Biological evaluation of an ornithine-modified ⁹⁹mTc-labeled RGD peptide as an angiogenesis imaging agent

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A B S T R A C T

Introduction: Radiolabeled RGD peptides that specifically target integrin α₅β₃ have great potential in early tumor detection through noninvasive monitoring of tumor angiogenesis. Based on previous findings of our group on radiodeptides containing positively charged aminoacids, we developed a new cyclic cRGD derivative, c(RGDfK)-(Orn)₃-CGG. This new peptide availing the polar linker (Orn)₃ and the ⁹⁹mTc-chelating moiety CGG (Cys-Gly-Gly) is appropriately designed for ⁹⁹mTc-labeling, as well as consequent conjugation onto nanoparticles.

Methods: A tumor imaging agent, c(RGDfK)-(Orn)₃-CGG-[⁹⁹mTc], is evaluated with regard to its radiochemical, radiobiological and imaging characteristics.

Results: The complex c(RGDfK)-(Orn)₃-CGG-[⁹⁹mTc] was obtained in high radiochemical yield (>98%) and was stable in vitro and ex vivo. It presented identical to the respective, fully analytically characterized ¹⁸⁵/¹⁸⁷Re complex retention time in RP-HPLC. In contrary to other RGD derivatives, we showed that the new radiopeptide exhibits kidney uptake and urine excretion due to the ornithine linker. High tumor uptake (3.87±0.48% ID/g at 60 min p.i.) was observed and was maintained relatively high even at 24 h p.i. (1.83±0.05 % ID/g), thus providing well-defined scintigraphic imaging. Accumulation in other organs was negligible. Blocking experiments indicated target specificity for integrin receptors in U87MG glioblastoma cells.

Conclusion: Due to its relatively high tumor uptake, renal elimination and negligible abdominal localization, the new ⁹⁹mTc-RGD peptide is considered promising in the field of imaging α₅β₃-positive tumors. However, the preparation of multifunctional SPECT/MRI contrast agents (RGD-conjugated nanoparticles) for dual modality imaging of integrin expressing tumors should be further investigated.
RGD peptides in the development of integrin-ανβ3-targeted radio-tracers in order to image rapidly growing and metastatic tumors in several tumor-bearing animal models [11–15]. In particular, it has been shown that cyclic (Arg-Gly-Asp-D-Phe-Lys) cRGDK presents high affinity to the ανβ3 receptor and can specifically accumulate in tumors including osteosarcomas, glioblastomas, melanomas, lung carcinomas, and breast cancer [11–15].

Over the last decade, a variety of radiolabeled cyclic RGD peptides have been evaluated as radiotracers for imaging tumors by single photon emission computed tomography (SPECT) or positron emission tomography (PET) techniques and some of them are already in clinical studies [12,16–19]. Especially, SPECT imaging is widely available and has significant imaging potential using tumor-specific radiopharmaceuticals. It should be noted that as far as diagnostic imaging is concerned, 99mTc is considered to be an ideal radionuclide and 99mTc-radiopharmaceuticals are in high demand in nuclear medicine due to their low cost, widespread availability and favorable physical and imaging characteristics (γ ray = 142 keV, half-life = 6.02 h) [20,21].

A traditional approach for radiolabeling RGD derivatives with 99mTc is via stabilization of the [TcO4]− metal core with ligands of the so-called N3S type [22–26]. Smith et al. have studied 99mTc-complexes containing a N3S chelating group [27]. Our group has also focused on the design of Bombesin (BN) derivatives in which the chelating moiety is comprised of an N3S-combination of amino acids, presenting the sequence CGG (Cys-Gly-Gly) [22,23,25]. We designed the new RGD derivative, namely c(RGDfK)-(Orn)3-CGG (Fig. 1), based on previous findings of our group on radiotracers that contain positively charged aminocids, such as ornithine and arginine in order to improve excretion kinetics and biodistribution profile [22,25]. The aim was to investigate the characteristics and the biological and imaging properties of this radiolabeled RGD compound, which contains in its moiety such a polar linker and a 99mTc-chelator.

Increased interest for RGD conjugated nanoparticles [28–30] has prompted researchers to design RGD derivatives as successful receptor integrin ανβ3 targeting moieties in SPECT/MRI dual-modality tumor imaging [31]. Nanoparticles produce multivalent effects due to multiple simultaneous interactions between the biomolecules conjugated on the nanoparticle surface and the specific cell surface receptors. Taking into consideration the advantages offered by a combined SPECT/MRI system, it is critical that multifunctional probe development is improved for future use of this bifunctional imaging modality and accessed for further investigation of c(RGDfK)-(Orn)3-CGG, after conjugation with magnetic nanoparticles. The new RGD derivative, containing the radionuclide chelating moiety CGG, is considered to be appropriately designed for both 99mTc-labeling, and conjugation with nanoparticles, via the Cys moiety.

Within the framework of the present study, the new RGD derivative, c(RGDfK)-(Orn)3-CGG, was complexed with both 99mTc and the respective 185/187Re (V) glauconate precursors. The non-radioactive rhenium complex was characterized by liquid chromatography-electrospray ionization mass spectrometry (ESI-MS). Radiochemical identification of the 99mTc-RGD derivative, metabolic stability studies, in vitro assays, in vivo evaluation in normal Swiss mice and tumor-bearing SCID mice are reported. The final goal was to evaluate the potential use of the new RGD derivative, c(RGDfK)-(Orn)3-CGG, as a tumor imaging agent in nuclear medicine, in order to head for the design and synthesis of a multifunctional SPECT/MRI contrast agent, RGD conjugated nanoparticles, for imaging of integrin expressing tumors [15,28–31]. The multimodal imaging approach could facilitate verification of the accuracy in tumor detection and provide additional information regarding the pathology of the tumor.

2. Materials and methods

2.1. General

The RGD derivative, c(RGDfK)-(Orn)3-CGG, was designed by our group and consequently obtained from Caslo Laboratory at the Technical University of Denmark. All other chemicals were of reagent grade and used without further purification. Ionization mass spectroscopy (ESI-MS) analysis was performed on a Finnigan AQA Navigator, using a Harvart syringe pump. The 185/187Re-complex was purified by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) on a Waters system (pump 600E, detector UV-484) equipped with a 10 Nucleosil 7 C18 column [250 × 12.7 mm ID; Macherey-Nagel]. Technetium-99m in the form of 99mNaTcO4 in saline, was eluted from a commercial 99Mo/99mTc generator (Drytec, GE Healthcare). The 99mTc-complexes were identified by comparative RP-HPLC analysis with their respective rhenium complexes, on a Waters 600 chromatography system coupled to both a Waters 2487 Dual λ absorbance detector and a Gabi gamma detector from Raytest. Radiochromatographic analysis was performed on a C-18 RP (25.4 cm × 2.5 cm, 5 μm porosity) column eluted with a binary gradient system at 1 mL/min flow rate. Instant thin layer chromatography (ITLC) measurements were performed by electronic autoradiography (Instant Imager Packard-Canberra). Scintigraphic images were acquired on a small mouse-sized camera manufactured by our team [32]. The detection device is based on two Position Sensitive Photomultiplier Tubes (HB5000, Hamamatsu, Japan), a parallel hole collimator and a Na(TI) pixedilated scintillator. The spatial resolution of the systems is ~1.5 mm at 0 mm distance from the collimator’s surface. The high resolution gamma camera is in ideal position for planar studies, minimizing the distance between the animal and the collimator, so that maximum resolution and sensitivity are achieved.

2.2. Conjugation and analysis of the c(RGDfK)-(Orn)3-CGG, 185/187Re(V) derivative (Nonradioactive Complex)

The Rhenium(V) complex of the RGD derivative was prepared by a ligand exchange reaction, between the Re(V) gluconate precursor and the RGD derivative according to a slightly modified literature method [33]. Briefly, an aqueous solution of crude c(RGDfK)-(Orn)3-CGG and the 185/187Re(V) glauconate precursor was allowed to react overnight at 37 °C. The crude c(RGDfK)-(Orn)3-CGG,185/187Re(V) complex was purified by semi-preparative RP-HPLC and characterized by ESI-MS and RP-HPLC analysis.

2.3. ESI-MS mass spectral analysis

The ESI-MS mass spectral analysis of the c(RGDfK)-(Orn)3-CGG and 185/187Re complexes with the peptide was performed at the "Mass Spectrometry and Dioxin Analysis Lab", NCSR “Demokritos”. A test solution in 50% aqueous ACN was infused at a flow rate of 0.1 mL/min, under a stream of hot nitrogen gas (Dominic-Hunter UHPLCMS-10) for desolvation at 170 °C. Negative or positive ion ESI-MS spectra were acquired by adjusting the needle and cone voltages.

2.4. Formation of the c(RGDfK)-(Orn)3-CGG, 99mTc(V) complex

Radiolabeling of the new RGD derivative, c(RGDfK)-(Orn)3-CGG [Fig. 1], was performed according to an already published method via the 99mTc-glucuonate precursor (sodium gluconate being used as an intermediate exchange ligand for 99mTc with stannous chloride as the reducing agent) [22]. Briefly, a solid mixture containing 1.0 g sodium gluconate (C6H12NaO6), 2.0 g sodium bicarbonate (NaHCO3) and 15 mg stannous chloride (SnCl2) was homogenized and kept dry. Then, 3.0 mg of the above mixture was homogenized and kept dry. Then, 3.0 mg of the above mixture...
Fig. 1. Radiochemical structure of c(RGDfK)-(Orn)$_3$-[CGG-$_{99m}$Tc].
Table 1
Analytical HPLC retention times (tR) for c(RGDfK)-(Orn)3-CGG and its complex.

<table>
<thead>
<tr>
<th>Compd</th>
<th>UV detector</th>
<th>Radioactivity detector</th>
</tr>
</thead>
<tbody>
<tr>
<td>c(RGDfK)·(Orn)3-CGG</td>
<td>16.6</td>
<td>17.8</td>
</tr>
<tr>
<td>c(RGDfK)·(Orn)3-[CGG-[99mTc]</td>
<td>17.7</td>
<td></td>
</tr>
</tbody>
</table>

was dissolved in 1.0 ml of a sodium pertechnetate solution (Na99mTcO4), containing 370–555 MBq of 99mTc and was left to react for 10 min at room temperature. An amount of the lyophilized RGD derivative was then dissolved in an aliquot of 500 μl of the above solution, the mixture (pH 7–8) was homogenized by vortexing, allowed to react at 37 °C for 30 min and subsequently, the radiolabeled formed peptide was purified by RP-HPLC. The effect of peptide concentration on the labeling yield was studied by using peptide solutions of different concentrations varying from 8.6×10−5 M to 8.3×10−2 M.

2.5. Radiochemical analysis

The labeling efficiency of the purified derivative was assessed up to 24 h post-labeling and determined by analytical RP-HPLC. Elution was performed with a solvent system consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in 90%CH3CN (solvent B) at a flow rate of 1 ml/min. The elution gradient was 95% A, followed by a linear gradient to 75% A for 20 min; this composition was maintained for another 5 min. After a column wash with 95% B for 5 min, the column was re-equilibrated by applying the initial conditions that is 95% A for 15 min prior to the next injection. The same system was used for the 185/187Re complex analysis. The radioactive RGD derivative was also analyzed by ITLC on Silica Gel (ITLC-SG) strips using saline (0.9% NaCl), acetone and pyridine/CH3COOH/H2O as the mobile phases.

2.6. Lipophilicity: logP values

To a mixture of 300 μl n-octanol and 300 μl phosphate-buffered saline (PBS, pH 7.4), 30 μl of purified radiolabeled RGD derivative were added and vortexed. The final mixture was centrifuged at 6000 rpm for 10 min at 4 °C. An aliquot of both saline and octanol layers was collected and the activity of each sample was measured in a γ-counter. The log P value was calculated as the log ratio of the radioactivity in the organic and aqueous phases (mean of three samples).

2.7. Cysteine challenge tests

In order to examine the in vitro stability of the new radiolabeled RGD derivative, the radiolabeled solution was challenged with cysteine. Aliquots of 100 μl of the purified radiolabeled peptide derivative were added to 900 μl of cysteine solutions in saline (10−3 M and 10−4 M). The samples were incubated at 37 °C for 2 h. At two time points (60 and 120 min), 100 μl of each sample were removed and analyzed by gradient RP-HPLC in order to determine potential transchelation effects.

2.8. Metabolic studies

2.8.1. In plasma

The metabolic stability of the radiolabeled RGD derivative was studied in human plasma ex vivo. Human blood (approximately 3 ml) was collected in heparinized polypropylene tubes and centrifuged at 5000 rpm at 4 °C for 5 min. The supernatant layer (450 μl plasma) was collected and incubated with 50 μl (1.85–3.70 MBq) of the purified radiolabeled RGD derivative at 37 °C. Fractions were withdrawn at 15, 30, 60 and 120 min, treated with ethanol (2:1 EtOH/ aliquot, v/v) and centrifuged at 14000 rpm for 30 min. Supernatants were filtered through Millex GP filters (0.22 μm) and analyzed by RP-HPLC using the conditions described for radiochemical analysis.

2.8.2. In urine

To detect metabolites excreted in urine, 100 μl (22.2 MBq) of c(RGDfK)-(Orn)3-[CGG-[99mTc] were injected via the tail vein in female Swiss Albino mice. Animals were kept at metabolic cages for 30 min and then were sacrificed by cardiac puncture under mild ether anesthesia. Urine was immediately collected from their bladder with a syringe and analyzed by ITLC and HPLC for detection of metabolites and free pertechnetate.

2.9. Cell culture

Human glioblastoma cancer cells (U87MG) were maintained in Dulbecco’s DMEM-high glucose supplemented with 10% FBS, 1% L-Glutamine, 1% penicillin/streptomycin, 1% GlutaMAX. The cells were incubated in a controlled humidified atmosphere containing 5% CO2 at 37 °C and were subcultured weekly. They were detached from the flask surface by using a trypsin/EDTA solution (0.25%).

2.10. Animal models

All animal experiments were performed in compliance with the European legislation for animal welfare. Animal protocols have been approved by the Greek Authorities. Normal Swiss and SCID mice (average weight of 20 g) were obtained from the breeding facilities of the Institute of Biology NCSR “Demokritos” and were used for biodistribution and dynamic imaging studies. SCID mice were inoculated subcutaneously with 100 μl of cell suspension (approximately 107 U87MG cells/animal) just above the left anterior leg, under sterile conditions. Tumors were allowed to grow for up to 4 weeks. All experiments were carried out in compliance with the relevant national laws relating to the conduct of animal experimentation.

2.11. Biodistribution evaluation in normal Swiss mice

Normal Swiss mice were injected via the tail vein with 100 μl (-3.70 MBq per animal) of the purified radiolabeled RGD derivative, diluted in saline (pH 7.0). Different groups of animals were sacrificed 5, 30, 60 min p.i. (post injection) by cardiac puncture under mild ether anesthesia.

Table 2
Data for c(RGDfK)-(Orn)3-CGG peptide and its 185/187Re complex by MS Analysis.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Calculated MW</th>
<th>Charged ion (m/z)</th>
<th>Theoretically expected m/z</th>
<th>Found m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>c(RGDfK)·(Orn)3-CGG</td>
<td>1163.4</td>
<td>[M + H]+</td>
<td>1164.4</td>
<td>1164.0</td>
</tr>
<tr>
<td>c(RGDfK)·(Orn)3-[CGG-[185/187Re]]</td>
<td>1362.6</td>
<td>[M + 2 H]+</td>
<td>582.7</td>
<td>582.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[M + H]+</td>
<td>1363.6</td>
<td>1363.4</td>
</tr>
</tbody>
</table>

Table 3
Stability data obtained by the cysteine challenge test. The % intact c(RGDfK)-(Orn)3-[CGG-[99mTc] in the presence of different concentrations of cysteine (10−3 M) and (10−4 M), incubated at 37 °C for 60 min and 120 min, respectively.

<table>
<thead>
<tr>
<th>% Intact c(RGDfK)-(Orn)3-[CGG-[99mTc]]</th>
<th>Time (min)</th>
<th>Cys (10−3 M)</th>
<th>Cys (10−4 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>92.3</td>
<td>80.8</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>92.7</td>
<td>62.1</td>
</tr>
</tbody>
</table>
ether anesthesia. The main organs were subsequently removed and, together with samples of muscles and urine, were weighed and counted in a NaI well counter. Stomach and intestines were not emptied before the measurements. Uptake of the radiotracer in each tissue was calculated and expressed as the percentage injected dose per gram of the tissue (%ID/g). The %ID in whole blood was estimated assuming a whole-blood volume of 6.5% of the total body weight. Biodistribution data are reported as %ID/g and are means ± SD (n = 3–5).

2.12. Biodistribution study in U87MG tumor bearing SCID mice

SCID mice bearing U87MG tumors were injected via the tail vein with 100 μl (~3.70 MBq) of the purified radiolabeled RGD derivative, diluted in saline, pH 7.0 and were sacrificed at 5, 30, 60, 120 min and 24 h p.i.. Receptor blocking studies were also carried out, as follows: A group of mice was co-injected additionally with an excess amount (100 μl, 1 mg/mL) of unlabeled RGD derivative for receptor blocking. The main organs and tissues were subsequently removed, weighed, and counted as previously described.

2.13. Dynamic imaging studies in normal and tumor bearing mice

Evaluation of the new radiolabeled RGD derivative, c(RGDfK)-(Orn)3-CGG and c(RGDfK)-(Orn)3-[CGG-185/187Re] along with the theoretically expected charged ion values are summarized in Table 2. The retention time of the 185/187Re(V)O-N3S-RGD derivative, compared to the respective radioactive complex, was similar after their co-injection into the RP-HPLC column, confirming that both 185/187Re and 99mTc form complexes of similar structure (Table 1).

3. Results

3.1. Rhenium(V) complex analysis

Chemical purity of the peptide, c(RGDfK)-(Orn)3-CGG, was checked by HPLC and was found higher than 95%. The nonradioactive complex, c(RGDfK)-(Orn)3-[CGG-185/187Re], of the crude RGD derivative was obtained via the 185/187Re-gluconate precursor. The purification and characterization of the peptide was performed by semi-preparative RP-HPLC, and analytical RP-HPLC and ESI-MS respectively (Tables 1 and 2). The ESI-MS results for c(RGDfK)-(Orn)3-CGG and c(RGDfK)-(Orn)3-[CGG-185/187Re] along with the theoretically expected charged ion values are summarized in Table 2. The retention time of the 185/187Re(V)O-N3S-RGD derivative, compared to the respective radioactive complex, was similar after their co-injection into the RP-HPLC column, confirming that both 185/187Re and 99mTc form complexes of similar structure (Table 1).

3.2. Radiolabeling and quality control

The new RGD derivate was efficiently labeled with 99mTc with a high specific activity of 5.55 GBq/μmol. HPLC analysis indicated that the purified product remained stable at a yield higher than 98% for at least 24 h post labeling when stored at room temperature. The retention time of the purified 99mTc complex is similar to that of the analogous 185/187Re complex (Table 1). The peptide could be efficiently radiolabeled at a yield higher than 95% even at low concentrations down to 8.3×10^-7 M. The lipophilicity of the radiolabeled RGD derivative was evaluated by determining the log P partition coefficient values between n-octanol and PBS at pH 7.4. Partition coefficient for the radiolabeled compound was logP(n-Oct/PBS) = -2.45 ± 0.01, a value compatible with a hydrophilic molecule [34].

3.3. In vitro stability studies

*In vitro* stability was examined by the cysteine challenge test, using two different concentrations of cysteine (10^-3 M and 10^-4 M). The purified c(RGDfK)-(Orn)3-[CGG-99mTc] remained stable, at a yield higher than 90 % up to 120 min in the presence of excess cysteine (10^-4 M) and >60 % until 120 min in the presence of an even higher concentration of cysteine (10^-3 M) (Table 3).

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**Fig. 2.** RP-HPLC radioactivity detector analysis of mouse urine, collected 30 min post injection of c(RGDfK)-(Orn)3-[CGG-99mTc].

**Fig. 3.** Biodistribution results of c(RGDfK)-(Orn)3-[CGG-99mTc] in normal mice at 5, 30, 60 min (% dose/g). Each value is the average of three to five animals.
3.4. Metabolic stability

The human plasma stability study, performed as described above, showed that c(RGDfK)-(Orn)3-[CGG-99mTc] remained stable in plasma (~90%) after a 120 min incubation at 37 °C. Specifically, almost the 30% value of radioactivity remained in the supernatant after treatment with ethanol and centrifugation. Moreover, HPLC analysis of a urine sample collected 30 min after injection of the radiopeptide in mice, indicated metabolism in the form of hydrophilic products. Injected activity was 22.2 MBq/μl and proved to be sufficient to show the metabolic profile of the radiotracer in urine by HPLC. A representative radiochromatogram of c(RGDfK)-(Orn)3-[CGG-99mTc] and its metabolites in mouse urine is presented (Fig. 2), showing that a significant portion of intact radiopeptide is still detected in the urine (tR = 17.8 min), in addition to catabolic products, which have tR = 2.0–8.5 min.

3.5. Biodistribution evaluation in normal Swiss mice

An in vivo study in physiological experimental mice was carried out and the biodistribution results are presented in Fig. 3. Rapid blood clearance was observed with blood levels of about 0.28 ± 0.07% ID/g at 60 min p.i. Low values were observed in the stomach, indicating that there is minimal, if any, reoxidation to free 99mTcO4−. In addition, liver uptake was low even at 60 min p.i. (0.77 ± 0.02% ID/g) indicating no hepatobiliary excretion of the radiopeptide. It is worth noting that even at 60 min p.i., kidney uptake was high (77.54 ± 1.68% ID/g).

3.6. Biodistribution analysis in U87MG tumor-bearing SCID mice

The results of the in vivo analysis in tumor-bearing SCID mice are presented in Table 4. The biodistribution study was performed in animals bearing experimentally-induced U87MG tumors that overexpress the αvβ3 receptors. The new radiopeptide, c(RGDfK)-(Orn)3-[CGG-99mTc], was capable of targeting the αvβ3-positive U87MG tumor at 30 min p.i. (3.32 ± 0.09% ID/g) and remained at high values at 120 min p.i. (3.39 ± 1.05% ID/g). It is worth noting that even 24 h p.i. tumor uptake was significant (1.83 ± 0.05% ID/g). Regarding the tumor-bearing SCID mice, the predominant excretion route was via the urinary system, which is in agreement with the biodistribution results in normal mice. High kidney values (59.16 ± 8.80% ID/g) were observed even at 24 h p.i. Insignificant uptake was observed in all other organs such as liver, intestines and muscles.

The tumor/blood, tumor/muscle, tumor/intestines and tumor/liver ratios at 5, 30, 60 and 120 min p.i. for the radiolabeled derivative c(RGDfK)-(Orn)3-[CGG-99mTc] are presented in Fig. 4. The tumor to background ratio was significantly high, due to high tumor uptake and low uptake in the rest of the organs. As can be observed, the tumor/blood and tumor/muscle contrast ratios are high with values of 4.39 and 10.08 at 60 min and 120 min p.i., respectively, indicating high tumor uptake and retention of the new radiopeptide.

In vivo blocking experiments, performed by co-administration of an excess amount of native RGD, resulted in a significantly reduced uptake of c(RGDfK)-(Orn)3-[CGG-99mTc] in tumor 60 min p.i.. Tumor uptake was decreased from 3.87 ± 0.48 %ID/g to 1.23 ± 0.48 %ID/g, advocating for specificity of the radiotope under study for the above-referenced experimental tumor (Fig. 5).

3.7. Dynamic imaging studies in normal and in tumor-bearing mice

Dynamic imaging of anesthetized normal mice was performed at a high resolution, dedicated small animal γ-camera, up to 65 min p.i.. Data were continuously recorded and images were stored every 10 min, providing fast and low-cost dynamic planar view of the biodistribution of the radiopeptide. It has been possible to record data in list mode format and construct images at shorter intervals. The respective images are shown in Fig. 6. The localization of c(RGDfK)-(Orn)3-[CGG-99mTc] in a U87MG tumor-bearing mouse in dynamic imaging is presented in Fig. 7. The experimental tumor at the left anterior leg was delineated with high accumulation. Regions of Interest (ROI) analysis was performed in the collected images to obtain "semi-quantitative" information at 3.5 min intervals from 5 min up to 82 min p.i., as shown in Fig. 8. Due to the high values observed in kidneys and in order to provide better visualization, the kidneys’ values are reported to the secondary y-axis on the right, while the tumor, muscle and bladder values to the primary y-axis on the left. Comparitive imaging of the new radiolabeled derivative, c(RGDfK)-(Orn)3-[CGG-99mTc], in normal mice and in U87MG tumor-bearing models, was assessed in representative planar γ-camera images in Fig. 9. The same tumor was not visualized in another SCID mouse, which had received a high dose of unlabeled RGD along with the radiolabeled RGD derivative, for receptor blocking (Fig. 9). It is worth noting that imaging data were recorded for 24 h p.i. indicating

<table>
<thead>
<tr>
<th>Organ</th>
<th>5 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>24 h</th>
<th>60 min/blocked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>7.21±0.11</td>
<td>0.91±0.11</td>
<td>0.88±0.07</td>
<td>0.79±0.13</td>
<td>0.21±0.11</td>
<td>0.24±0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>3.09±0.38</td>
<td>1.53±0.38</td>
<td>1.60±0.22</td>
<td>1.62±1.06</td>
<td>1.09±0.38</td>
<td>1.33±0.22</td>
</tr>
<tr>
<td>Heart</td>
<td>4.12±0.05</td>
<td>0.87±0.05</td>
<td>0.97±0.06</td>
<td>0.75±0.22</td>
<td>0.44±0.05</td>
<td>1.46±0.06</td>
</tr>
<tr>
<td>Kidneys</td>
<td>36.43±6.80</td>
<td>58.44±8.80</td>
<td>72.17±11.31</td>
<td>59.39±11.65</td>
<td>59.16±8.80</td>
<td>38.89±10.79</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.50±0.03</td>
<td>1.21±0.03</td>
<td>1.19±0.15</td>
<td>0.58±0.12</td>
<td>0.21±0.03</td>
<td>1.09±0.15</td>
</tr>
<tr>
<td>Intestines</td>
<td>2.23±0.21</td>
<td>0.94±0.21</td>
<td>1.10±0.30</td>
<td>0.99±0.44</td>
<td>0.91±0.21</td>
<td>2.81±0.30</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.26±0.01</td>
<td>1.57±0.01</td>
<td>1.98±0.55</td>
<td>0.82±0.35</td>
<td>1.24±0.01</td>
<td>3.64±0.55</td>
</tr>
<tr>
<td>Muscles</td>
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<td>0.38±0.06</td>
<td>0.40±0.02</td>
<td>0.34±0.15</td>
<td>0.31±0.06</td>
<td>0.80±0.02</td>
</tr>
<tr>
<td>Lungs</td>
<td>7.60±0.27</td>
<td>1.99±0.27</td>
<td>2.27±0.16</td>
<td>1.29±0.48</td>
<td>0.62±0.27</td>
<td>4.18±0.16</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2.09±0.09</td>
<td>0.55±0.09</td>
<td>0.44±0.12</td>
<td>0.48±0.16</td>
<td>0.33±0.09</td>
<td>3.10±0.12</td>
</tr>
<tr>
<td>Tumor</td>
<td>4.00±0.05</td>
<td>3.32±0.09</td>
<td>3.87±0.48</td>
<td>3.39±1.05</td>
<td>1.83±0.05</td>
<td>1.23±0.48</td>
</tr>
</tbody>
</table>
significant retention into the experimental U87MG tumor overexpressing the ανβ3 integrin receptors (Fig. 10). Planar imaging study is in agreement with biodistribution results.

4. Discussion

Integrin ανβ3 is overexpressed on the surface of activated endothelial cells as well as cancer cells during neovascularization induced by carcinogenesis. Radiolabeled peptides based on the Arg-Gly-Asp (RGD) sequence have been reported as radiopharmaceuticals with high affinity and selectivity for the ανβ3 integrin and are therefore useful in the noninvasive monitoring of tumor angiogenesis by molecular imaging techniques. For the past decade, many RGD peptides and non-peptide RGD mimetics have been labeled with different radioisotopes to develop angiogenesis-targeting radiocompounds for both diagnosis (125I, 99mTc, 111In, 18F, and 64Cu) and tumor-specific radiotherapy (90Y and 177Lu), some of them being already introduced in clinical trials [12,16–19,34–41]. In general, the radiolabeled RGD peptide radiotracer of clinical importance should have high affinity and high specificity for integrin ανβ3, a good elimination profile and no toxicity. The pharmacokinetic requirement for an optimal radiotracer depends largely on its medical applications (diagnostic or therapeutic). For therapeutic radiopharmaceuticals, high uptake in kidneys and prolonged kidney retention of radiotracers imposes a serious challenge for radiolabeled cyclic RGD peptides [39,42]. In the last several years, a variety of pharmacokinetic modifying linkers have been used to improve excretion kinetics of radiolabeled RGD peptides. It has been reported that introduction of the polyethylene glycol (PEG) linker can improve not only excretion kinetics but also tumor uptake of 125I- and 18F-labeled c(RGDyK) and 64Cu-labeled E[c(RGDyK)]2 [36,43,44]. We designed a new RGD derivative, as a hydrophilic molecule, namely c(RGDfK)-(Orn)3-CGG (Fig. 1), based on previous findings of our group on radiopeptides containing non-natural aminoacids, such as ornithine and arginine [22,25]. To the best of our knowledge, there are no other studies that have evaluated the radiolabeled cyclic RGDfK sequence conjugated to an N3S chelator via a spacer containing the triaminoacid sequence of ornithine. The intention was to obtain a radiolabeled RGD derivative with main excretion route, the renal system, and consequently reducing the upper abdominal area radioactivity [17,19,45–47]. In the present study, the new radiopeptide, c(RGDfK)-(Orn)3-[CGG-99mTc] showed high and stable 99mTc-labeling, high tumor uptake, and rapid blood clearance, while the main route of excretion was found to be the renal system. Moreover, the new RGD derivative,
containing the radionuclide chelating moiety CGG, is considered to be appropriately designed for both 99mTc-labeling and conjugation with nanoparticles.

In the present study, the 99mTc-labeled peptide was HPLC-purified in order to be sufficient pure for further in vitro and in vivo investigation. HPLC analysis indicated that the purified product remained stable at a yield higher than 98% for at least 24 h after labeling when stored at room temperature. The above results were consistent with previous studies, which demonstrated that N3S-type ligand systems such as CGG form stable 99mTc-peptide conjugates [22,23,25,48]. According to the literature, the addition of a positively charged spacer chain did not seem to affect the labeling efficiency of the CGG chelator [25]. The claim that c(RGDKI)-(Orn)3-[CGG-185/187Re] and c(RGDKI)-(Orn)3-[CGG-99mTc] derivatives have the same structure was also verified by their similar Rt after co-injection on a RP-HPLC column (Table 1).

Challenge stability studies showed that the radiopeptide remained stable when incubated with excess of a strong competitor for the 99mTc-core such as Cys. A worth noticing degree of transmetalation occurred only when the radiopeptide was incubated with a high Cys.

![Fig. 7. Gamma-ray dynamic images of U87MG tumor-bearing mice, administered with 3.70 MBq of c(RGDfK)-(Orn)3-[CGG-99mTc]. Images were stored 3.5 min per frame 5 min up to 82 min p.i.. Mouse was anesthetized by the subcutaneous injection of a proper anesthetizing solution at a dose of 300 μl per 30 g body weight.](image)

![Fig. 8. Time activity curves showing the percentage of radioactivity in major organs, using 3.5 min frames, from 5 to 82 min post injection in the U87MG tumor-bearing mice shown in Figure 7. The kidney values correspond to the secondary y-axis on the right, while the tumor, muscle and bladder values to the primary axis on the left. Concentration in tumor is ~4.5 times higher than concentration in normal tissue.](image)
concentration ($10^{-3}$ M) for 2 hours. Metabolic stability studies, which ran in parallel, interestingly showed that metabolites detected in human plasma and urine did not include free $^{99m}$TcO$_4^-$ ($t_{1/2} = 3.5$ min) expected to be released in the case of metal chelate degradation, which is consistent with previous studies reporting the high in vivo stability of CGG chelates [22,23,25]. This finding was additionally verified by ITLC tests, running in parallel.

It is worthwhile noting that predominant excretion route of radioactivity for c(RGDfK)-(Orn)$_3$-[CGG-$^{99m}$Tc] took place via the kidneys to the urine as indicated by the obtained kidney values ($58.44\pm8.80\%$ ID/g) at 30 min p.i.. High kidney values may be attributed to the high overall positive charge of the peptide. The overall charge of the radiometal–chelator–spacer conjugate affects the hydrophilicity of the molecule, especially when we refer to peptides with similar spacer moieties[22,25]. Lipophilicity of the radiolabeled RGD derivative was evaluated by determining the log $P$ partition coefficient values between $n$-octanol to PBS, with partition coefficient $\log P_{(n\text{-octanol/PBS})} = -2.45\pm0.01$, a value compatible with a hydrophilic molecule. Other RGD derivatives with similar lipophilicity, such as $^{99m}$Tc-PGC-c(RGDyK) showed hepatobiliary excretion and high intestinal uptake ($22.16\pm5.66\%$ ID/g) at 120 min p.i., while the new RGD derivative, c(RGDfK)-(Orn)$_3$-[CGG-$^{99m}$Tc], exhibited urine excretion and high kidney uptake ($59.39\pm11.65\%$ ID/g) at 120 min p.i. [34]. This is due to the ornithine linker and is in agreement with previous work from our group with BN peptides bearing (Orn)$_3$ linker [22,23,25].

Several groups have developed radiolabeled RGD compounds that have shown promise in terms of tumor uptake and tumor-to-background ratios [34–40]. Tumor uptake of c(RGDfK)-(Orn)$_3$-[CGG-$^{99m}$Tc] was relatively high at 30 min p.i. ($3.32\pm0.09\%$ ID/g) and remained at high values at 120 min p.i. ($3.39\pm1.05\%$ ID/g). Even though a direct comparison among radiolabeled RGD derivatives was not appropriate since a wide variety of linkers and chelating groups for $^{99m}$Tc were used, we can note that tumor uptake of c(RGDfK)-(Orn)$_3$-[CGG-$^{99m}$Tc] is relative high in comparison to other RGD monomers with tumor uptake ($2.8\pm1.5\%$ ID/g for $^{99m}$Tc-His-cRGDfK and $1.38\pm0.30\%$ ID/g for $^{99m}$Tc-PGC-c(RGDyK) at 30 min p.i. and 120 min p.i. respectively) [15,34]. It is worth noting that tumor accumulation of c(RGDfK)-(Orn)$_3$-[CGG-$^{99m}$Tc] was significantly high ($1.83\pm0.05\%$ ID/g) at 24 h p.i. compared to other $^{99m}$Tc-labeled RGD peptides [34]. The tumor to background ratio is significantly high, due to high tumor uptake and also to low uptake in the rest of the organs allowing clear nuclear imaging (Fig. 4). As can be observed, the tumor/blood and tumor/muscle ratios are high with values 4.39 and 10.08 at 60 min and 120 min p.i., respectively, indicating high tumor uptake and retention of c(RGDfK)-(Orn)$_3$-[CGG-$^{99m}$Tc]. The addition of the charged spacer in the structure was advantageous for biodistribution and tumor targeting ability, because it reduced the upper abdominal radioactivity levels observed elsewhere [17,19,34] and increased tumor/muscle tissue contrast ratio. High kidney uptake observed at 30 min p.i. remained stable ($59.16\pm8.80\%$ ID/g) even at 24 h p.i.. This can be attributed to kidney tubular reabsorption, which is a process that can influence the residence time of radiolabeled peptides into the kidneys [48,49] and can be manipulated by making use of the electrostatic interaction between the negatively charged surface of the proximal tubular cells and the cationic portion of peptides. Accumulation in other organs...
such as muscle and spleen was insignificant. It is worth examining the pharmacokinetic influence of a hydrophilic RGD derivative, c(RGDfK)-(Orn)3-[CGG-\(^{99m}\text{Tc}\)] in the tumor, while tumor uptake was reduced to 1.23 ± 0.48 %ID/g in the blockage group, compared to 3.87 ± 0.48 %ID/g in the non-blocked group at 60 min p.i. (Fig. 5). Therefore, blocking experiments showed that target specificity could be obtained for integrin receptors in U87MG glioblastoma cells. It is worth noting that high kidney uptake (72.17 ± 11.31 %ID/g) was decreased (38.89 ± 10.79 %ID/g) after receptor blocking. According to the literature, the receptor mediated endocytosis and the peptide reabsorption could be prevented by blocking the receptor by the administration of positively charged amino acids [52,53]. High kidney accumulation of the radiolabeled peptide, c(RGDfK)-(Orn)-(CGG-\(^{99m}\text{Tc}\)] is under investigation and may be overcome by co-administration of gelofusine or hyaluronic acid.

The biodistribution and the dynamic \(\gamma\)-camera imaging studies in normal and U87MG tumor-bearing SCID mice have shown significant early tumor uptake, combined with fast blood clearance and renal elimination. The predominant excretion route for the tumor-bearing SCI mice was also via the kidneys to the urine, in agreement with the biodistribution results in normal mice. Insignificant uptake was observed in all other organs such as liver, intestines and muscles. The images also showed high background activity in the kidneys and bladder, verifying that the predominant excretion route of radioactivity is via the urinary tract, in full accordance to the biodistribution data. Dynamic imaging study in U87MG tumor-bearing mouse showed accumulation to the tumor 5 min p.i., in clear contrast to the adjacent background. The tumor remained clearly observable up to 82 min p.i. (Fig. 7). Since in planar imaging organs overlap, ROIs were drawn only on the tumor, kidneys, bladder and muscle (left foot) (Fig. 8). These data provide radioactivity concentration per organ and are complementary to the biodistribution results presented above. Concentration in kidneys is slowly increased from 31.5% to 39%, while an almost stable concentration in bladder (-4.5%) is observed. Tumor concentration presented a slow decrease from ~7% to 5.9%, while muscle values ranges from ~1.7% to ~1.3%. The tumor to muscle ratio has an average value of ~4.5, which is in good agreement with the value of 4.39 reported in biodistribution studies 1 h p.i. Finally, ROI analysis also shows a slow increase in tumor to muscle ratio. Comparative imaging of c(RGDfK)-(Orn)3-[CGG-\(^{99m}\text{Tc}\)] in normal mice and U87MG tumor-bearing mice was represented in representative planar \(\gamma\)-camera images (Fig. 9). It is worth noting that the U87MG tumor is delineated with clear contrast, while the other organ radioactivity uptake is the same in both normal mice and experimental tumor models. In contrast, the same tumor was not visualized in another SCID mouse, which had received a high dose of unlabeled RGD along with the radiolabeled RGD derivative, for receptor blocking. Moreover, it is worth noting that imaging data recorded for 24 h p.i., indicating significant uptake into the experimental U87MG tumor over-expressing the \(\alpha\_v\beta\_3\) integrin receptors (Fig. 10).

Planar imaging study is in agreement with biodistribution results.

5. Conclusion

We designed a new RGD derivative, c(RGDfK)-(Orn))3-[CGG-\(^{99m}\text{Tc}\)]. Labeling with \(^{99m}\text{Tc}\) led to a radioactive compound which remained stable up to 24 h post-labeling. Administration of the \(^{99m}\text{Tc}\)-RGD derivative in tumor-bearing mice resulted in scintigraphic detection of experimentally induced tumors. The primary route of clearance was the renal system. Tumor uptake remained high and it is worth noting that even 24 h p.i., tumor uptake values were significant. Low tumor uptake was obtained in the receptor blocking study, indicating high tumor specificity of the radiopeptide. The final aim was to evaluate the potential use of the new derivative as a tumor imaging agent in nuclear medicine, as well as to apply it further in nanoparticle \(^{99m}\text{Tc}\)-labeling. Taking into consideration the advantages offered by a combined SPECT/MRI system, it is critical that multifunctional probe development is improved for the future use of this bifunctional imaging modality and accessed for further investigation of the RGD derivative, c(RGDfK)-(Orn)3-[CGG-\(^{99m}\text{Tc}\)], after conjugation with magnetic nanoparticles. Investigation of the functionalized magnetic nanoparticles with c(RGDfK)-(Orn)3-[CGG-\(^{99m}\text{Tc}\) peptide is currently in progress.

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