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BIOLOGICAL PROFILE OF ^{99m}Tc-HYNIC-βALA-NT(8-13) IN MDA-MB-231 BREAST CANCER CELL LINE

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ABSTRACT

Introduction: Neurotensin (NT) is a tridecapeptide involved in several growth-steps of human cancers. Recent studies postulated the role of NT and NT-receptor subtype 1 in breast cancer progression. However, the main drawback of natural NT is its rapid degradation in plasma. In an effort to develop a NT peptide-based radiopharmaceutical for the detection of breast cancer, the aim of this study was the radiolabeling of the double-stabilized NT(8-13) peptide using HYNIC as chelating agent. **Methods:** Conjugated HYNIC-βAla-NT(8-13) was labeled with ^{99m}Tc using tricine and EDDA as coligands. Radiochemical purity was checked by TLC and confirmed by RP-HPLC. ^{99m}Tc-HYNIC-βAla-NT(8-13) (0.1 mL/74 MBq) was administered in *Nude* mice bearing MDA-MB-231 breast cancer cells and biodistribution studies were carried out at 30 and 90 min post-injection (pi). Blocking evaluation was also conducted by co-injection of 115 nmol of cold NT (8-13) analog. Planar gamma-camara imaging was acquired at the earlier time point studied. **Results:** Radiochemical purity of the radioconjugate was higher than 99%. Biodistribution studies revealed a very fast accumulation in tumor (1.97±0.18% ID/g, 30 min pi) with a sharply decrease at the later time point studied (0.44±0.02% ID/g). The specificity of the radioconjugate was evaluated with blockade studies. A reduction of 45.94%, 27.73% and 36.39% was found for tumor, large and small intestines, respectively, at 30 min pi. Otherwise, a less impressive blockade was observed for tumor and small intestine (28.68% and 24.90%, respectively) at the later time point studied. **Conclusion:** The results provide encouraging evidence in the development of radiolabeled NT(8-13) analogues for breast cancer diagnosis.

1. INTRODUCTION

Peptide-based radiopharmaceuticals have been employed as a powerful tool in the early prognosis of neoplastic tissues. Among the advantages of its use are the favorable pharmacokinetics characteristics *in vivo* and high tissue to background ratios due their rapid clearance from plasma [1].

The past nearly 30 years have revealed a continued interest in Neurotensin (NT), since it is known to play an important role in the Central Nervous System (CNS) as well as in the periphery. Besides that, NT exerts a wide spectrum of physiological functions by interactions with specific receptors found at the cell membrane of normal and cancer cells [2]. The biochemical functions played by NT is well-documented, however, particularly in breast cancer cell progression so far, its action is only reported as an anti-apoptoptic agent [3] as well as an inducer of breast tumorigenesis by the interaction with Neurotensin receptor-1 (NTR1) [4].

Many reports postulate that the C-terminal hexapeptide sequence of NT **(Fig. 1)** contains all the structural requirements for full binding activity of the peptide at the Neurotensin receptor

subtype-1 (NTR1) [5]. Over-expressed NTR could be targeted with radiolabeled NT analogues and would offer an interesting tool for tumor imaging and subsequent therapeutic intervention. However, NT as well as other small neuropeptides is rapidly metabolized in plasma by endogenous peptidases, limitating its clinical application [6]. In order to circumvent this drawback, several stabilization strategies in the cleavage sites (Arg^8-Arg^9, Pro^{10}-Tyr^{11} and Tyr^{11}-Ile^{12}) have been done, including the synthesis of linear peptides, non-peptides and cyclic peptides. Moreover, a large body of evidence indicates that a double-stabilization in the cleavage sites Arg^8-Arg^9 and Tyr^{11}- Ile^{12} showed long plasma stability, enhanced tumor-to-background ratios and higher tumor uptake than native NT [7].

pGlu^1-Leu^2-Tyr^3-Gly^4-Asn^5-Lys^6-Pro^7-**Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}-OH**

Fig. 1. Aminoacid sequence of Native Neurotensin. Bold corresponds to the active part of the Native peptide.

Several strategies for ^{99m}Tc radiolabeling of NT have been described[6,7], and none was found in the literature using 2-hydrazinonicotinamide, HYNIC, as bifunctional chelator. In this sense, the aim of this study was the radiolabeling of the double-stabilized NT(8-13) peptide, using HYNIC as chelating agent, for breast cancer diagnosis.

2. MATERIALS AND METHODS

2.1. General

Technetium-99m was obtained from an alumina-based ^{99}Mo/^{99m}Tc generator, supplied by the Diretoria de Radiofarmácia of the Instituto de Pequisas Energéticas e Nucleares (IPEN/CNEN, Sao Paulo, Brazil). HYNIC-βAla-NT(8-13) was obtained from piCHEM (Vienna, Austria). All other reagents such as tricine, N´,N´-ethylenediaminediacetic acid (EDDA) and stannous chloride ($SnCl_2.2H_2O$) were purchased from Sigma-Aldrich (Sao Paulo, Brazil). Culture media RPMI-1640, fetal bovine serum (FBS), antibiotic/antimycotic solution, Trypsin were obtained from Emcare® (Sao Paulo, Brazil). The MDA-MD-231 human breast cancer cell line was a donation of Federal University of Sao Paulo (UNIFESP, Sao Paulo, Brazil). *Nude* mice for biodistribution and imaging studies were provided by the Animal Facility (IPEN-CNEN, Sao Paulo, Brazil).

The investigation was conducted at the Diretoria de Radiofarmácia (IPEN/CNEN, Sao Paulo, Brazil) and the protocol was approved by the local Animal Welfare Committee.

2.2. Radiolabeling and radiochemical control

The labeling procedure was done as previously described by our group [8]. Briefly, 0,96mM of HYNIC-βAla-NT (8-13) peptide was added to a sealed reaction vial containing 20 mg tricine and 5 mg of EDDA in 500μL of 0.1 M of nitrogenated phosphate buffer solution. Then, 8.9 mM $SnCl_2.2H_2O$ solution in 0.1 N HCl (nitrogen-purged) followed by 500 μL of

$Na^{99m}TcO_4$ (1110-1480 MBq) was added. The labeled mixture was heated at 100 °C for 15 minutes and cooled to room temperature. The pH of the reaction mixture was 7.

Radiochemical analysis of ^{99m}Tc-HYNIC-βAla-NT(8-13) was performed by thin-layer chromatography (TLC) on silica gel strips (ITLC-SG, Gelman Sciences, Ann Arbor, MI) using a two solvent system, namely methylethylketone (MEK) for detection of $^{99m}TcO_4^-$ and 50% Acetonitrile (ACN) for detection of $^{99m}TcO_2$. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) was performed using a system composition of H_2O containing 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.1% trifluoracetic acid (Solvent B). A Symmetry C-18 column (5.0 μm, 100 Å, 4.6 x 250 mm, Waters, Milford, MA) was used at a flow rate of 1 ml/min. The HPLC gradient system began with a solvent composition of 95% A and 5% B and followed a linear gradient of 30%A:70%B from 0-25 min, and 5%A:95%B from 25-30 min.

2.3. Cell culture and inoculation of MDA-MB-231 cancer cells in Nude mice

Human MDA-MB-231 breast cancer cells were grown in RPMI-1640 culture media with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were kept in humidified air containing 5% CO_2 at 37°C. Cells were grown to confluency and then harvested by trypsinization. After centrifugation (5 min at 100xg), cells were resuspended in PBS, counted, centrifuged once again and resuspended in PBS (100 μL) for inoculation into animals.

Athymic male *Nude* mice (18-22g) were injected subcutaneously on the upper part of the back with a suspension containing 5 x 10^6 cells. MDA-MB-231 cells were allowed to grow *in vivo* for two to three weeks post inoculation, thus forming tumors with a diameter of 1 cm approximately.

2.4. Biodistribution studies

^{99m}Tc-labeled peptide (74-111 MBq/mL/0.1 mL) was administrated into the tail vein of animals bearing MDA-MB-231 cancer cells.

Biodistribution studies were carried out at 30 and 90 min time points (N=6 per group). γ-Camara images were taken at the earlier time point studied. All animals were sacrificed by cervical dislocation. Organs and tissues 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. In blockade studies, *Nude* female mice MDA-MB-231 xenografts received 115 nmol of cold NT(8-13) along with the radiopeptide.

2.5. Statistical Analysis

Results are expressed as average of %ID/g±SD, analyzed using Student's unpaired *t*-test and significance was assumed for p value less than 0.05. The hypothesis test was formulated assuming equal variances:

*H*o : $\mu 1 = \mu 2$ *(*null hypothesis*)*
Hi : $\mu 1 > \mu 2$ *(*alternative hypothesis*)*
where $\mu 1$ is mean of variables from non-blocked mice; $\mu 2$ is mean of variables from blocked mice.

3. RESULTS AND DISCUSSION

3.1. Radiolabeling and radiochemical control

Radiochemical purity of ^{99m}Tc-HYNIC-βAla-NT(8-13) was greater than 99%. $^{99m}TcO_4^-$ and $^{99m}TcO_2$ represented a maximum of 0.5% and 0.2%, respectively, of the total activity and the latter was not detectable in HPLC analysis. The radiochromatogram showed a single peak for ^{99m}Tc-HYNIC-NT (8-13), with a retention time of 12.50 min **(Fig. 2)**.

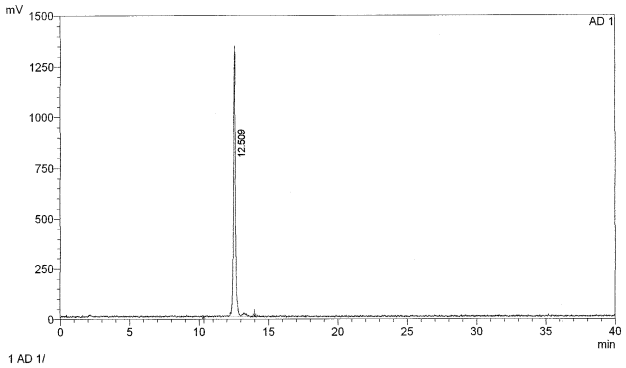

Fig. 2. RP-HPLC radiochromatogram of ^{99m}Tc-HYNIC-βAla-NT(8-13).

The high number of isomers and the relatively low stability of ^{99m}Tc-labeled HYNIC biomolecules are still object of investigation [9]. The only peak in the radio-HPLC profile indicated the absence of isomers. However, nothing can be ensured about the final structure of the radioconjugate.

3.2. Biodistribution studies

In the biodistribution studies performed in *Nude* female mice xenografts **(Fig. 3)**, the radioconjugate showed a rapid clearance in all the organs tested, except for kidneys in both time points evaluated (12.03±6.38% and 6.63±0.96% ID/g at 30 and 90 min pi, respectively). Moreover, the accumulation of radioactivity in the liver was low with values of 0.88±0.44% and 0.60±0.04% ID/g at 30 and 90 min pi, respectively, suggesting a renal-system excretion and highlighting the hydrophilic character of the ^{99m}Tc-labeled conjugate.

Tumor activity washout (1.97±0.18% and 0.44±0.02% ID/g at 30 and 90 min pi, respectively) was also rapid, which may be explained by the mechanism of accumulation of the analog in this cell line. After binding the receptor, as it has been widely reported for radiolabeled NT analogues [6,7,10], internalization studies showed a time-dependent accumulation of the radioactivity in different colon cancer cell lines, which maintained high until 120 min of incubation time. Preliminary internalization studies conducted by our group in MDA-MB-231 breast cancer cells (data not shown), revealed that the radioconjugate did not follow the same mechanism, where a sharply decrease was observed after 30 min of incubation time.

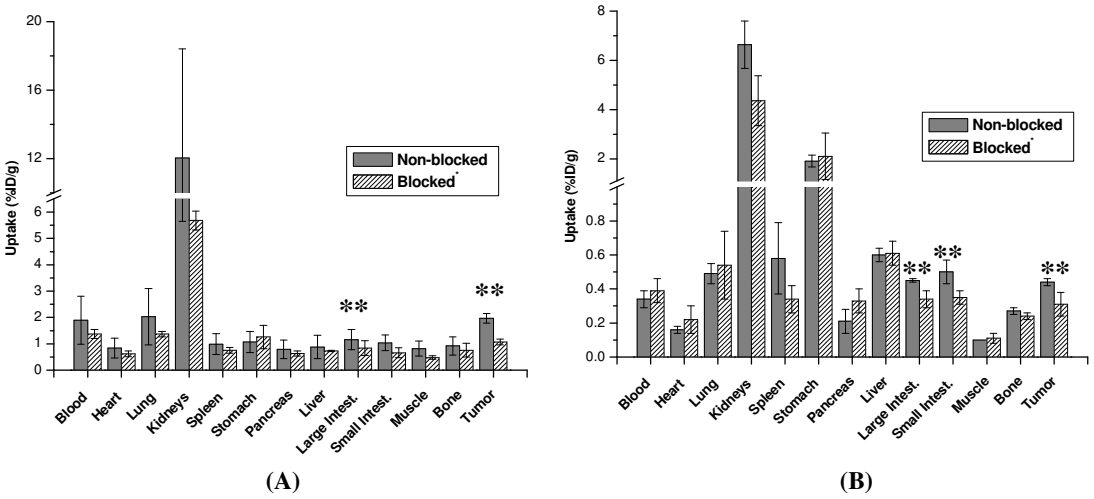

Fig. 3. Biodistribution of ^{99m}Tc-HYNIC-βAla-NT(8-13) in *Nude* mice bearing MDA-MB-231 human breast cancer cells at (A) 30 min pi and (B) 90 min pi. (Results are reported as %ID/g± SD, N=6). *Blocked. Animals received the radiolabeled analog coinjected with 100 μg/mouse of cold NT(8-13).($p < 0.05$ vs. non-blocked mice)**

In blockade studies (Fig. 3), a similar accumulation of the radioactivity was found in most organs, with the exception of the large/small intestines and tumor (NTR1-positive tissues), in which the inhibition was 27.73%, 36.39% and 45.94%, respectively at the earlier time point studied. However, large intestine reduction was not statistically significant. A less impressive blockade was observed at 90 min pi for large intestine and tumor (24.90% and 28.68%, respectively), which was not observed for small intestine (30.38%). This fact could be explained since a fast enzimatic degradation of the radioconjugate may occur *in vivo* due the short half-lives of NT analogues already reported [6,7].

Tumor-to-non-tumor ratios results are expressed in Fig. 4. After 30 min pi, the highest values were obtained for tumor/muscle, tumor/bone and tumor/liver (2.40, 2.14 and 2.24, respectively). Tumor/muscle ratio (4.29) was also high at the later time point studied, whereas for the other organs evaluated values did not reach 2% at this time point.

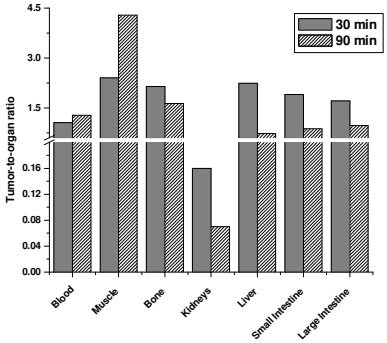

Fig. 4. Tumor/non-tumor ratios of ^{99m}Tc-HYNIC-βAla-NT(8-13) at 30 and 90 min post-injection.

Image acquisition **(Fig. 5)** was performed based on the set of data obtained in biodistribution studies, thus allowing a clear visualization of tumor. Although the best tumor/non-tumor ratios were obtained at the later time point studied, the fast washout from tumor was not favorable for tumor visualization.

Fig. 5. Scintigraphic image of mouse bearing tumor xenografts at 30 min after intravenous injection of ^{99m}Tc-HYNIC-βAla-NT(8-13).

4. CONCLUSIONS

The radiochemical and *in vivo* evaluation of ^{99m}Tc-HYNIC-βAla-NT(8-13) as breast cancer diagnostic agent are presented in this study. Blockade studies confirmed a significant reduction of the uptake in NTR-positive tissues. The highest uptake of the radiotracer in tumor was achieved 30 min p.i, thus permitting a clear visualization of tumor. Finally, the results provide encouraging evidence in the development of radiolabeled NT(8-13) analogues for breast cancer diagnosis.

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