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Clinical translation of a PSMA inhibitor for ^{99m}Tc-based SPECT

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ABSTRACT

Background: Prostate-specific membrane antigen (PSMA) is highly over-expressed in advanced prostate cancers. ^{68}Ga-labeled PSMA inhibitors (iPSMA) are currently used for prostate cancer detection by PET imaging. The availability of simple, efficient and reproducible radiolabeling procedures is essential for developing new SPECT radiopharmaceuticals for clinical translation. The aim of this research was to prepare ^{99m}Tc-EDDA/HYNIC-Lys(Nal)-Urea-Glu (^{99m}Tc-EDDA/HYNIC-iPSMA) obtained from lyophilized kit formulations and evaluate the *in vitro* and *in vivo* radiopharmaceutical binding to prostate cancer cells over-expressing PSMA, as well as the ^{99m}Tc-EDDA/HYNIC-iPSMA normal biodistribution in humans and the preliminary uptake in patients with prostate cancer.
Methods: ^{99m}Tc labeling was performed by adding sodium pertechnetate solution and a 0.2 M phosphate buffer (pH 7.0) to a lyophilized formulation containing HYNIC-iPSMA, EDDA, tricine, mannitol and stannous chloride. The radiochemical purity was evaluated by reversed-phase HPLC and ITLC-SG analyses. Stability studies in human serum were performed by size-exclusion HPLC. *In vitro* cell uptake was tested using prostate cancer cells (LNCaP) with blocked and non-blocked receptors. Biodistribution and tumor uptake were determined in LNCaP tumor-bearing nude mice with blocked and non-blocked receptors, and images were obtained using a micro-SPECT/CT. Whole-body images from three healthy men and two patients with histologically-confirmed prostate cancer (one of them with a previous ^{68}Ga-PSMA-617scan) were acquired at 1 h and 3 h after ^{99m}Tc-EDDA/HYNIC-iPSMA administration with radiochemical purities of >98%.
Results: In vitro and *in vivo* studies showed high radiopharmaceutical stability in human serum, specific recognition for PSMA, high tumor uptake (10.22 ± 2.96% ID/g at 1 h) with rapid blood clearance and mainly kidney elimination. Preliminary images in patients demonstrated the ability of ^{99m}Tc-EDDA/HYNIC-iPSMA to detect tumors and metastases of prostate cancer as well as ^{68}Ga-PSMA-617 does.
Conclusions: The results obtained in this study warrant further dosimetry and clinical studies to determine the specificity and sensitivity of ^{99m}Tc-EDDA/HYNIC-iPSMA.

1. Introduction

The glutamate carboxypeptidase II enzyme, also known as prostate-specific membrane antigen (PSMA), is expressed in epithelial cells of the prostate and highly overexpressed in 95% of advanced prostate cancers (PCa). The expression levels of PSMA correlate directly to androgen independence, metastasis and progression of PCa [1]. Therefore, PSMA is an appropriate molecular target for imaging and radiotherapy of metastatic prostate cancer using specific radiopharmaceuticals.

PSMA contains Zn in the active center of the enzyme, which has allowed for the Lys(β-naphthyl alanine)-NH-CO-NH-Glu (Lys(Nal)-Urea-Glu) molecule to be proposed as an effective inhibitor of its activity [2]. In the specific chemical interaction, the three carboxylic groups of the Glu-Urea-Lys fragment interact electrostatically with the side chain peptides of the active site of PSMA; oxygen from urea is coordinated to zinc and the aromatic structure in Nal interacts with the hydrophobic active sites of the enzyme. In recent clinical trials, the application of two different ^{177}Lu-labeled PSMA inhibitors (iPSMA) has demonstrated a significant decrease in PSA levels in 70–80% of patients with PCa, without severe side effects, and with a significant increase in survival [3–7].

However, before the radiotherapeutic treatment, the radiopharmaceutical uptake in tumors or metastases should be evaluated by imaging to confirm whether the treatment would be useful for the patient. It is therefore necessary to use diagnostic PSMA inhibitors for molecular

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imaging by positron emission tomography (PET) or single photon emission computed tomography (SPECT). Of these two techniques, PET is the one with higher spatial resolution and sensitivity, and thus most iPSMA derivatives have been developed based on ^{68}Ga [8–11].

For SPECT imaging, several preclinical studies of ^{99m}Tc-iPSMA have been reported using the $[^{99m}Tc(H_2O)(CO)_3]^+$ and $[^{99m}TcO(N_3S)]^{-1}$ labeling approaches [12–14]. In order to match the sensitivity of SPECT with that of PET, it is necessary to develop more competitive SPECT molecules in affinity and feasibility of being labeled with ^{99m}Tc.

The successful translation of ^{99m}Tc-iPSMA to clinical application also depends on the transfer of expertise in the development of kits for the fast labeling of peptides. The technology of ^{99m}Tc-hydrazinonicotinyl (^{99m}Tc-HYNIC) involves an indirect labeling procedure, where HYNIC is conjugated to the peptide and ethylenediamine-N, N″-diacetic acid (EDDA) is used as a coligand to complete the technetium coordination sphere. Moreover, the labeling is easily obtained from freeze-dried kit formulations [15–17].

From fragment-based prediction calculations (ChemBioDraw Ultra program), it can also be evidenced that the heterocyclic ring of HYNIC could act as an additional lipophilic site in the HYNIC-Lys(Nal)-Urea-Glu molecule (CLogP of −1.53 for Lys(Nal)-Urea-Glu and CLogP of 0.70 for the heterocyclic ring of HYNIC). Therefore, a new iPSMA derivative containing HYNIC could improve the coupling to the hydrophobic active site of PSMA and the iPSMA affinity, besides the conventional use of HYNIC as a chelating agent for the ^{99m}Tc radiometal.

The aim of this research was to prepare ^{99m}Tc-EDDA/HYNIC-Lys(Nal)-Urea-Glu (^{99m}Tc-EDDA/HYNIC-iPSMA) obtained from lyophilized kit formulations and evaluate the *in vitro* and *in vivo* radiopharmaceutical binding to prostate cancer cells over-expressing PSMA, as well as the ^{99m}Tc-EDDA/HYNIC-iPSMA normal biodistribution in humans and the preliminary uptake in patients with prostate cancer.

2. Materials and methods

2.1. Synthesis of HYNIC-iPSMA

The HYNIC-iPSMA (hydrazinonicotinyl -Lys(Nal)-Urea-Glu) (MW 651.94 g/mol) peptide conjugate was designed at ININ (Instituto Nacional de Investigaciones Nucleares, Mexico) and synthesized with the support of Ontores Biotechnology Co., Ltd. (Zhejiang, China) with a purity >98% as analyzed by reversed phase HPLC (RP-HPLC). Briefly, the synthesis started with the di-tert-butyl ester of glutamic acid, which was reacted with carbonyldiimidazole (CDI) in the presence of triethylamine (TEA) to form the acyl imidazole derivative; this last compound was activated with triflate methyl (MeOTf) to react with (S)-ter-butyl-2-amino-6-(benzyloxycarbonylamine) hexanoate (Cbz-Lys-Ot-Bu) with the subsequent deprotection of Cbz *via* hydrogenolysis, obtaining the derivative Glu-Urea-Lys. The latter was reacted in solid phase (MBHA resin) with the Fmoc-β-naphthyl alanine (HBTU/HOBt) amino acid, followed by addition of the 6-Boc-hydrazinepyridin-3-carboxylic acid (Boc-HYNIC) in the presence of diisopropylethylamine (DIPEA) and dimethylformamide (DMF). The compound was deprotected with TFA, purified by HPLC and lyophilized. Finally, the molecular structure was characterized by ^{1}H–NMR (300 MHz, tetramethylsilane as internal reference), IR (ATR-FTIR) and mass (MALDI as ionization technique) spectroscopies.

2.2. HYNIC-iPSMA lyophilized formulations

Technetium-99 m-labeled HYNIC-iPSMA was prepared from a lyophilized formulation in which the amount of coligands and mannitol (diluent) added does not affect the characteristics of the peptide [16,18]. The procedure was performed under aseptic conditions in a GMP-certified facility. Briefly, 5 mg of HYNIC-iPSMA were dissolved in 98 mL of injectable-grade water (stirring and heating at 50 °C). Said solution was added to a solution of ethylenediamine-N,N′-diacetic acid (EDDA)-N-tris[hydroxymethyl]methylglycine (Tricine)-Mannitol that had previously been prepared by mixing 1.0 g of EDDA, 2.0 g of tricine and 5 g of mannitol in 100 mL of sterile, apyrogenic water and stirring at low heat. Finally, 2.0 mL of a freshly prepared stannous chloride solution (1 mg/mL in 0.012 M HCl) was added under a nitrogen atmosphere. The mixture was sterilized by membrane filtration (Millipore, 0.22 μm) and 2.0 mL was dispensed into 100 pre-sterilized serum vials and lyophilized for 24 h.

Additionally, 150 mL of a 0.2 M buffer phosphate (pH 7) solution was prepared under aseptic conditions, sterilized by membrane filtration (Millipore, 0.22 μm). Then, 5.0 mL was dispensed into pre-sterilized serum vials and stored at 4 °C.

Kits were tested for sterility and apyrogenicity using conventional pharmaceutical procedures. Kits were stable up to one year.

2.3. HYNIC-iPSMA techenetium-99 m labeling

^{99m}Tc-pertechnetate was obtained from a ^{99}Mo/^{99m}Tc generator (ININ-Mexico). Radiolabeling was performed by adding 1 mL of 0.2 M phosphate buffer (pH 7.0) to the freeze-dried kit formulation, followed by immediately adding 740–1110 MBq (1 mL) of ^{99m}Tc-pertechnetate and incubating at 95 °C in a block heater or in a boiling water bath for 10 min.

2.4. Evaluation of radiochemical purity

Radiochemical purity analyses were performed by instant thin-layer chromatography on silica gel (ITLC-SG, Gelman Sciences) and reversed-phase high-performance liquid chromatography (HPLC).

For the ITLC-SG analysis, 3 different mobile phases were used: 2-butanone, to determine the amount of free $^{99m}TcO_4^-$ ($R_f = 1$); 0.1 M sodium citrate (pH 5), to determine the amount of ^{99m}Tc-coligand and $^{99m}TcO_4^-$ ($R_f = 1$); methanol: 1 M ammonium acetate (1:1 *v*/v), to determine the amount of ^{99m}Tc-colloid ($R_f = 0$). R_f values of the radiolabeled peptide in each system were 0.0, 0.0 and 0.7–1.0, respectively.

HPLC analyses were performed with a Waters instrument running Empower software with both radioactivity and UV-photodiode array in-line detectors and a μBondapak C_{18} column (5 μm, 3.9 × 300 mm). A gradient using 0.1% TFA/water as solvent A and 0.1% TFA/acetonitrile as solvent B was used at a flow rate of 1 mL/min. The gradient began at 100% solvent A for 3 min, changed to 50% solvent A over 10 min and was maintained for 10 min, changed to 30% solvent A over 3 min and finally returned to 100% solvent A over 4 min. In this system, retention times for free $^{99m}TcO_4^-$, ^{99m}Tc-coligand and ^{99m}Tc-EDDA/HYNIC-iPSMA were 3–3.6 min, 4.0–5.0 min and 11–12 min respectively.

2.5. In vitro studies

2.5.1. Serum stability

Size exclusion HPLC analysis (ProteinPak 300SW column, Waters, 0.01 M PBS, flow 1 mL/min) and ITLC-SG were used to estimate the serum stability of ^{99m}Tc-EDDA/HYNIC-iPSMA. A 50 μL volume of labeled peptide solution (0.5 μg/50 μL) was incubated at 37 °C with 1 mL of fresh human serum. Radiochemical stability was determined from 10 μl samples taken at 1 h and 24 h for analysis. A shift in the HPLC radioactivity profile to higher molecular weight indicated protein binding, while peaks of lower molecular weight indicated labeled catabolites or serum cysteine binding.

2.5.2. Cell uptake and non-specific binding

LNCaP (PSMA-positive) human prostate cancer cells and PC-3 (PSMA-negative) human prostate cancer cells were originally obtained from ATCC (USA). The cells were routinely grown at 37 °C with 5% CO_2 and 85% humidity in Roswell Park Memorial Institute medium (RPMI, Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum and