Original Article

Labeling and Biological Evaluation of ^{99m}Tc-HYNIC-Trastuzumab as a Potential Radiopharmaceutical for *In Vivo* Evaluation of HER2 Expression in Breast Cancer

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Abstract

The amplification of HER2 gene has been described in several tumor types, mainly breast cancer with a subsequent increase in HER2 protein expression. Trastuzumab is a humanized monoclonal antibody that recognizes selectively the HER2 extracellular domain. The objective of the present work was to standardize the conjugation of Trastuzumab with Succinimidyl-hydrazinonicotinamide (HYNIC) and labeling with ^{99m}Tc to obtain ^{99m}Tc-HYNIC-Trastuzumab for use as in vivo tracer of the HER2 expression in breast cancer. The labeling procedure involved derivatization of 0.067 μmol of Trastuzumab with 0.33 μmols of HYNIC in dimethyl sulfoxide (DMSO). The mixture was incubated for 30 min. A mixture of Tricine and $SnCl_2.2H_2O$ was prepared by add a solution of 44.6 μmols Tricine in 0.05 mL HCl 2.0 M and a similar volume of another solution containing 44.3 μmols $SnCl_2.2H_2O$ in 0.5 mL HCl 2.0 M. Then, 0.05 mL of this mixed was added to the conjugated with 296 MBq of 99mTcO-4. The final mixture was incubated at room temperature (18-25°C) for 30 min. Radiochemical purity of the labeled solution was studied by chromatography, to evaluate ^{99m}Tc-Tricine, $^{99m}TcO_2.H_2O$, and free $^{99m}TcO_4^-$. Radiochemical purity was also evaluated by HPLC. Stability studies were tested in solution at 4°C and lyophilized at 4°C. Biodistribution studies were performed in healthy CD-1 female mice at 2, 5, and 24 h (n = 3) and CD-1 female mice spontaneous breast adenocarcinoma (n = 3). Scintigraphic images of spontaneous breast adenocarcinoma in female CD-1 mice were acquired in a gamma camera at 2, 5, and 24 h post-injection. Labeling was easily performed with high yields (>90%) and radiopharmaceutical stability for 24 h post-labeling. Stability studies revealed that antibody derivative must be lyophilized for undamaged storage. Biodistribution studies and imaging revealed excellent uptake in the tumor. Based on the results it was concluded that ^{99m}Tc-HYNIC-Trastuzumab could be a promising radiopharmaceutical for *in vivo* diagnosis of the HER2 status in breast with impact on treatment planning.

Keywords: Breast cancer, hydrazinonicotinamide, scintigraphy, ^{99m}Tc-labeling, trastuzumab

Introduction

Breast cancer is the most common malignant disease in women being responsible for 16% of all female cancers types.[1] Several prognostic factors have been tested to define risk groups. These include tumor size, histological degree, level of steroids receptors, proliferative index, and expression of epidermal growth factor receptors (EGFR).[2,3] EGF receptor family constitutes one of the most widely studied and best characterized protein kinase families. The family of EGFR tyrosine kinase includes four members: HER1, HER2, HER3, and HER4.[4-6] HER receptors are localized at the cell membranes sharing similar structures. These consist of a glycosylated cysteine rich extracellular ligand binding domain, a lipophilic transmembrane domain, and an intracellular domain with kinase activity. The HER proteins produce dimers in the cell membrane, particularly HER2 and HER3 proteins, as they are

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functionally incomplete, HER2 due to lack of a known ligand binding activity and HER3 has nonfunctional kinase domain. Therefore, it has been postulated a ranked interaction among the members of the HER family where HER2 is the favorite co-receptor for dimer formation.[7,8] Consequently, a cascade of signaling transductions are initiated, conducting to biochemical changes, and finally to cell proliferation. In particular, heterodimers which which HER2 participates are those possessing higher signaling and higher ligand binding efficiencies.[7-9]

HER2 is poorly expressed in normal tissue, including normal breast. Despite, HER2 plays an important role in pathogenesis and biological aggressiveness of a significant percentage of breast cancers. HER2 amplification and over-expression are correlated with disease progression and poor prognosis in patients with negative axillary lymph nodes.[3,10-13] The development of monoclonal antibodies directed to the HER2 receptor extracellular domain, such as Trastuzumab, is currently being used for the treatment of breast cancer with HER2 over-expression.[14-18]

Molecular imaging has made rapid strides in recent years; it means to the characterization and measurement of biological processes at the molecular level.[19,20] It take advantages of traditional diagnostic imaging techniques and introduces molecular probes to measure the expression of indicative molecular markers at different stages of diseases. Molecular imaging modalities include molecular MRI, magnetic resonance spectroscopy (MRS), optical bioluminescence, optical fluorescence, targeted ultrasound, single photon emission computed tomography (SPECT), and positron emission tomography (PET).[21-23]

The aim of this study was to develop a novel ^{99m}Tc labeled Trastuzumab throughout HYNIC[24-27] for its use as a potential radiopharmaceutical for *in vivo* minimally invasive evaluation of HER2 receptor expression in breast cancer.

Materials and Methods

Trastuzumab purification

Trastuzumab (Herceptin®, Roche Laboratories) 0.067 µmols was purified from the Herceptin® Kits by size exclusion chromatography using PD-10 column (GE Healthcare), equilibrated and eluted with NaCl 0.9%, and detected by UV Spectrophotometry at 280 nm and the final monoclonal concentration determined.

Hydrazinonicotinamide-Trastuzumab

Suc-HYNIC was synthesized according to protocol from literature.[28] To a solution containing purified Trastuzumab, 33 µL de $NaHCO_3$ 1 M and 0.33 umol of HINIC in 7.1 µL DMSO were added. The mixture was incubated at 18-25C for 30 min in the dark. The solution was added in a PD10 column and was eluted with sodium acetate 0.15M pH 6.4, and detected by UV Spectrophotometry at 280 nm. The purified Trastuzumab-HYNIC solution was freeze dried at 0.05hPa, −49ºC for 2 h and stored at 4ºC.

Radiolabeling

An amount of 44.6 umol of Tricine (Sigma) was dissolved in 0.8 mL of water and the pH was adjusted to 4.5 with mL HCl2.0 M (vial A). In another vial 44.3 µmol $SnCl_2.2H_2O$ was dissolved in 0.5 mL HCl 2.0 M and 0.05 mL) (vial B). The volume is then increased to 10 mL with saline. To vial A was added 50 µL of Vial B and 296-555 MBq of $Na^{99m}TcO_4$, in not more than 2 mL volume, were added and incubated at 18-25°C for 30 min.

Quality control

Radiochemical purity of the labeled biomolecule was assessed by chromatography on ITLC-SG using NaCl 0.9% as mobile phase, ITLC-SG (Pall Corporation) saturated with bovine serum albumin (BSA) using ETOH: NH_3:H_2O (2:1:5) as mobile phase and Whatman 3 MM (Whatman International Ltd) with acetone as mobile phase to identify the different possible species: ^{99m}Tc-HYNIC-Trastuzumab, ^{99m}Tc-Tricina plus free $^{99m}TcO_4^-$, $^{99m}TcO_2.H_2O$, and free $^{99m}TcO_4^-$, respectively.

Radiochemical purity was also assessed by HPLC (Varian 5000 Liquid Chromatograph, integrator 4290 Varian, simultaneous detection by NaI (Tl) crystal detector (ORTEC)) using a molecular exclusion column (Waters SW300), isocratic mode, with phosphate buffer 0.01 M, pH 7.0, and 1 mL/min flow rate.

Storage of HYNIC-trastuzumab conjugate

Two storage conditions of the conjugate were evaluated: solution at 4ºC and lyophilized with further storage at 4ºC. Both were labeled with ^{99m}Tc and the products were controlled by the protocol described above.

Stability of ^{99m}Tc-HYNIC-trastuzumab

In vitro stability of ^{99m}Tc-HYNIC-Trastuzumab in saline was evaluated for 24 h post labeling. Maximum labeling activity was assessed by addition of 74 to 550 MBq and the labeling yield determined by the physicochemical controls described.

Inmunoaffinity studies

Immunoreactive fraction was determined by affinity thin layer chromatography (ATLC). Receptors

extracted from fresh human placenta donated from Laboratorio de Oncología Básica y Biología Molecular (LOBBM, Faculty of Medicine, Universidad de la República) were used. ITLC-SG was activated by 30 min heating at 110°C. The positive affinity chromatograms were prepared by seeding 200 µL receptor solution 0.5 mg/mL a third of the origin and further blocking with aqueous solution BSA 50 mg/mL, washed with distilled water, and dried mildly with forced air at room temperature at 37°C. Negative affinity chromatograms were performed similarly, but seeding fetal serum instead.

The labeling of HYNIC-Trastuzumab with ^{99m}Tc and physicochemical control were performed following the described protocol. Immediately, before seeding the activity on the positive and negative affinity chromatograms, it was diluted with fetal serum to a protein concentration of 2 mg/mL. The chromatograms were developed in saline phosphate buffer with 4% ethanol up to a height of 9 cm. The layers were allowed to dry and cut and counted in a well counter. The percent of immunoreactivity (% IR) was determined by the following formula

$$\%IR = \left(O/T\right) * 100 - \%UI$$

were O and T means the origin and total net activity of the positive affinity chromatogram and UI is the non-specific activity measured in a similar way on the negative affinity chromatogram. The UI% could differ from 8 to 20%. The % IR is typically 60-70% considering an antigen: antibody molar relationship of 100:1.

Biodistribution studies

Biodistribution in normal CD-1 female mice (n = 3) and bearing spontaneous breast tumor CD-1 mice (n = 3) was done at 2, 5, and 24 h after injection. Eleven MBq of ^{99m}Tc-HYNIC-Trastuzumab was administered *via* the tail vein to CD-1 female mice and they were later killed by cervical dislocation. Organs were dissected, weighed, and counted for activity in a NaI (Tl) well counter at different times. Percentage of activity in each organ and percentage of activity per gram were calculated.

Scintigraphy images

Static scintigraphy studies were performed at 2 (n = 3), 5 (n = 3), and 24 h (n = 3) post-injection (of 3.7 MBq of ^{99m}Tc-HYNIC-Trastuzumab) *via* the tail vein to female mice diseased with breast spontaneous adenocarcinoma. Procedures were approved by our local Animal Experimentation Ethics Committee. Mice were anesthetized with pentothal 20 mg/kg intraperitoneally in order to perform the studies. Scintigraphic images (obtained by Sopha camera, DXR rectangular) were visually analyzed.

Results

Labeling of HYNIC-trastuzumab with ^{99m}Tc

The amounts and ratio between Tricine and $SnCl_2.2H_2O$ were optimized, being the best molar ratio1:1 for a 92% yield of ^{99m}Tc-HYNIC-Trastuzumab for an activity of 259 MBq ^{99m}Tc added. The maximum specific activity reached was 8.28 MBq/µmol.

Radiochemical stability of labeled conjugate

The stability of the conjugate was checked after labeling. It was observed that ^{99m}Tc-HYNIC-Trastuzumab was Stable for 24 h post labeling at least [Figure 1].

HYNIC-trastuzumab conjugation

Purification by PD-10 column was performed to aim of separate the conjugate of HYNIC-Trastuzumab from free HYNIC. More than 80% of the initial amount of monoclonal antibody was recovered. The conjugate was not stable in solution when stored at 4°C, although it showed good storage stability for to 7 months when lyophilized and stored at 4°C [Figure 2].

Quality control

ITLC-SG, Whatman 3 MM and BSA saturated ITLC-SG

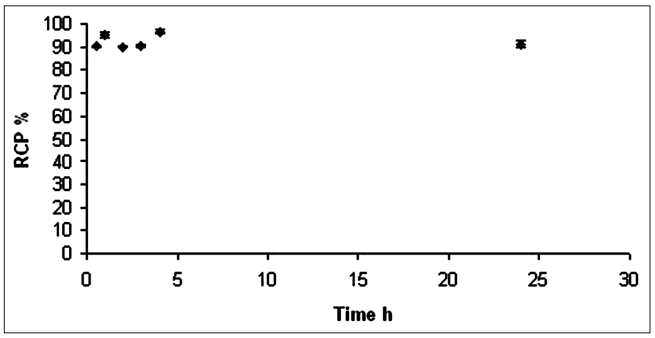

Figure 1: Radiochemical stability of ^{99m}Tc-HYNIC-trastuzumab. ^{99m}Tc-HYNIC-Trastuzumab was found to be stable for at least 24 h post-labeling

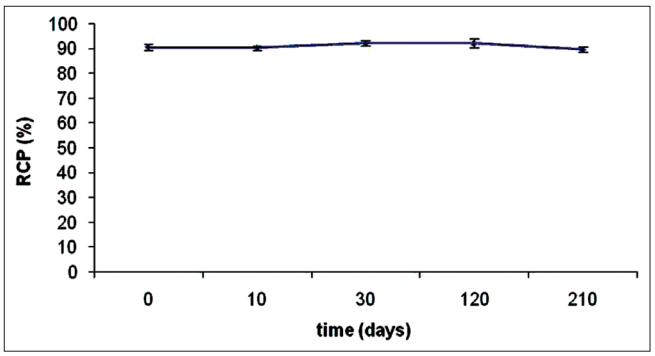

Figure 2: Stability of lyophilized HYNIC-trastuzumab. The conjugate showed good storage stability up 7 months when lyophilized and stored at 4°C

controls evidenced that ^{99m}Tc-HYNIC-Trastuzumab maintain a radiochemical purity higher than 90% under storage conditions as previously mentioned; both gamma detection and UV, shows one single peak corresponding to ^{99m}Tc-HYNIC-Trastuzumab [Figure 3].

Immunoaffinity studies

Immunoreactivity was performed in triplicate with a value of immunoreactivity of 66 ± 0.8% indicating a good affinity of ^{99m}Tc-HYNIC-Trastuzumab for the HER2 receptors extracted from placenta, therefore showing a good affinity for the HER2 receptors.

Biodistribution studies

Biodistribution data obtained in normal female CD-1 mice are shown in [Figure 4a]. This study in normal mice evidenced a low blood clearance with mainly hepatic excretion without significant uptake in other organs. Biodistribution of spontaneous adenocarcinoma diseased female CD-1 mice showed a significant uptake at tumor level even at 2 h post injection [Figure 4b]. However, the best tumor/muscle and tumor/blood was observed at 5 h.

Images

Scintigraphic images in mice bearing spontaneous adenocarcinoma also show a high liver uptake at 2 h, as well as significant tumor uptake.

Discussion

It has been found that 20-30% of breast cancers over-express HER2.[28] These patients with HER2 overexpression are associated with a poor prognosis, more disease relapse, and distant metastasis. Trastuzumab has been approved as a therapeutic agent for treatment of patient with overexpressed HER2 breast cancer.[29] Unfortunately, Trastuzumab treatment is expensive and has been associated with cardiac toxicity.[30] For these reasons, it is very important to evaluate HER.2 expression. Currently, It is evaluated by FISH or immunohistochemistry from a biopsy sample.

A minimally invasive *in vivo* characterization of the HER2 status of these patients would represent a qualitative advantage. This approach would be possible with the development of radiolabeled Trastuzumab. Such minimally invasive strategy could provide instant information about HER2 expression, as well as the receptor distribution within the tumor and enable a whole body scan looking for HER2 positive metastasis.

There are currently different strategies to label monoclonal antibodies with ^{99m}Tc;[31-33] several chelators are reported for ^{99m}Tc-labeling of proteins and biomolecules. A bifunctional agent widely used currently is the HYNIC. The use of aromatic hydrazine was first reported as bifunctional coupling agents for the ^{99m}Tc-labeling of polyclonal IgG for imaging inflammation and infection diseases.[23-27] Since then HYNIC technology has successfully been used for the ^{99m}Tc-labeling of antibodies and small biomolecule.[34-37]

Several factors could affect the labeling efficiency of a HYNIC, such as the chelator concentration, and reaction

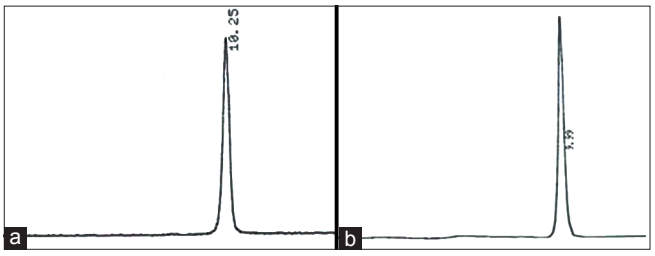

Figure 3: (a) HPLC with UV detection (λ = 280 nm) of HYNIC-Trastuzumab, (b) - HPLC with gamma detection of ^{99m}Tc-HYNIC-Trastuzumab. Both procedures revealed one single peak

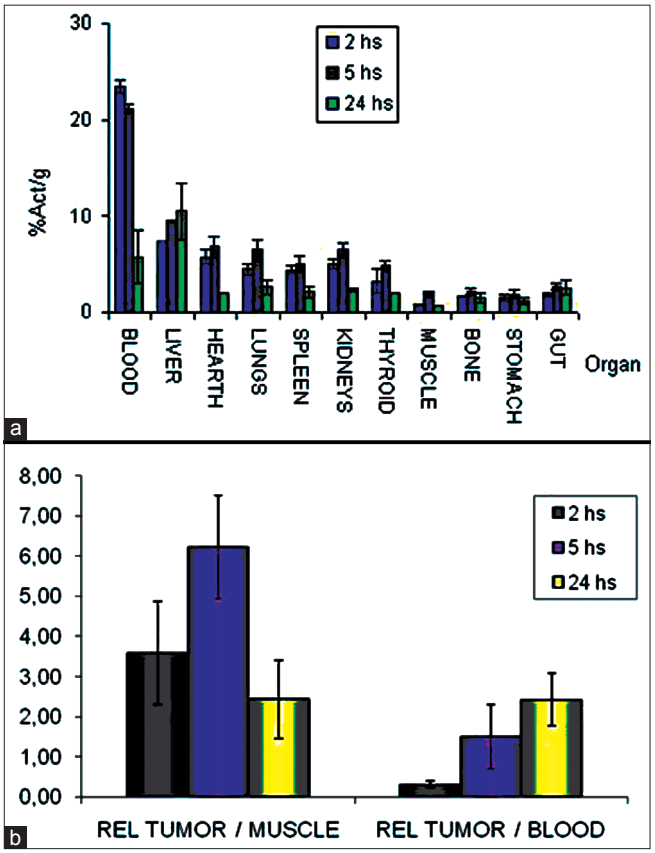

Figure 4: (a) Biodistributions at 2, 5, and 24 h in normal female CD-1 mice and (b) Biodistributions at 2, 5, and 24 h in mice with spontaneous breast adenocarcinoma. The results in normal mice revealed low blood clearance with mainly hepatic excretion without significant uptake in other organs; while the results in female CD-1mice carrying adenocarcinoma showed significant uptake in the tumor [Figure 4b]. The best tumor/muscle and tumor/blood ratios were observed at 5 h

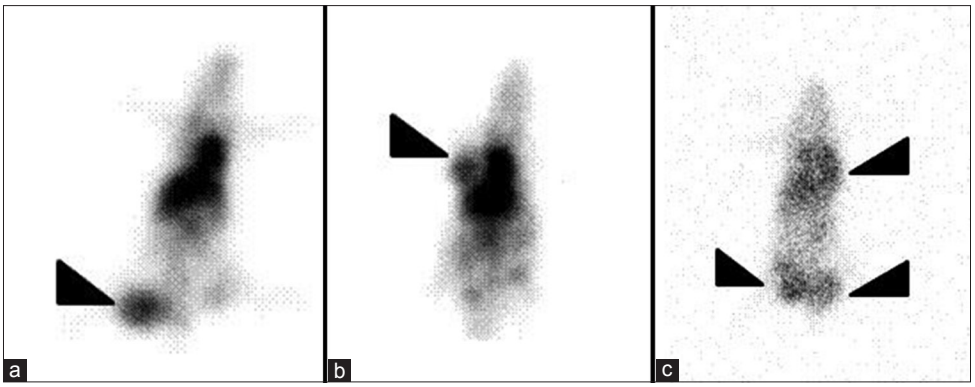

Figure 5: (a) Female CD-1 mouse with spontaneous breast adenocarcinoma at 2 h post injection of 11 MBq of ^{99m}Tc-HYNIC-Trastuzumab, (b) Female CD-1 mouse with spontaneous breast adenocarcinoma at 5 h post injection of 11 MBq of ^{99m}Tc-HYNIC-Trastuzumab and (c) Female CD-1 mouse with spontaneous breast adenocarcinoma at 24 h post injection of 111 MBq of ^{99m}Tc-HYNIC-Trastuzumab. Arrows indicate tumor sites in different mice

conditions (e.g., temperature, time, and the pH). The main advantages of HYNIC as a bifunctional agent is its high labelling efficiency, rapid and high-yield radiolabeling.

In this study, different conditions were assessed to optimize labeling procedure of ^{99m}Tc-HYNIC-Trastuzumab, achieving radiochemical purity higher than 90%. ^{99m}Tc-HYNIC-Trastuzumab showed *in vitro* stability for 24 h post-labeling, allowing enough time for managing controls and clinical application.

Stability of HYNIC-Trastuzumab conjugate was increased after lyophilization and storage at 4°C (controlled during 210 days) which would enable kit formulations. *In vitro* immunoaffinity control was optimized to evaluate the integrity of the antibody in a fast and easy assay. This approach allowed us to evaluate ^{99m}Tc-HYNIC-Trastuzumab before each injection.

Biodistribution in normal CD-1 mice showed a slow blood clearance, high liver uptake without significant kidney excretion, being significantly absent in other organs.

Biodistribution studies in female CD-1 mice with spontaneous breast adenocarcinoma, showed a significant uptake in the tumor from 2 h post-injection. However, best tumor/muscle and tumor/blood ratios were observed at 5 and 24 h, respectively, with a better contrast at 24 h [Figure 5].

Scintigraphic images showed good tumor uptake that was evidenced from 2 h until even 24 h. Liver uptake was consistent with the biodistributions at different times.

Conclusion

The use of 99mTc-HYNIC-Trastuzumab allow minimally invasive *in vivo* evaluation of breast tumors HER2 status in mice. This has the potential to substitute breast biopsy methods to establish HER2 status and also to be used to detect HER2 distant metastasis and evaluate Trastuzumab therapy response.

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