrFSC(succ-RGD)3 (Figure 5). Again images confirmed receptor-specific activity accumulation.

**Table 1. LogD and Binding Properties of 89ZrDFO, 89ZrTAFC, and 89ZrFSC-RGD Conjugates**

| 89Zr complex          | logD (pH 7.4) | incubation time (h) | protein binding (%) |
|-----------------------|--------------|---------------------|--------------------|
| 89ZrDFO               | −3.0 ± 0.1   | 168                 | 8.7 ± 1.0          |
| 89ZrTAFC              | −2.0 ± 0.0   | 168                 | 6.8 ± 0.5          |
| 89ZrFSC(RGDfE)3       | −3.0 ± 0.0   | 4                   | 5.0 ± 0.8          |
| 89ZrFSC(succ-RGD)3    | −2.9 ± 0.1   | 4                   | 7.3 ± 0.5          |
| 89ZrFSC(Mal-RGD)3     | −2.9 ± 0.2   | 4                   | 6.0 ± 1.5          |
in the αvβ3 integrin positive M21 tumors, and more importantly, no bone uptake was observed, confirming high in vivo stability of the compound. The M21 tumors could be clearly visualized over the whole monitoring period of 24 h, whereas the αvβ3 integrin negative M21-L tumors showed no uptake confirming the high specificity of [89Zr]FSC(succ-RGD)3. Besides the M21 tumors, only kidneys were visible, which is related to excretion and kidney retention.

**DISCUSSION**

The positron-emitting radionuclide 89Zr is increasingly used for molecular imaging with PET; its clinical application is currently limited to conjugates using DFO as chelator. Even though several antibodies conjugated to DFO and radiolabeled with 89Zr achieved some successful results, dissociation of 89Zr in vivo has been observed indicating limited in vivo stability. Therefore, increasing efforts are made to design and synthesize novel robust and stable chelators for 89Zr. The design of an ideal chelator is affected by many factors, such as the type and number of coordinated atoms, acyclic vs cyclic constructs, ring size, etc. Even the charge of the metal-complex may influence the biodistribution of a corresponding tracer labeled with a radiometal.

Based on the knowledge on structure, synthesis, and labeling of DFO-conjugates, the natural siderophore FSC was chosen as the starting point for our research. FSC is produced by fungi for acquiring iron from the environment and has been proven to be an excellent bifunctional chelator for 68Ga in our laboratory. Therefore, FSC, having three hydroxamic acid moieties similar to those of DFO, embedded in a cyclic structure, was expected to be an interesting alternative for 89Zr labeling. In our study, TAFC was initially chosen to explore the labeling procedure and to evaluate the in vitro stability. It was labeled with 89Zr-

| starting complex | competitor (1000-fold excess) | pH | 1 h | 4 h | 1 d | 3 d | 5 d | 7 d |
|------------------|------------------------------|----|-----|-----|-----|-----|-----|-----|
| [89Zr]DFO        | EDTA                         | 7  | 95.5 ± 0.5 | 76.0 ± 1.0 | 55.4 ± 3.2 | 41.0 ± 5.2 | 43.3 ± 2.5 | 42.2 ± 2.3 |
|                  | TAFc                         | 6  | 94.6 ± 0.4 | 63.5 ± 3.6 | 38.2 ± 8.4  | 6.4 ± 2.0   | 6.0 ± 1.8   | 6.0 ± 1.8   |
| [89Zr]TAFC       | EDTA                         | 7  | 99.6 ± 0.1 | 99.6 ± 0.1 | 99.3 ± 0.2  | 96.9 ± 0.3  | 98.3 ± 0.1  | 97.2 ± 0.2  |
|                  | DFO                          | 6  | 99.0 ± 0.1 | 98.1 ± 0.2 | 96.0 ± 0.6  | 94.7 ± 0.3  | 95.9 ± 0.3  | 94.4 ± 0.5  |
| [89Zr]FSC(succ-RGD)3 | EDTA                     | 7  | 98.2 ± 0.1 | 98.2 ± 0.1 | 96.5 ± 0.5  | 96.0 ± 0.4  | 94.5 ± 0.3  | 93.9 ± 0.7  |

**Figure 3.** Internalization of [89Zr]FSC(RGDfE)3, [89Zr]FSC(succ-RGD)3, and [89Zr]FSC(Mal-RGD)3 in αvβ3 integrin positive M21 tumor cells.

**Figure 4.** Biodistribution of [89Zr]/[68Ga]FSC(succ-RGD)3 at 1, 2, and 4 h p.i. in BALB/C nude mice bearing the αvβ3 integrin positive M21 cell tumor on the right flank and the αvβ3 integrin negative control tumor M21-L on the left flank, and [89Zr]TAFC at 6 h in normal BALB/c mice. Significant differences in uptake are marked with an asterisk (P = 0.05). Each data point represents an average of biodistribution data in four animals (n = 4).
oxalate following the established protocol found for $^{89}$Zr-DFO. The cyclic structure of TAFC exhibits excellent complexation ability with quantitative complexation of $^{89}$Zr from $^{89}$Zr-oxalate between 30 to 90 min. This resulted in highly acidic conditions (pH 6) may be beneficial for cancer imaging.

In vitro and in vivo studies of all three octadentate ligands showed improved stability. However, HOPO and DFO lack the pendant group for coupling it to a targeting molecule. DFO* was linked to the bombesin peptide analogue [Nle14]BBS(7-14), but was only investigated in vitro. $^{89}$Zr]TAFC and C7, both have a 36-membered ring but different coordination numbers for $^{89}$Zr. Nevertheless, transchelation found in an EDTA challenging experiment was lower for TAFC (3-5% TAFC vs 13% C7 after 7 days) despite its lower coordination number (6 vs 8).

Although further research is warranted, our study shows that the use of macrocyclic structure leads to considerably improved

affinity and imaging properties of this tracer class benefit from the so-called multimerization approach. Due to the fact that FSC includes three primary amine functions, this chelator allows conjugation of up to three ligands making them, besides working as chelator for the radiometal, an ideal scaffold for such approaches. Thus, this option additionally increases potential applications of FSC and derivatives. Anyway, it has to be pointed out that only small peptides may benefit from such a multimerization approach and not antibodies, which is the main field of application of $^{89}$Zr-labeling. Nevertheless, it has been demonstrated by using, e.g., $^{64}$Cu-NODAGA-RGD that tumor-to-background ratios further improve with prolonged imaging time making labeling of this peptide class with $^{89}$Zr not per se uninteresting.

As a proof of concept, different cyclic RGD pentapeptides were synthesized, and versatile conjugation strategies were explored. FSC(succ-RGD)$_3$ was synthesized using a succinic acid linker by amidation with a yield of 40%. In another approach c(RGDfE) was directly conjugated via the glutamic acid carboxylate to the chelator. This approach reduces the synthesis steps but also resulted in a lower yield possibly related to steric effects. In a third approach the maleimide-thiol strategy was introduced. This is a well-established technique and allows fast and mild reaction conditions in aqueous solution, facilitating the site specific conjugation of peptides and antibodies, which might be not stable under harsh conditions. Thus, the PEG-modified RGD-peptide could be coupled with even higher yield as found for the other two derivatives under mild conditions with the FSC derivative.

Biological properties of $[^{89}$Zr]FSC(succ-RGD)$_3$ were comparable to the properties of $[^{68}$Ga]FSC(succ-RGD)$_3$, which has clearly shown the advantage of multivalency through remarkable improvement of internalized activity in vitro and tumor uptake in vivo in our previous study. An exception was the lower activity concentration in blood resulting in better tumor/blood ratios for the $^{89}$Zr compound in comparison with the $^{68}$Ga-labeled analogue, indicating an excellent in vivo stability of $[^{89}$Zr]FSC(succ-RGD)$_3$. The high stability was confirmed by microPET/CT imaging using the same murine tumor model. These studies clearly demonstrated no uptake in bone and high uptake in α,β3 integrin positive M21 tumors. A reason for the superior properties of $[^{89}$Zr]FSC(succ-RGD)$_3$, compared with $[^{68}$Ga]FSC(succ-RGD)$_3$, could partially be to the difference in the charge, having been reported to potentially influence excretion patterns of such biomolecules.

The preferred coordination number for $^{89}$Zr is eight, which was confirmed recently. Certain developments aim on the design and synthesis of novel both octadentate and oxygen-rich Zr complexes with high activity concentration in blood resulting in better tumor/blood ratios. The PEG-modified RGD-peptide could be coupled with even higher yield as found for the other two derivatives under mild conditions with the FSC derivative.

However, HOPO and C7 lack the pendant group for coupling it to a targeting molecule. DFO* was linked to the bombesin peptide analogue [Nle14]BBS(7-14), but was only investigated in vitro. $^{89}$Zr]TAFC and C7, both have a 36-membered ring but different coordination numbers for $^{89}$Zr. Nevertheless, transchelation found in an EDTA challenging experiment was lower for TAFC (3-5% TAFC vs 13% C7 after 7 days) despite its lower coordination number (6 vs 8).
in vitro stability compared to DFO. A limitation of the study is that DFO cannot be used to generate a trimeric structure. Thus, in vivo comparison of both chelating systems is difficult because not only the chelator system would change but also the number of targeting moieties. Based on these changes the in vivo properties are not only correlated to the different chelators but also are influenced by the different structures. Therefore, it was decided for this proof of principle study to restrict comparison to in vitro assays where it is assumed that these differences have only minor effects. Anyway, to our knowledge this is the first report where a \( \text{\textsuperscript{89}Zr} \)-labeled macrocyclic bifunctional chelation system conjugated to a target vector is studied in vivo including biodistribution and PET imaging. Both, the in vitro and in vivo studies of \([\text{\textsuperscript{89}Zr}]\text{TAF}\) and \([\text{\textsuperscript{89}Zr}]\text{FSC(succ-RGD)}_3\) demonstrated very promising results. However, small peptides, even though an excellent model for this evaluation based on their rapid pharmacokinetics, are certainly not the most suitable application for this concept. Nevertheless, for larger targeting vectors (such as antibodies or antibody fragments), due to their slower pharmacokinetics, FSC system could be a very interesting alternative for \( \text{\textsuperscript{89}Zr} \)-labeling to currently used bifunctional chelators. At the moment, mono- and diacetylated FSC are under investigation, which may, due to the reduced free valences, better suit the application with higher molecular weight targeting vector systems.

**CONCLUSION**

Versatile conjugation strategies of FSC were explored using RGD as model peptide and quantitative labeling with \( \text{\textsuperscript{89}Zr} \) was achieved successfully. Excellent in vitro and in vivo stability of \([\text{\textsuperscript{89}Zr}]\text{TAF}\) and \([\text{\textsuperscript{89}Zr}]\text{FSC-RGD}\) conjugates were demonstrated. These results led us to conclude that FSC is a novel promising chelator for the development of \( \text{\textsuperscript{89}Zr} \)-based PET imaging agents.

**ASSOCIATED CONTENT**

*Figure S1 showing the representative chromatograms of ITLC of \( \text{\textsuperscript{89}Zr} \)-oxalate and \( \text{\textsuperscript{89}Zr} \)-TAF. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmolpharmaceut.5b00128.*

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**Notes**

The authors declare no competing financial interest.

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