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RADIOCHEMICAL AND BIOLOGICAL EVALUATION OF Ter-Cys-RGD DERIVATIVE LABELED WITH THE PRECURSOR $[^{99m}Tc(H_2O)_3(CO)_3]^+$

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ABSTRACT

Introduction: Endothelial $\alpha_v\beta_3$ integrin is considered a marker of tumor-induced angiogenesis. This integrin can bind to the arginine-glycine-aspartic acid (RGD) aminoacid sequence present in extracellular matrix proteins. Cyclic peptides containing RGD sequences have high affinity and selectivity for $\alpha_v\beta_3$ integrin. *Aim*: The aim of this study was the labelling of Ter-Cys-RGD derivative using the ^{99m}Tc-tricarbonyl technique and the exploration of its potential as a radiopharmaceutical *in vivo*. *Methods*: The preparation of the ^{99m}Tc-precursor consisted in flushing the mixture of 4.4 mg of sodium carbonate, 5.5 mg of sodium borohydride and 20 mg of sodium-potassium tartrate tetrahydrate with CO gas during 30 min. Pertechnetate was added and the vial was heated for 35 min at 75 °C. The reaction was stopped in ice bath, and pH was adjusted to 7. Then 100 µL of the precursor was added to 1.1 mM (50 µg) of the ligand and heated at 75 °C for 60 min. The reaction was stopped in ice bath. Radiochemical purity of the precursor and the final product was assessed by paper and thin layer chromatography, as well as by HPLC. Biodistribution studies were performed in healthy *Swiss* mice at 1 and 4 hours post-injection and also in nude mice bearing A549 lung-cancer cells, 4 hours post-injection. Planar gamma-camera imaging was also done in nude athymic mice bearing tumor. *Results*: The precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was quantitatively formed by carbonylation with high yield (94.8 ± 2.6%). Radiochemical purity of $^{99m}Tc(CO)_3$-Ter-Cys-RGD was 86.9% ± 4.3 %. A specific activity of 6.7 MBq/nmol was achieved. Biodistribution findings 4 hours post-injection revealed the highest uptake by respectively kidneys (12.1 ± 0.7 %ID/g), liver (11.8 ± 0.8 %ID/g) and intestines (10.0 ± 1.5 %ID/g). Biodistribution in mice bearing lung tumor also showed highest uptake by kidneys and liver, similarly to studies in healthy mice, suggesting that ^{99m}Tc-$(CO)_3$-Ter- Cys-RGD was excreted by both hepatobiliary and renal systems. Tumor uptake was 5.73 ± 1.4% ID/g. Scintigraphic imaging in nude mice bearing A549 cells was also documented. *Conclusions*: 1) Labeling conditions permitted a good yield, nevertheless a purification step is advised before carrying out biological studies. 2) The high tumor uptake encourages further studies with this complex.

1. INTRODUCTION

Tumors must established a neovasculature to grow. To do so, the endothelial cells need to organize themselves into capillaries anchored to the extravascular matrix. The organization of the vascular endothelial cells is accomplished by an interaction of dimerized single-transmembrane glycoproteins named $\alpha_v\beta_3$ integrin, with triplet peptides Arg-Gly-Asp (RGD) sequenced in the proteins of the extracellular matrix [1].

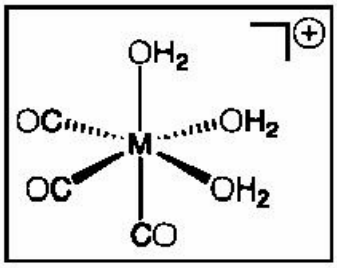
The $\alpha_v\beta_3$ integrin are also expressed on cancer cells [2] and therefore play an important role in the invasion, metastasis, proliferation and apoptosis of cancer [3].
Because the expression of $\alpha_v\beta_3$ integrins is reported to be strong on activated endothelial cells but restricted on normal cells synthetic RGD containing small peptides have been proposed as antagonists against vascular endothelial cell and tumor growth[4].

The detection of early stage cancer is important in preventing cancer cell metastasis. However, the sensitivity and specificity of current methods for cancer detection especially in early stages are not ideal [5]. Radiolabeled RGD peptides could be used as a class of tumor (i.e. tumor angiogenesis) specific markers in cancer detection [6].

Recently new ^{99m}Tc moieties have been introduced as potentially adequate for labeling small biomolecules.

The precursor complex ^{99m}Tc-carbonyl $[^{99m}Tc(OH_2)_3(CO)_3]^+$ is one of the promising cores for the development of new ^{99m}Tc based radiopharmaceuticals[7]. Many ligands for the stable binding of the *fac*-$[^{99m}Tc(CO)_3]^+$ core to biomolecules have been described[8].

The ^{99m}Tc-carbonyl core allows the labeling of even the smallest biomolecules with high specific activity and without damaging the biological activity and specificity. This precursor is not only readily water soluble but reveals good stability in aqueous solutions over a broad pH range, for several hours. The three water molecules coordinated to the highly inert $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core are readily substituted by a variety of functional groups (Fig. 1).



$M(H_2O)_3(CO)_3$, M=Tc, Re

Figure 1. ^{99m}Tc-carbonyl core

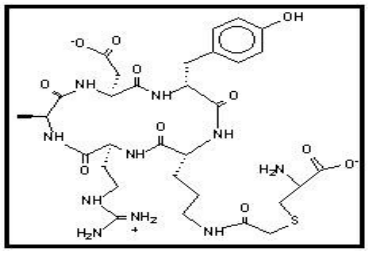


Figure 2. Ter-Cys-RGD

Alberto et al. 1998 [9], developed a fully aqueous-based kit preparation of the organometallic technetium precursor under mild reaction conditions in the presence of gaseous carbon monoxide and sodium borohydrate, which is here investigated.

In this study a cyclic RGD peptide analog X-RGDyK (Cyclo[Arg-Gly-Asp-D-Tyr-Lys) derivative was synthesized and coupled to Ter-Cys to allow the coordination to ^{99m}Tc-carbonyl core (Fig. 2)

2. MATERIAL AND METHODS

2.1 Material

- ^{99}Mo/^{99m}Tc generator – Radiopharmacy Center (IPEN-CNEN/SP)- Brazil.
- Ter-Cys-RGD peptide – Biosynthan, Berlim, Germany
- Lung carcinoma cells – A549 (ATCC Number CCL-185, USA).
- Animals for imaging and biodistribution studies: *Swiss* mice and *Nude* mice are provided by the Animal facility of IPEN-CNEN/Sao Paulo, Brazil.
- Monoxide carbon gas – White Martins Gases Industriais AS, Sao Paulo, Brazil

2.2 Methods

2.2.1 Labeling procedures and radiochemical control

The organometallic precursor was prepared according to the procedure published by Alberto et al.,1998 [9] with minor modifications. Briefly, 4.4 mg Na_2CO_3, 20 mg Na/K-tartrate.H_2O and 5.5 mg $NaBH_4$ were purged for 1 hour with monoxide carbon gas. Then 1 mL of $Na^{99m}TcO_4$ (3700 – 5550 MBq) was added to the vial containing the mixture of salts purged with CO gas. Then the vial was heated at 75-80 °C for 35 min. The reaction was stopped in an ice bath. Then the pH was adjusted to 7 using 0.2 mL of 1M HCl / 0.1 M Phosphate buffer solution (2:1).

In the vial containing 1.1 mM (50 μg) of Ter-Cys-RGD derivative it was added first 100 μL of $^{99m}Tc(CO)_3$. The vials were heated at 75 °C for 60 minutes. The reaction was stopped in an ice bath.

The quality control was done using paper and thin layer chromatography with Whatman n.1 and TLC-Aluminium as support and the mixture of methanol/HCl 6 M (99.5:0.5) was used as mobile phase.

The purification was performed in a C_{18}-SepPak-Mini cartridge (Waters, Milford, MA). The column was activated with 5 mL of ethanol and 5 mL of water. The impurities were eluted with

2 mL of water and the radiolabeled peptide with 2 mL of ethanol. For biological studies and other evaluations the ethanol solvent was slowly evaporated under nitrogen atmosphere. The residue was taken up with physiological saline solution.

2.2.2 Biological studies

The studies were approved by the local Animal Welfare Committee.

Biodistribution studies, after purification step. ^{99m}Tc-labeled preparation (0.1 mL) were i.v. administrated via the tail vein in Swiss male mice (body mass 20-25 g) in a radioactive concentration of 185-370 MBq/mL. Mice were sacrificed at 1 and 4 hours post injection. Organs and tissues (blood, heart, lung, spleen, kidneys, liver, pancreas, stomach, large and small intestines, muscle, bone) were excised, weighed and the radioactivity was determined by γ-counting. Results were expressed as percentage of injected dose per gram (% ID/g) of tissue.

2.2.3 Biological studies with mice-bearing tumor cells

A549 cells, a human non-small-cell lung carcinoma cell line, were grown in RPMI growth medium, enriched with penicillin at 50 IU/mL, streptomycin 50 μg/mL, amphotericin-B 1.25 μg/mL, L-glutamine 2 mM and fetal bovine serum [FBS; 10% (v/v)].

The cells were grown to confluency and then harvested by trypsinization. After centrifugation for 5 min at 100xg, cells were resuspended in PBS. Cells were counted without staining and one more time centrifuged and resuspended again in PBS (70 μL) for innoculation in animals.

Athymic male nude mice (18-22g) were injected subcutaneously on the right higher back with a suspension of 6.5 x 10^6 cells. Tumor-bearing mice were used in the biodistribution 4h post injection of ^{99m}Tc(CO)$_3$-Ter-Cys-RGD and for planar gamma-camera imaging.

3. RESULTS AND DISCUSSION

The development in ^{99m}Tc chemistry is considered of great importance for investigating new ^{99m}Tc labeled molecules with potential applications. In this sense the tricarbonyl technology which is currently being exploited is remarkable [10].

One of the major advantages of the carbonyl approach is the availability of a well defined complex with very high specific activity only depending from the ligand type.

The synthesis of the precursor ^{99m}Tc-carbonyl is a convenient method producing three tighly bound CO radicals and three labile water ligands. The metal centre (^{99m}Tc) is in the low oxidation state +1 and is therefore chemically inert [11].

The three water molecules coordinated to the fac-[$M(CO)_3$]-core can be readily substitute by a variety of functional groups using mono-, bi--, and tridentate ligand systems.
The RGD analogue peptide used in this study was derivatized with Ter-Cys to allow the formation of the complex under mild reaction conditions.

The precursor [$^{99m}Tc(H_2O)(CO_3)]^+$ was formed quantitatively by carbonylation with high yield 94.8 ± 2.6%. Radiochemical purity of $^{99m}Tc(CO)_3$-Ter-Cys-RGD was 86.9% ± 4.3 %. A specific activity of 6.7 MBq/nmol was achieved.

Biodistribution findings studies in healthy *Swiss* male mice revealed the highest uptake by respectively kidneys (14.5 ± 0.4 %ID/g), liver (12.2 ± 0.2 %ID/g) and intestines (12.9 ± 1.6 %ID/g), 1 h post administration of the radiotracer (Table 1).

Table 1. Biodistribution studies of $^{99m}Tc(CO)_3$-Ter-Cys- RGD in healthy *Swiss* mice (%ID/g)

Organ/	$^{99m}Tc(CO)_3$Ter-Cys-RGD	
tissue	1 h	4 h
Blood	2.6 ± 0.1	1.3 ± 0.0
Heart	2.6 ± 0.0	1.6 ± 0.2
Lung	5.2 ± 0.3	3.5 ± 0.8
Kidneys	14.5 ± 0.4	12.1 ± 0.7
Spleen	2.9 ± 0.1	2.8 ± 0.0
Stomach	3.3 ± 0.2	1.6 ± 0.1
Pancreas	1.2 ± 0.5	0.9 ± 0.2
Liver	12.2 ± 0.2	11.8 ± 0.8
L. intestine	3.2 ± 0.1	3.6 ± 0.4
S. intestine	9.7 ± 1.5	5.4 ± 1.1
Muscle	0.8 ± 0.2	0.7 ± 0.2
Bone	2.6 ± 0.5	2.1 ± 0.1
Total Blood	5.1	2.2
Total Muscle	9.1	6.8
Total Bone	8.5	5.4

Biodistribution in healthy mice and in mice bearing tumor cells 4h post injection of the tracer showed a similar profile as can be observed in Fig. 3.

Highest uptake by kidneys and liver, suggest that ^{99m}Tc-(CO)$_3$-Ter- Cys-RGD was excreted by both hepatobiliary and renal systems. Tumor uptake was 5.7 ± 1.4% ID/g.

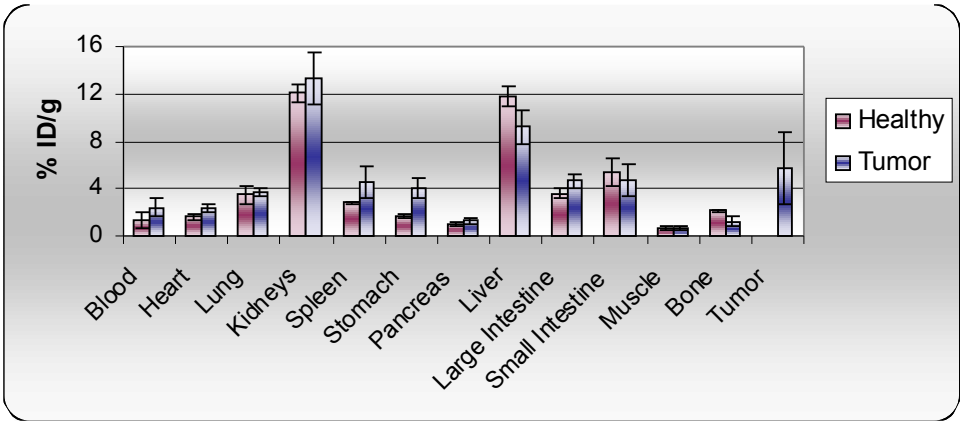


Figure 3. Biodistribution studies of ^{99m}Tc-Ter-Cys-RGD 4 hours post-injection in healthy *Swiss* mice and in *Nude* Mice bearing tumor cells

Scintigraphic static image in nude mice bearing A549 lung cancer cells in the right upper back 4 hours post-injection can be observed in Figure 4. Quantification data described by ROI gave 0.81% in tumor nodule.

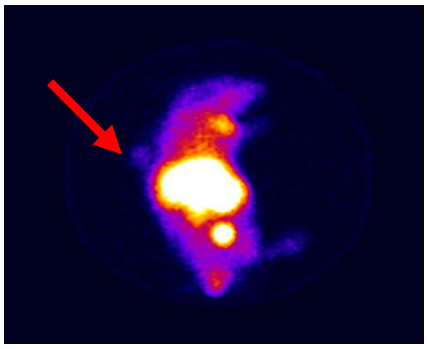


Figure 4. Image of mice bearing lung cancer cells

4. CONCLUSIONS

1) Labeling conditions permitted a good yield, nevertheless a purification step is advised before carrying out biological studies. 2) The high tumor uptake encourages further studies with this complex.

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