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^{99m}Tc-HYNIC-RGD ANALOG FOR MONITORING OF INTEGRIN EXPRESSION IN ISCHEMIC ANIMAL MODEL

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ABSTRACT

Introduction: The development of new blood vessels, from existing network of angiogenesis, occurs during embryonic development, cancer, arthritis and in response to ischemia. Ischemia in muscle tissue increases expression of integrin in the form of proliferating endothelial cells, including the $\alpha_v\beta_3$ receptor. Cyclic peptides containing RGD sequences have high affinity and selectivity for $\alpha_v\beta_3$ integrin, and may thus be used for angiogenesis monitoring. *Aim*: The aim of this study was the investigation of neovascularization in an ischemic hindlimb model using RGD conjugated with HYNIC and labeled with technetium-99m. *Methods*: ^{99m}Tc-HYNIC-RGD analog was prepared using tricine and EDDA as coligands. Unilateral hindlimb ischemia was induced in *Wistar* rats by surgical ligation of the femoral artery. Radiolabeled RGD was injected in rats after 2h, as well as 1, 3, 5, 7, 10 and 14 days of ischemic induction. Evaluation of uptake by ischemic limb and normal contralateral limb was done by planar imaging and biodistribution studies. Pertechnetate distribution, which has no affinity for regeneration tissue and in the circumstances indicates just blood flow, was also assessed for confirmation. *Results*: The highest uptake ratio between ischemic hindlimb and control side was achieved at the 7th day (2.62 ± 0.95), with substantial decreased by the 14th day. For pertechnetate the 7th day ratio was 0.87 ± 0.23, consistent with reduced perfusion following arterial ligation. Scintigraphic imaging confirmed the RGD results. Both renal and hepatic elimination was observed, with noticeable uptake also by intestine, spleen and bone. *Conclusions*: ^{99m}Tc-HYNIC-RGD analog corresponded to the expectations in this animal model, displaying elevated uptake in ischemic tissue by the time of widespread angiogenesis. Utilization in other ischemic models can be recommended.

1.INTRODUCTION

The development of new blood vessels from an existing network of vessels, angiogenesis, occurs during embryonic development, cancer, and arthritis and in response to ischemia [1,2].

Integrins are heterodimers composed of 1 α and 1β subunit. $\alpha_v\beta_3$ is a member of the integrin family or receptors which are involved in adhesion and signaling of many types of cells.

Recently, increased $\alpha_v\beta_3$ integrin expression in injured vasculature and infarcted myocardium has been successfully imaged with ^{111}In-labeled quinolone [3,4].

There has been increasing interest in peptides containing the Arg-Gly-Asp (RGD) sequence for targeting of $\alpha_v\beta_3$ integrins to image angiogenesis [5-7].
As far as imaging is concerned, ^{99m}Tc is considered to be the ideal diagnostic radionuclide. We focused in the use of the bifunctional chelator HYNIC approach for the radiolabeling of the target-specific biomolecule [8].

Linear peptides containing RGD have been shown to antagonize the binding of native ligands to $\alpha_v\beta_3$ [6].

It has been reported that the aspartic acid residue of RGD is highly susceptible to chemical degradation leading to the loss of biological activity. This degradation was prevented when the RGD- containing peptide was cyclized via disulfide linkage [9,10].

In this study cyclic RGD peptide analog RGDyK (Cyclo[Arg-Gly-Asp-D-Tyr-Lys) was synthesized and conjugated to HYNIC (Figure 1) and then labeled with the radioisotope technetium-99m (^{99m}Tc).

2-hydrazinonicotinamide, HYNIC, is a bifunctional chelating ligand development for technetium and rhenium-based radiopharmaceuticals [11].

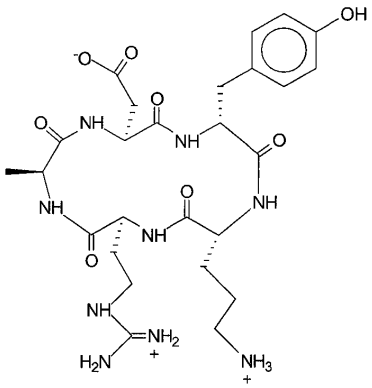

Figure 1. Molecule of HYNIC- RGDyK

The aim of the study was the evaluation of neovasculatization in ischemic region using the radiolabeled peptide that is an imaging agent for $\alpha_v\beta_3$ integrin.

2. MATERIAL AND METHODS

2.1. Material

- RGD derivatives was synthesized and purchased from Biosynthan, Berlin.
- $^{99}Mo/^{99m}Tc$ generator – Institute of Energetic and Nuclear Research (IPEN-CNEN/SP)-Brazil
- Animals for imaging and biodistribution studies: *Wistar* rats are provided by the animal facility of IPEN-CNEN/Sao Paulo, Brazil.
- Tricine, EDDA, Stannous Chloride and other reagentes - Sigma-Aldrich, Brazil, Ltda.

2.2. Labeling Procedure Using EDDA/Tricine

Labeling procedure was reported before [11]. Briefly, the coligands Tricine and EDDA were dissolved in 0.1M phosphate buffer solution, previously nitrogenated. Then the peptide [c[Arg-Gly-Asp-D-Tyr-(HYNIC)-Lys] plus 8.9 mM $SnCl_2{\cdot}H_2O$ solution in 0.1N HCl (nitrogen-purged) and 500µL of $Na^{99m}TcO_4^-$ was added to the solution. The solution was heated for 15 minutes in a water bath at 100°C and cooled to room temperature. The pH of the reaction was 7.

2.3. Radiochemical Control

Radiochemical analysis of ^{99m}Tc-HYNIC-RGD was performed by thin-layer chromatography (TLC) using instant thin layer chromatography on silica gel strips (ITLC-SG, Gelman Sciences, Ann Arbor, Mich.) with a two solvent system. Methylethylketone (MEK) was used to determine the amount of $^{99m}TcO_4^-$ (R_f =1), and 50% acetonitrile solution to determine ^{99m}Tc-colloid (R_f=0).

The ^{99m}Tc-HYNIC-RGD was also characterized by reverse phase high performance liquid chromatography (RP-HPLC).

2.4. Biological studies

The study was approved by the local Animal Welfare Committee. The *in vivo* studies were performed in male *Wistar* rats submitted to femoral artery occlusion. The study was performed in groups of 5 animals.

2.4.1. Surgical technique

The animals were anesthetized with ketamine and shaved for surgical intervention. The right femoral vessels were exposed through a longitudinal skin incision, and the femoral artery was

carefully separated and ligations in two locals were done. Then the incision was sutured. Rats were allowed to recover for 2h and 1, 3, 5, 7, 10 and 14 days when the radiolabeled peptide was injected.

2.4.2. Biodistribution studies

Each animal was injected with 0.1 mL of ^{99m}Tc-HYNIC-RGD analog via a tail vein. The animals were sacrificed 2 hours post-injection. Evaluation of the uptake by ischemic limb and normal contralateral limb was done as well biodistribution in others tissues and organs of healthy animals. Pertchnetate distribution, which has no affinity for regenerating tissue and in the circumstances indicates just bloow flow, was also assessed for confirmation.

3. RESULTS AND DISCUSSION

Angiogenesis process is not only involved in cancer progression but also plays an important part in the improvement and healing of ischemic lesions [12,13]. Accordingly, elevated $\alpha_v\beta_3$ integrin expression has been observed in ischemic tissue of the brain [14], retina [15], and muscles [16].

Neovascularization is a physiological response to ischemia that often produces insufficient collateral vessels to resolve its symptons or signs [17,18]. Various studies in recent years have shown that $\alpha_v\beta_3$ integrin is upregulated on angiogenic endothelial cells, and can therefore be considered as a target molecule.

3.1. Labeling Procedure Using EDDA/Tricine

Radiochemical purity of ^{99m}Tc-HYNIC-RGD was 99.4 ± 0.1 %. ITLC findings were confirmed by HPLC with a retention time for the product of 12.86 minutes. Only traces (< 0.6 %) of $^{99m}TcO_4^-$ could be detected, with a retention time of 5.33 min. Specific activity was 142.3 MBq/nmol.

Technetium-99m binds to the hydrazine-moiety forming a ^{99m}Tc-N bond. As HYNIC alone cannot satisfy the coordination requirements of Tc (V) (HYNIC can only occupy one or two coordination sites on the radionuclide), coligands are necessary to complete the coordination sphere of the technetium (V) core.

Faintuch et al. (2005) [11] reported that conjugates prepared by tricine/EDDA exchange labeling has high specific activity and excellent radiochemical stability.

3.2. Surgical Technique

Experimental methods to create hindlimb ischemia are not standardized. Method of occluding artery varies from ligation, or cutting, to excision of the artery. The targeted artery varies also from the iliac artery, the femoral artery, or the femoral with saphenous artery [19,20]. We opted to make the ligation of the femoral artery.

3.3. Biodistribution Studies

The biodistribution in tissues and organs expressed in %ID/g (Table 1), 2 hours post injection of ^{99m}Tc-HYNIC-RGD analog showed higher uptake by the kidneys, reflecting a renal excretion, followed by intestine and liver. The conjugated cleared the bloodstream very fast.

Table 1. Biodistribution of ^{99m}Tc-HYNIC-RGD

Organ	2 h post injection
Blood	0.07 ± 0.03
Heart	0.41 ± 0.04
Lung	0.64 ± 0.12
Kidneys	2.45 ± 0.15
Spleen	1.24 ± 0.33
Stomach	1.12 ± 0.13
Pancreas	0.30 ± 0.01
Liver	1.53 ± 0.25
Large Intestine	1.57 ± 0.16
Small Intestine	1.48 ± 0.12
Muscle	0.27 ± 0.02

(%ID/g ± SD)

The highest uptake ratio between ischemic hindlimb and control side was achieved at the 7th day (2.62), with substantial decreased by the 14th day. This results show that not only ^{99m}Tc-HYNIC-RGD can be a good radiopharmaceutical for detecting neovascularization but also that the ischemic model used by us permitted this evaluation.

Pertechnetate distribution, which has no affinity for regenerating tissue and in the circumstances indicates just blow flow, was also assessed for confirmation and the ratio obtained in this case for the 7th day was 0.87, consisted with reduced perfusion following arterial ligation. Scintigraphic imaging confirmed the RGD results.

4. CONCLUSIONS

^{99m}Tc-HYNIC-RGD analog corresponded to the expectations in this animal model, displaying elevated uptake in ischemic tissue by the time of widespread angiogenesis. Utilization in other ischemic models can be recommended.

REFERENCES

1. CARMELIET, P. and JAIN, R.K., "Angiogenesis in cancer and disease", *Nature,* **407**, pp.249-257 **(**2000).

2. ZHONG, J.; ELICERI, B.; STUPACK, D.; PENTA, K.; SAKAMOTO, G.; QUERTEMOUS, T.; COLEMAN, M.; BOUDREAU, N.; VARNER, J.A., "Neovascularization of ischemic tissues by gene delivery of the extracellular matrix protein Del-1", *J. Clin Invest*., **112**, pp.30-41 (2003).

3. SADEGUI, M.M.; KRASSILNIKOVA, S.; ZHANG J. et al., "Detection of injury-induced vascular remodeling by targeting activated $\alpha_v\beta_3$ integrin in vivo", *Circulation*, **110**, pp.84-90 (2004).

4. MEOLI, D.F.; SADEGUI, M.M.; KRASSILNIKOVA, S. et al., "Noninvasive imaging of myocardial angiogenesis following experimental myocardial infarction", *J. Clin. Invest.*, **113**, pp.1684-1691 (2004).

5. DECRISTOFORO, D.; FAINTUCH, B.L.; REY, A.; von GUGGENBERG, E.; RUPPRICH, M.; HERNANDEZ-GONZALES, I.; TEODORO, R.; HAUBNER, R., "[^{99m}Tc]HYNIC-RGD for imaging integrin $\alpha_v\beta_3$ expression" *Nucl. Med. Biol.*, **33**, pp.945-952 (2006).

6. MCQUADE, P. AND KNIGHT, L. C., "Radiopharmaceutical for targeting the angiogenesis marker $\alpha_v\beta_3$", *The Quarterly Journal of Nuclear Medicine*, **47(3)**, pp.209-220 (2003).

7. PSIMADAS, D.; FANI, M.; ZIKOS, C.; XANTHOPOULOS, S; ARCHIMANDRITIS, S.C.; VARVARIGOU, A.D., "Study of the labeling of two novel RGD-peptidic derivatives with the precursor $[^{99m}Tc(H_2O)(CO)_3]^+$ and evaluation for early angiogenesis detection in cancer" *Applied Radiation and Isotopes,* **64**, pp.151-159 (2006).

8. von GUGGENBERG, E.; BEHE, M.; BEHR, T.M.; SAURER, M.; SEPPI, T.; DECRISTOFORO, C., "^{99m}Tc-labeling and in vitro and in vivo evaluation of HYNIC- and (N alpha-His) acetic acid-modified [D-Glu1]-mini-gastrin", *Bioconjug Chem*., **15**, pp.864-871 (2004).

9. SU, ZI-FEN; LIU, G.; GUPTA, S.; ZHU, Z.; RUSCKOWSKI, M. and HNATOWICH, D. J., " In vitro and in vivo evaluation of a techenetium-99m-labeled cyclic RGD peptide as a specific marker of $\alpha_v\beta_3$ integrin for tumor imaging", *Bioconjugate Chem.*, **73**, pp.561- 570 (2002).

10. BOGDANOWICH-KNIPP, S.J.; CHAKRABARTI, S.; WILLIAMS,T.D.; DILLMAN, R.K.; SIAHAAN, T.J., " Solution stability of linear vs cyclic RGD peptides", *J. Pept.Res.* **53**, pp.530-41 (1999).

11. FAINTUCH, B.L.; SANTOS, R.L.S.R.; SOUZA, A.L.F.M.; HOFFMAN, T.J.; GREELEY, M.; SMITH, C.J., "^{99m}Tc-HYNIC-bombesin (7-14)NH2: Radiochemical evaluation with co-

ligands EDDA (EDDA=ethylenediamine-N,N'-diacetic acid), tricine, and nicotinic acid", *Synthesis and reactivity in Inorganic, Metal-Organic and Nano-metal Chemistry*, **35**, pp.43-51 (2005).

12. BAUMGARTNER, I.; PIECZEK, A.; MANOR, O., "Constitutive expression of VEGF 165 following intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia" *Circulation*, **97**, pp.1114-1123 (1998).

13. LEE, K.H., JUNG, K.H.; SONG, S.H; KIM, D.H.; LEE, B.C.; SUNG, H.J.; HAN, Y.M.; CHOE, Y.S.; CHI, D.Y.; KIM, B.T., "Radiolabeled RGD uptake $\alpha_v\beta_3$ Integrin expression is enhanced in ischemic murine hindlimbs" *J. Nucl. Med*., **46**, pp.472-478 (2005)

14. ABUMIYA, T.; LUCERO, J.; HEO, J.H. et al., "Activated microvessels express vascular endothelial growth factor and integrin $\alpha_v\beta_3$ during focal cerebral ischemia", *J. Cereb. Blood Flow Metab.*, **19**, pp.1038-1050 (1999).

15. TAKAGI, H.; SUZUMA, K.; OTANI, A. et al., "Role of vitronectin receptor-type integrins and osteopontin in ischemia-induced retinal neovascularization", *Jpn. J. Ophthalmol.*, **46**, pp.270-278 (2002).

16. KONTTINEN, Y.T.; MACKIEWICZ, Z.; POVILENAITE, D.; SUKURA, A.; HUKKANEN, M.; VIRTANEN, I., "Disease-associated increased HIF-1, $\alpha_v\beta_3$integrin, and Flt-1 do not suffice to compensate the damage-inducing loss of blood vessels in inflammatory myopathies", *Rheumatol. Int*. **24** 333-339 (2004).

17. CARMELIET, P., "Angiogenesis in health and disease", *Nat. Med.*, **9**, pp.653-660 (2003).

18. YOON, CHANG-HWAN; HUR, J.; OH, IL-YOUNG; PARK, KYUNG-WOO; KIM, TAE-YOUN; SHIN, JAE-HOON; KIM, JI-HYUN; LEE, CHOON-SOO; CHUNG, JUNE-KEY; PARK, YOUNG-BAE and KIM, HYO-SOO, "Arteriosclerosis, Thrombosis, and Vascular biology", *Arterioscler. Thromb. Vasc. Biol.*, **26**, pp. 1066-1072 (2006).

19. SKJELDAL, S.; GROGAARD, B.; REIKERAS, O.; MULLER, C.; TORVIK, A.; SVINDLAND, A., "Model for skeletal muscle ischemia in rat hindlimb: evaluation of reperfusion and necrosis" *Eur. Surg. Res.*, **23**, pp. 355-365 (1991).

20. GOTO, T.; FUKUYAMA, N.; AKI, A.; KANABUCHI, K.; KIMURA, K.; TAIRA, H.; TANAKA, E.; WAKANA, N.; MORI, H.; INOUE, H. "Search for appropriate experimental methods to create stable hindlimb ischemia in mouse", *Tokai J. Exp. Clin. Med.*, **31**, pp.128-132 (2006).