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Chemical and biological evaluations of an ^{111}In-labeled RGD-peptide targeting integrin Alpha(V) Beta(3) in a preclinical tumor model.

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ABSTRACT

Angiogenesis plays a central role in tumor growth and metastasis. Quantification or evaluation of angiogenesis is crucial for anti-angiogenic therapeutic strategies. Since integrin $\alpha_v\beta_3$ over-expression appears specific of angiogenesis at the adult stage, it became a target of choice over the past decade and labeled RGD-based compounds therefore constitute promising agents for non-invasive tumor visualization and targeting. We evaluated the chemical and biological properties of a new tetrameric RGD-based tracer named RAFT-RGD. RAFT-RGD was radiolabeled with indium-111 using the chelating agent [(1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid] (DOTA). Labeling reaction parameters such as time, temperature, solvent or molar ratio were investigated in order to optimize the final properties of the labeled RGD peptide. A 97.7±0.7% binding efficiency was achieved. ^{111}In-DOTA-RAFT-RGD was injected intravenously in a cohort of $\alpha_v\beta_3$-positive tumors-bearing nude mice. We visualized non-invasively the *in vivo* distribution of the tracer using a small animal gamma camera. *In vivo* distribution and stability were also studied after organs removal. *In vivo*, the radiolabeled peptide showed rapid blood clearance and tumor uptake. Whole body non-invasive planar imaging allowed tumor visualization from 1 hour post-injection. However, renal uptake must be reduced to increase the therapeutic potential of RAFT-RGD.

Key words: cancer, molecular imaging, radiopharmaceuticals

Introduction

Angiogenesis is the process of generating new capillaries out of pre-existing blood vessels. This is fundamental to many physiologic and pathologic processes, such as tumor progression and metastasis formation.[1,2] Angiogenesis-based approaches for cancer treatment have focused on the development of agents that could inhibit angiogenesis, tumor growth and metastasis formation without affecting pre-existing blood vessels.[3] Because of its particularly limited expression pattern and its exceptionally important role in angiogenesis, the $\alpha_v\beta_3$ integrin is a promising target in recently investigated anticancer strategies.[4] In this context, the development of a non-invasive method to quantify $\alpha_v\beta_3$ is crucial.[5,6] Radiotracers targeting $\alpha_v\beta_3$, such as radiolabeled RGD-based peptides, are attractive agents for nuclear medicine imaging as well as therapy. In the past decade, several radiolabeled $\alpha_v\beta_3$ ligands have been developed in this objective.[7-10]

The $\alpha_v\beta_3$ integrin mediates the attachment of endothelial cells to submatrix proteins, especially to vitronectin, a component of the basal capillaries membrane.[11] $\alpha_v\beta_3$ integrin is not or weakly expressed on quiescent endothelial cells, but they are significantly over-expressed on activated endothelial cells.[11,12] Furthermore, tumoral cells, as well as metastatic tumor cells, can express various levels of $\alpha_v\beta_3$.[5,13] As integrin $\alpha_v\beta_3$ expression is a major determinant of the angiogenic phenomenon that is responsible for tumor aggressiveness[13,14], the molecular imaging of angiogenesis through $\alpha_v\beta_3$ integrin detection is of great clinical importance. The importance of $\alpha_v\beta_3$ in tumor angiogenesis is also demonstrated by the fact that $\alpha_v\beta_3$ antagonists, including cyclic RGD-peptides, were successfully used to inhibit blood vessel development and tumor growth in different models.[15,16] It is noteworthy that $\alpha_v\beta_3$ antagonists have very little effect on pre-existing blood vessels, indicating the usefulness of targeting this molecule for therapeutic benefit with limited adverse side effects.

We developed a new $\alpha_v\beta_3$ ligand: RAFT-RGD.[17] RAFT (Regioselectively Addressable Functionalized Template) is a cyclo-decapeptide scaffold with 2 faces: 4 cyclo-[-RGDfK-] were grafted on the addressable face, in order to specifically target $\alpha_v\beta_3$. On the functionalized side, drugs, isotopes or contrast agents could be grafted. RAFT-RGD has previously been described to bind specifically to $\alpha_v\beta_3$ *in vitro* and *in vivo*.[18-20]

The aims of the current study were 1) to develop an indium-111 (^{111}In)-labeled derivative of RAFT-RGD and to evaluate its potential as an angiogenesis and $\alpha_v\beta_3$-expressing tumor imaging agent *in vivo*; 2) to determine the biodistribution of this molecule and 3) to determine whether RAFT-RGD could be used for therapeutic purposes or not following the replacement of ^{111}In by a β^--emitter isotope such as Ytrium-90 (^{90}Y) or Lutetium-177 (^{177}Lu). To radiolabel RAFT-RGD with ^{111}In, a 1,4,7,10-tetraazacyclododecane-N tetra acetic acid (DOTA) group was added to the peptide to allow the formation of an ^{111}In-DOTA-RAFT-RGD complex. A tumor-bearing mice model was used to study the biodistribution of the tracer and for non-invasive nuclear imaging of tumor angiogenesis.

Materials and methods

Chemicals and reagents

DOTA-RAFT(cyclo[-RGDfK-])$_4$ (DOTA-RAFT-RGD) was used as ligand for integrin $\alpha_v\beta_3$ and was obtained using a previously described procedure.[21] After synthesis, peptides were analyzed by Reverse Phase - High Performance Liquid Chromatography (RP-HPLC), and purified on a preparative column when necessary. The DOTA-RAFT-RGD molecular weight, without TFA, is 4470.9 g.mol^{-1}.

Radiolabeling

An aliquot of [^{111}In]$InCl_3$ (Mallinckrodt Medical, Petten, Netherland) was added to a solution of DOTA-RAFT-RGD in ammonium acetate buffer. The pH of the reaction mixture was adjusted to 4.8 using HCl. Several labeling parameters were investigated. The reaction was performed by incubating DOTA-RAFT-RGD (concentration range: 10^{-6} M to 10^{-4} M) with [^{111}In]$InCl_3$ in ammonium acetate buffer (0.01 M to 1 M) at pH 4.8 for 10 to 50 minutes. Different peptide / indium molecular ratios were used. Temperature varied from 70 to 90°C. For the optimized reaction, 55 MBq [^{111}In]$InCl_3$ were added to 100 µg of DOTA-RAFT-RGD in an ammonium acetate buffer (pH 4.8), heated at 70°C for 30 minutes. The structure of the ^{111}In-DOTA-RAFT-RGD is described in figure 1. Radiolabeling control was performed by HPLC and Thin-Layer Chromatography (TLC).

Labeling efficiency

^{111}In-DOTA-RAFT-RGD labeling efficiency was determined using HPLC and TLC. For HPLC analysis, a Merck L-6200 pump and a Lambda-Max, 481 LC-spectrophotometer UV-Vis detector systems were used. Analytical RP-HPLC analysis was performed with a

LICHROSORB, RP-C18 (5 µm, 4.6 x 250 mm) column using a gradient H_2O / trifluroacetic acid 0.1% (A), acetonitrile 90% / trifluroacetic acid 0.1% (B), 0-5 minutes 20% B, 5-25 minutes from 20% to 80% B, 25-30 minutes 80% B. Elution was performed at 0.7 ml/min. The eluted radioactivity was monitored using a NaI probe (auto-gamma counting system, COBRA™ II). Collected fractions were also measured by a gamma counter. For TLC analysis, ITLC™-SG (Pall Life Sciences, Michigan, USA, silica gel impregnated glass fibber sheets) was used.

^{111}In-DOTA-RAFT-RGD stability

In vitro stability of the compound was evaluated in human serum. Thirty-seven (37) MBq of ^{111}In-DOTA-RAFT-RGD were incubated up to 24 hours with 0.8 ml (40 kBq/µL) of human serum at 37°C under shaking conditions. At different time points (30 minutes, 1, 2, 4, 6 and 24 hours), a 100 µL aliquot of the incubation mixture was centrifuged and filtered through a 10 Komega™ membrane (Nanosep®, Pall Life Science, New York, NY) at 14,000 g for 20 minutes. The filtered solutions were analysed by HPLC to determine the percentage of unmodified tracer and its potential metabolites. Electrophoretic analysis was performed to measure the binding of the tracer to serum proteins.

Cell lines and culture conditions

Murine mammary carcinoma cells (TS/A-pc, $\alpha_v\beta_3$ positive) from Balb/c mice were generously provided by JL Coll (INSERM, U823, University of Grenoble, France) and were cultured in RPMI 1640 medium supplemented with 10% FCS, L-Glutamine (200 mmol/L), penicillin (10,000 UI/ml), and streptomycin (10,000 µg/ml). Cells were maintained at 37°C in a 95% air / 5% CO_2 atmosphere. When culture reached approximately 90% confluency, medium was removed and replaced by FCS-free medium. Cells were then scrapped, counted

using a Malassez cell and centrifuged (5 minutes at 200 g). Cells were resuspended in sterile NaCl 0.9% for extemporaneous administration to the animals.

Animals

A total of seventeen animals were used for the present study. Six weeks-old female Balb/c mice were obtained from Charles River Laboratories (St Germain sur l'Arbresle, France). The animals were housed for one week prior to tumoral cell administration with free access to water and standard rodent food. All the experiments described in the present study were approved by the Animal Care and Use Committee of the Centre de Recherche et Service de Santé des Armées (CRSSA, La Tronche, France, Authorization # 2004/25.0) and the experiments were performed by an authorized individual (L. Sancey, authorization # 38 05 32).

Nuclear imaging and tracer biodistribution

10^6 TS/A-pc cells (in 50 to 100 µL PBS) were injected subcutaneously in the right hind leg of 8 Balb/c mice. Once tumors reached approximately 300 mm^3, the animals were anaesthetized by intraperitoneal injection of a ketamine (10% wt/vol - 75 mg/kg) / xylazine (2% g/vol - 15 mg/kg) (1:1) mix.

4 micrograms of ^{111}In-DOTA-RAFT-RGD were injected (3.7 MBq in 50 µL normal saline buffer) intravenously in the lateral tail vein of the mice. 1, 4, 24 and 48 hours following tracer injection, animals were placed under the collimator of a clinical gamma camera (SoftCam, DSX rectangular) and a 15-minutes whole-body planar image acquisition was performed. Animals were then euthanized and samples from the heart, lung, liver, spleen, kidney, brain, bones, skeletal muscle, intestine, stomach, blood and tumor were collected and weighed. Radioactivity was then counted using a gamma counter.

In vivo metabolic stability studies

To determine whether ^{111}In-DOTA-RAFT-RGD was metabolized *in vivo*, kidney, liver, blood and urine were collected at 2, 4 and 24 h following the injection of 2 MBq of tracer (n = 3 per time point, except 24h: n = 2). Blood was immediately centrifuged at 800 g for 15 minutes. Plasma was then filtered through a Komega™ membrane filter (Nanosep®, Pall Life Science, USA) and analyzed by RP-HPLC. Urine (100 µl) was directly analysed by RP-HPLC. For liver and kidney, the tissues were mixed in phosphate buffer (pH = 7.4). Homogenates were centrifuged for 5 minutes at 5,000 g. Supernatants were then filtered through a Nanosep® membrane and analyzed by RP-HPLC.

Results

Radiolabeling and quality control

RP-HPLC analysis of ^{111}In-DOTA-RAFT-RGD indicated the presence of a single major radioactive compound (Fig. 1B). The product was obtained at 25 minutes with a radiochemical purity of more than 97%. Non-bound indium (< 4%) was obtained at 5 minutes. More than 90% of injected radioactivity were recovered from the column in analysis. Radiolabeling efficiency of ^{111}In-DOTA-RAFT-RGD was also determined by TLC using silica gel impregnated glass fibber sheets as the solid phase and a mixture of ammonium acetate (NH_4Ac) 10%: methanol (80:20) as the mobile phase (see Table 1). In these conditions, non-bound indium migrated at the solvent front (R_f 0.7 - 1.0) whereas ^{111}In-DOTA-RAFT-RGD remained at the origin (R_f 0). Radiochemical purity was higher than 97%.

Optimization of indium-111 radiolabeling efficiency.

Radiolabeling efficiency in various conditions is shown in figure 2. The most efficient labeling was obtained between 30 and 40 minutes at 70°C and between 20 and 30 minutes at 90°C (> 97%) (Fig. 2A). Longer incubation time leads to a much lower labeling efficiency (10 to 15%). Because high temperature could damage the peptide, radiolabeling was performed at 70°C.

Radiolabeling efficiency was also influenced by ammonium acetate (NH_4Ac) buffer molarity. A NH_4Ac concentration range of 0.01 to 1 M was used to dilute DOTA-RAFT-RGD. The best efficiency was obtained with a concentration of 0.1 M (Fig. 2B).

In order to obtain the highest binding efficiency and optimal stability, we also used different DOTA-RAFT-RGD / indium-111 molecular ratios (Fig. 2C). The best radiolabeling

efficiencies were obtained with a DOTA-RAFT-RGD / indium-111 ratio of at least 500:1. For an optimal reaction, radiolabeling was performed using a ratio of 1000:1.

Finally, the labeling efficiency decreased as the peptide concentration was reduced. DOTA-RAFT-RGD could be labeled at a high yield (> 97%) at 7.10^{-5} M at least (see Table 2).

To summarize, using optimized conditions (NH_4Ac 0.1 M, heating time 30 min at 70°C), 30 µg (~ 6.5 nmol) of DOTA-RAFT-RGD could be labeled with 11.1 MBq of indium-111. Under the same conditions, 100 µg and 200 µg of peptide could be labeled with respectively 55.5 MBq and 135 MBq of indium-111 with a high efficiency (up to 97%).

Stability of ^{111}In-DOTA-RAFT-RGD in human serum

^{111}In-DOTA-RAFT-RGD was incubated in human serum and HPLC was performed at various time points (30 min to 24 hours) to study the stability of the compound (See Table 3). The HPLC radiochromatogram showed a single, major activity peak with an identical shape and retention time as the control peak of the radiolabeled peptide in the absence of serum. ^{111}In-DOTA-RAFT-RGD remained stable in human serum at 95.6% at 24 hours. Only 2.5% of non-bound indium-111 was detected in human serum at 24 hours, indicating a weak exchange reaction with plasma proteins and a high stability of the labeled complex.

***In vivo* biodistribution and imaging studies of ^{111}In-DOTA-RAFT-RGD**

^{111}In-DOTA-RAFT-RGD biodistributions in TS/A-pc tumor-bearing mice are presented in Table 4. Rapid clearance of the radioactivity from the blood circulation was observed. The kidneys represented the main route for tracer elimination. Furthermore, low tracer activity was observed in the skeletal muscle. Thus, tumor-to-blood (T/B) and tumor-to-muscle (T/M) ratios reached rapidly values above 1. Both ratios indicated that imaging contrast should be better at 24 or 48 hours after systemic injection. One hour after tracer injection, ^{111}In-DOTA-

RAFT-RGD uptake in TS/A-pc tumors was 1.63 ± 0.67% ID/g. Tumoral uptake decreased with time but stabilized between 4 and 24 hrs and remained at approximately 0.7% ID/g between 24 and 48 hours.

Non-invasive planar whole-body imaging confirmed those results. Scintigraphic images allowed the visualisation of all tumors between 1 and 48 hours. A representative image obtained at 24 hours is shown in Figure 3. As expected from the biodistribution results, high accumulation of the tracer was observed in the kidneys. Biodistribution studies showed that kidney accumulation was persistent with more than 30% ID/g at 48 hours after injection.

Stability of ^{111}In-DOTA-RAFT-RGD *in vivo*

The stability of ^{111}In-DOTA-RAFT-RGD was determined in mouse blood, liver and kidney extracts at 1, 4 and 24 hours. For all tissues, extraction efficiency was between 77% and 99% (See Table 5). More than 90% of injected radioactivity was recovered from the purification column. Analysis of urine and liver extracts showed two peaks at 5 and 25 minutes which corresponded respectively to non-bound indium-111 and ^{111}In-DOTA-RAFT-RGD. Urine and liver showed radiochemical purity for ^{111}In-DOTA-RAFT-RGD of respectively 90 and 85% at 4 hours. In the kidney, only 54.3% of radioactivity corresponded to the complete labeled tracer and about 25% of non-bound indium-111 was recovered. A third peak representing 11 % of the radioactivity was found at 7 minutes. HPLC demonstrated that the elution profile of this unidentified compound was the same as ^{111}In-DOTA we used as control (Data not shown). Finally, blood radioactivity was undetectable 1 hour and hours after the tracer injection.

Discussion

From the past decade, RGD-based tracers targeting tumoral angiogenesis represent an intensive field of experimental and clinical research. Some of these tracers are currently used in Nuclear Medicine departments where they provide invaluable information about tumor characterization and constitute a new tool for the assessment of treatment efficacy. Alternatively, anti-angiogenic compounds are also under development. For instance, Cilengitide, a high affinity cyclic RGD-based inhibitor of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, is currently in phase II clinical trial. The anti-VEGF-A compound Avastin has been approved for the treatment of advanced colorectal cancer, non-small cell lung cancers, breast and kidney cancers.[22] We contributed to the development of a multivalent RGD-tracer, RAFT-RGD, a synthetic peptide particularly efficient for the delivery of an imaging agent, a drug, or both.[18-21,23] We previously demonstrated that RAFT-RGD is highly specific of integrin $\alpha_v\beta_3$. Here, we focused on indium-111 labeling using a DOTA group with two objectives: (1) to develop and validate ^{111}In-DOTA-RAFT-RGD as a new tracer of tumoral $\alpha_v\beta_3$ expression and (2) to determine its biodistribution, with the perspective of a future labeling with ^{90}Y or ^{177}Lu for radiotherapy purposes.

Other investigators previously demonstrated that RGD-tracers could accumulate in $\alpha_v\beta_3$-positive tumor-bearing animals. Haubner *et al.* labeled a cyclo-RGD (c(-RGDyK-)) peptide with iodine-125 for tumor $\alpha_v\beta_3$ targeting.[24] This tracer was mainly cleared by the hepatobiliary route. Since then, many RGD-based tracers, from monomers to multimers, were developed for tumor $\alpha_v\beta_3$ imaging and therapy.[8,14,20,25-28]

Among other isotopes, indium-111, associated with different chelating agents, has been investigated for tumor angiogenesis imaging and for radionuclide therapy studies.[10,14,25,29] The macrocyclic chelating agent DOTA has become a subject of widespread interest because it forms extremely stable complexes with a variety of metals. DOTA is the most stable chelator

of ^{90}Y for clinical application [29,30] and is an attractive candidate for conjugation of radiometals such as ^{111}In. The radiolabeling reaction parameters influenced the reaction equilibrium and therefore the formation and stability of the complex. It has been shown by several investigators that peptide to radioisotope ratio had to be carefully controlled for optimal binding and stability.[31,32] Important factors were temperature, incubation time, solvent, DOTA-RAFT-RGD / indium ratio and concentration of the different components. The reaction was thus optimized to obtain the highest efficiency. Heating time was kept as short and temperature as low as possible to prevent peptide degradation. In our conditions, heating 30 minutes at 70°C was sufficient to obtain an excellent radiolabeling efficiency. Importantly, ammonium acetate buffer molarity must be set at 0.1 M to obtain the best results. Finally, the peptide concentration and the peptide/indium-111 ratio have to be carefully controlled. Indeed, a lower amount of DOTA-RAFT-RGD was associated with a poor labeling efficiency. Furthermore, a lower peptide/indium-111 ratio would lead to an excess of non-bound indium and would partially inhibit the reaction. It has been shown that non-bound indium binds to the surface of glass vials and containers or react with other unwanted compounds, thus leading to a large loss of radioactivity.[33] On the contrary, a higher ratio might affect the properties of long-term stability of the radiolabeled compound and would increase the volume of ^{111}In-DOTA-RAFT-RGD solution to inject per animal. In this study, peptide/indium-111 ratio ranged from 10 to 10000. A ratio of at least 500 to 1 was required to obtain an optimal radiolabeling efficiency. For each reaction, radiolabeling was controlled by RP-HPLC and TLC analyses. The final radiochemical purity was above 97%.

Before being injected into mice, the stability of the tracer was studied in human serum and controlled by HPLC. A 24 hours incubation in serum confirmed the stability of the compound with a purity above 95%. With such stability, we hypothesized that the tracer would be bioavailable *in vivo*. *In vivo* experiments confirmed these preliminary *in vitro* results. Blood

activity decreased rapidly and was undetectable 4 hours after tracer injection. The low binding of the tracer to plasma proteins was reflected by a fast elimination rate of the ^{111}In-DOTA-RAFT-RGD radioactivity from the blood.

Skeletal muscle uptake was weak with less than 0.7% ID/g 1 hour after injection. However, tumoral uptake was still above 1% ID/g 4 hours after injection. These uptake values were weaker than those obtained with ^{99m}Tc-RAFT-RGD (2.77 ± 0.8% ID/g at 1 hr).[20] But unlike the ^{99m}Tc labeled tracer, ^{111}In labeled RAFT-RGD showed rapid blood clearance and low muscle uptake allowing T/B and T/M ratios suitable for nuclear imaging. This is in accordance with the literature where weak muscle uptake has been described for similar compounds.[14] Dijkgraaf *et al.* developed several indium-111 labeled RGD-tracers.[25,34,35] They obtained a tumor uptake of approximately 4% ID/g 2 hours after injecting 3 µg of ^{111}In-DOTA-RGD-tracer (^{111}In-DOTA-E-c(RGDfK)). In the present study, tumors were easily visualised using non-invasive whole body nuclear imaging. The images confirmed that the tracer was rapidly eliminated through the kidneys, in accordance with the rapid blood clearance. However, renal uptake remained significant after 24 hours. The renal uptake of radiolabeled peptides has long been described and reduces the sensitivity for detection around the kidneys.[34,36] Here, the radiolabeled species found in the kidney after extraction were the initial compound (< 50%) and non-bound indium (~ 25%), but also a third compound (~ 11%). This last compound was trapped in the kidneys as it was not detected in urine. HPLC confirmed that this metabolite has the same profile as ^{111}In-DOTA alone, indicating a partial degradation of the tracer, leading to the entrapment of ^{111}In-DOTA in the kidneys. The reaction leading to the partial cleavage of the molecule might be triggered by its intrinsic properties and its protection could be achieved by functionalizing the DOTA cycle. ^{111}In-DOTA has not been found in other tissues. Thus, it is possible that the cleavage occurs in the kidney itself. The cause remains to be elucidated. However, this represents only 11% of the

renal activity. If the high renal uptake is not necessarily an issue for nuclear imaging, it is unsuitable for radiotherapy. Therefore, this uptake has to be reduced as it limits the maximum tolerated activity dose that can be safely administered without inducing radiation nephrotoxicity. To achieve this, the ^{111}In-DOTA-RAFT-RGD could be chemically modified. Alternatively, amino acids such as Lysine or gelatine-based plasma expander or a combination of both could reduce kidney uptake. Gelofusine successfully reduced ^{111}In-DOTA-Octreotide renal uptake in mice and rats when administered before the tracer.[36]

Other tissues, such as liver, spleen, stomach and intestine, also accumulated a part of the tracer. This has previously been described for other indium-111 labeled RGD-tracers.[14,35] Localization of the radiolabeled peptides in non-tumor tissues may limit their imaging potential, as well as their therapeutic application. The physiological uptake in those organs may hamper imaging of abdominal tumor lesions and will limit the activity dose that can be administered safely.

In summary, RAFT-RGD can be radiolabeled with indium-111 through the chelating agent DOTA, with high radiochemical purity and stability. ^{111}In-DOTA-RAFT-RGD allowed non-invasive tumor visualization of $\alpha_v\beta_3$-positive tumors with good T/B and T/M ratios. Renal uptake must be reduced for Yttrium-90 or Lutetium-177 radiotherapy.

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