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Development and evaluation of a ^{99m}Tc(V)-nitrido complex derived from estradiol for breast cancer imaging



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Emilia Tejería, Javier Giglio**, Leticia Fernández, Ana Rey*

Área de Radioquímica, Facultad de Química, General Flores 2124, Universidad de La República, 11800, Montevideo, Uruguay

HIGHLIGTHS

G R A P H I C A L A B S T R A C T

- The uptake of the complex in breast cancer cells was comparable with that of tritiated estradiol.
- The blocking study suggest specific interaction with the receptors.
- Biodistribution studies show lower liver uptake than [¹⁸F]FES.
- The evaluation in nude mice with ER + tumors showed a good tumor/ muscle ratio.

A novel estradiol derivative bearing a dithiocarbamate group has been synthesized. Dithiocarbamate acts as bidentate ligand to generate a Tc(V) nitride symmetric complex with two units of pharmacophore. The radiolabelled compound has adequate physicochemical and biological properties, superior to other previously reported technetium labeled ER receptor ligands.



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ABSTRACT

Estrogen receptors are overexpressed in 70% of breast cancer and identification of their presence is important to select the appropriate treatment. This work proposes the preparation and evaluation of an estradiol derived as potential ER imaging agent. Ethinylestradiol was derivatized to introduce a dithiocarbamate function for Tc coordination. Labeling was achieved through the formation of a symmetric Tc(V)-nitrido complex with a radiochemical purity (RCP) > 95%. Physicochemical evaluation, cell uptake, biodistribution in normal animals and in nude mice bearing induced ER + breast tumors showed promising results.

1. Introduction

Cancer is a relevant health problem in Uruguay since almost 16,000 new cases and 8000 deaths are registered every year. In particular, breast cancer has the highest incidence and mortality among women with around 9000 new cases and more than 600 death in the period 2010–2014 (CHLCC, 2018). Sex hormones play an important role in the development of breast cancer through the formation of hormone-receptor complexes in tumor cells that act as transcription factors

signaling pathways that induce proliferation and tumor growth (De Vries et al., 2010). 70% of the breast cancers overexpress hormone receptors, specially estrogen receptors (ER) (Munnink et al., 2009; Van Kruchten et al., 2012; Nayak et al., 2008).

The ER is a very important biomarker in breast cancer often guiding treatment planning. Tumors with higher concentration of ERs (ER+) tend to respond to anti-estrogen hormone therapy, whereas ER-negative (ER-) tumors require a different treatment approach (Xiaotian et al., 2016; Takahashi et al., 2007).

* Corresponding author. Área Radioquímica, Facultad de Química, Universidad de la República, C.C. 1157, Montevideo, Uruguay.

- ** Corresponding author. Área Radioquímica, Facultad de Química, Universidad de la República, C.C. 1157, Montevideo, Uruguay.
- E-mail addresses: jgiglio@fq.edu.uy (J. Giglio), arey@fq.edu.uy (A. Rey).

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Received 29 May 2019; Received in revised form 2 August 2019; Accepted 11 August 2019 Available online 13 August 2019 0969-8043/ © 2019 Published by Elsevier Ltd. Molecular imaging plays a very important role in the characterization of breast cancer overcoming the problem of tumor heterogeneity that severely compromises treatment outcome. A better understanding of tumor biology may be obtained by Nuclear Medicine procedures which provide information of the whole tumor mass in comparison to the limitations of classical biopsies (Kurdziel et al., 2008; Hargreaves, 2008; Youk et al., 2007; Lee, 2013; Greene and Wilkinson, 2015).

Since 1980s many radiolabeled steroids have been evaluated as PET and SPECT tracers for ER imaging but most of them failed in preclinical or early-clinical evaluation (De Vries et al., 2010). So far only 16α -[¹⁸F] fluoro-17 β -estradiol ([¹⁸F]FES) has been successfully used as PET tracer for ER imaging in breast cancer patients (De Vries et al., 2010; Neto et al., 2012). [¹⁸F]FES has become the reference method due to its high sensitivity and specificity (Linden et al., 2006). However, the use of ^{99m}Tc radiopharmaceuticals is still desirable due to the widespread availability and low comparative cost. Furthermore, the development of analogous ^{186/188}Re compounds for therapy is an additional advantage (Jürgens et al., 2014).

The design of potential ^{99m}Tc radiopharmaceuticals takes advantage of the rich coordination chemistry of the metal, which can be found in oxidation states from -I to VII and combined with a variety of chelators. Katzenellenbogen and co-workers first used the N2S2 donor atom system in the form of bisamino-bisthiol (BAT) and monoamine-monoamide (MAMA) derivatives as well as N2S chelators for the design of estrogen and progestin derivatives. Lipophilicity and overall size are the main properties influencing the biological behavior of the resulting Tc (V) = O complexes (Dizio et al., 1991a; Dizio et al., 1992b, O'neil et al., 1994a, O'neil et al., 1994b). A similar approach was followed by Spies and Johannsen with the introduction of the SXS (X = N,O,S) 3 + 1mixed ligand approach and the NS₃ 4 + 1 complexes rendering less voluminous species (Spies and Johannsen, 1995; Wüst et al, 1996a, 1998b; Skaddan et al., 1999) (Fig. 1). More recently, Alberto et al. introduced the Tc(I) tricarbonyl core that combined with various monodentate and chelating ligands has extensively been applied for the development of novel radiopharmaceuticals due to its high in vivo stability (Alberto et al., 1998; Schibli et al., 2000).

All these efforts demonstrate that linking a radiometal chelate to a pharmacophore without affecting the biological properties is not a simple task. The design of the suitable chelator is crucial in obtaining the desired biological stability and pharmacokinetics of the radio-pharmaceutical (Le Bideau and Dagorne, 2013; Pasqualini and Duatti, 1992; Bandoli et al., 2001; Bolzati et al., 2004a,b).

Our group has been working on the design of a family of complexes for breast cancer imaging based on the structure of ethinylestradiol, agonist of ER (Tejería et al., 2017). The terminal alkyne has been chemically modified to introduce different donor atom sets to coordinate the 99m Tc in the search of a derivative with enhanced biological properties. In this paper we present the preparation and evaluation of a novel $^{99m}Tc(V)$ symmetric nitrido complex bearing 2 estradiol moieties. Complexes bearing the $[TcN]^{+2}$ core can be easily obtained by reduction of pertechnetate in the presence of succinyl dihydrazide and offer the advantage to be virtually redox inert and stable over a wide range of pH values, thereby constituting a class of interesting candidates for nuclear medicine applications. Furthermore, symmetric nitrido complexes bear two units of the pharmacophoric group, which according to results reporter by other researchers can potentially increase the affinity with the target receptors due to the positive effect in binding of dimerization or multimerization.

An estradiol derivative bearing a dithiocarobamate for Tc coordination has been synthesized labeled with ^{99m}Tc. The physicochemical evaluation of the labeled compound included stability studies in labeling milieu and in the presence of human plasma, protein binding, and lipophilicity. In vitro evaluation was performed to study the interaction with ER receptors in MCF7 cells. Biodistribution studies in normal rats were also developed to evaluate pharmacokinetic properties.

2. Experimental

2.1. General

All chemicals were reagent grade and were used without further purification. Thin-layer chromatography (TLC) was performed on percolated silica gel plates (Sigma). The developer used was commercial anisaldehyde. 1H Nuclear magnetic resonance (NMR) was obtained at 400 MHz in deuterated dimethyl sulfoxide or methanol (Bruker DPX-400), ¹³C NMR was recorded in deuterated chloroform at 125.8 MHz (Bruker DRX500). The chemical shifts, δ , are reported in ppm (parts per million) relative to residual solvent peaks. The multiplicity in the ¹H NMR is defined by s(singlet), dd(Doublet doublet), or m (multiplet). Mass spectra were determined on an Applied Biosystem API 2000 (ESI-MS). IR spectra were determined on Fourier transform infrared spectrophotometer, Shimadzu IR-Affinity-1, using KBr tablets. [99mTc] NaTcO₄ was obtained from a commercial ⁹⁹Mo/^{99m}Tc generator (Tecnonuclear, Argentina). Solvents for chromatographic analysis were HPLC grade. The HPLC analysis was developed on a LC-10 AS Shimadzu Liquid Chromatography System using a reversed-phase column (Waters 10 µ, C18, 30 cm). Elution was performed with a binary gradient system at 1.0 mL/min Flow rate using trifluoroacetic acid 0.1% in water (A) and trifluoroacetic acid 0.1% in Acetonitrile (B). The elution profile was as follows: 0-3 min 100% A; 3-22 min 0%A and 22-24 min 100%A. Detection was accomplished either with a photodiode array detector (SPDM10A, Shimadzu) that recorded UV-Vis spectra on flux or with a 3×3 in. NaI (Tl) crystal scintillation detector. Activity measurements



Fig. 1. Examples of previously reported estrogen derivatives bearing different chelators and complexation chemistry.

were performed either with a dose calibrator (Capintec CRC-5R) or with a scintillation counter (3×3 in. NaI (Tl) crystal detector) attached to an ORTEC monochannel analyzer. Tritiated estradiol used was (Estradiol [6.7-³H(N)]), American Radiolabeled Chemicals, Inc, Siracusa, USA. Activity measurements of tritiated estradiol were performed in a Liquid scintillation counter, MicroBetaTriLux Wallac-1450.

2.2. Synthesis of (sodium ((1S)-1-carboxy-2-(4-((8S,9S,14S,17S)-3,17dihydroxy-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a] phenanthren – 17-yl)-1H-1,2,3-triazol-1-yl)ethyl)carbamodithioate) (L)

2.2.1. Synthesis of 17[Boc-2-amino-3-(1H-1,2,3-triazol-1-yl)propanoic acid] estradiol (A)

Equimolecular amounts of N-Boc-azidoalanine (200 m- 0.48 mmol) and ethinylestradiol (144 mg-0.48 mmol) in tert-Butanol, were mixed with ascorbic acid (19 mg-0.096 mmol) and copper acetate (38.4 mg-0.21 mmol) in water and reacted at room temperature for 6 days with continuous stirring. After completion of the reaction, extractions were performed with ethyl acetate. The organic fractions were dried and the solvent was evaporated under vacuum. (MW: 526.6 g/mol). ¹H NMR (400 MHz,(CD₃OD) δ (ppm): 7.8 (s,1H), 7.0 (d, 1H), 6.5 (dd, 2H), 4.6 (m, 3H), 2.8 (m,3H), 2.0 (m,2H), 1.4 (m, 19H), 1.1 (s,3H).MS (EI, 10 eV) m/z: 524.9 (M) 426.7.

2.2.2. Synthesis of 17 [2-amino-3-(1H-1,2,3-triazol-1-yl)propanoic acid] estradiol (B)

A mixture of A (242 mg, 0.46 mmol), dry dichloromethane (CH2Cl2) (20 mL) and trifluoroacetic acid (TFA) (436 μ L- 5.69 mmol) was stirred a room temperature for 2 days in nitrogen atmosphere protected from light. After completion of the reaction the solvent was evaporated under vacuum. (MW: 426.5 g/mol)

2.2.3. Synthesis of 17[sodium 1-carboxy-2-(1H-1,2,3-triazol-1- yl) ethyl) carbamodithioate] estradiol (L)

B (204 mg- 0.48 mmol) and dry THF (20 mL) was cooled to 0 °C, and carbon disulfide (CS₂) (0.4 mL - 6.63 mmol) and sodium hydride (NaH) (90 mg - 3.75 mmol) were added. The mixture was heated for 2 h at 65 °C. The solvent was evaporated under vacuum. (MW: 524.6 g/mol). ¹H NMR (400 MHz, (CD₃)₂SO) δ(ppm): 7.6 (s,1H), 6.9 (d, 1H), 6.5 (dd, 2H), 3.6 (m, 3H), 2.7 (m,3H), 2.1 (m,2H), 1.2 (m, 10H), 0.9 (s,3H). ¹³C (125 MHz, CDCl₃)δ(ppm): 166.97, 155.29, 149.92, 147.59, 145.26, 144.19, 142.93, 141.85, 98.75, 84.20, 82.17, 73.08, 69.29, 62.46, 60.26, 58.84, 57.69, 56.90, 55.71, 55.09, 54.28, 51.89, 43.00, 42.59. MS (EI, 10 eV) m/z: 525.4 (M+) 505.3, 496.4. IR (KBr): (CS₂) Dithiocarbamate bands 1139, 1207 cm⁻¹.

2.3. Radiolabeling

2.3.1. Synthesis of the nitrido precursor

The nitrido precursor was obtained by reacting succinic dihydrazide (5 mg in 0.5 mL of 0.9% NaCl) with 0.1 mL of stannous chloride (SnCl₂) solution (1 mg/mL in water) and [^{99m}Tc]NaTcO₄- (185–1850 MBq in 0.5–1 mL) at room temperature for 20 min. The RCP was determined by thin layer chromatography on Whatman 1 solid phase using acetone as mobile phase (Rf = 0 nitrido precursor, Rf = 1 [^{99m}Tc]TcO₄-) (Pasqualini and Duatti, 1992; Giglio et al., 2011).

2.3.2. Preparation of complex C

Substitution by L (3 mg, 0.0057 mmol, 300 μ L methanol) was achieved by incubation with 0.1 mL of nitrido precursor (50–350 MBq) for 30 min at 65 °C. RCP was assessed by HPLC using the chromatographic conditions described in Section 2.1.

2.4. Physicochemical evaluation

2.4.1. Stability in labeling milieu

Stability of the $^{99\rm m}{\rm Tc}$ -complex (C) was assessed by HPLC using the chromatographic conditions described in 2.1 for up to 3 h after labeling. $_{20}$

2.4.2. Stability in human serum

C (100 μ L) was incubated in 1000 μ L of human plasma at 37 °C. Samples (200 μ L) were extracted at 1 h, 2 h and 3 h. Proteins were precipitated with 200 μ L cold absolute ethanol, shaken on a Vortex shaker and cooled in the freezer for 5 min. Samples were centrifuged at 100g for 5 min at 0 °C and RCP of the supernatant was evaluated by HPLC using the conditions described in 2.1 (Tejería et al., 2017).

2.4.3. Lipophilicity

The lipophilicity was studied at pH 7.4 through the determination of the distribution coefficient octanol/buffer phosphate 0.1M. C (100 μ L) was mixed in a centrifuge tube with 2 mL of n-octanol and 1.9 mL of phosphate buffer 0.1M pH = 7.4. The mixture was shaken on a Vortex shaker for 2 min and finally centrifuged for 5 min. Two samples (100 μ L) of each phase were counted in a gamma counter. The distribution coefficient was calculated as the log of the mean counts per mililiter in the octanol phase divided by that of the buffer (Tejería et al., 2017).

2.4.4. Protein-binding studies

Plasma protein-binding was assessed by the incubation of C ($25 \,\mu$ L) in human serum ($475 \,\mu$ L) for 30 and 60 min at 37 °C. The protein bound activity was determined by molecular exclusion using microspin G50 columns (GE Health care). Aliquots ($25 \,\mu$ L) were added to the column which had been pre-spun at 716 g for 1 min. Columns were centrifuged again at 716 g for 1 min, and the collected eluate and the column were counted in a gamma counter. Protein bound fraction was calculated as the percentage of activity eluted from the column (Tejería et al., 2017).

2.5. Biological evaluation

2.5.1. Studies in MCF7 cells

The cell culture studies were performed using the adherent cell line MCF7 (ATCC HTB-22) corresponding to human mammary adenocarcinoma. Cells were cultured in Dulbecco's Modified Eagle's medium (A1316, 9050 PanReac AppliChem, ITW Reagents) supplemented with 10% fetal bovine serum (Gibco, TermoFisher Scientific), penicillin 100 U/mL (Sigma) and streptomycin 100 µg/mL (Sigma) in T75 culture flasks (Greiner bio-one) at 37 °C and 5% CO₂ until confluence in the monolayer.

2.5.1.1. Cell uptake studies. The cells were incubated with C for 1, 2 and 4 h at 37 $^{\circ}$ C and 5% CO₂. After the incubation time had elapsed, the culture medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) and treated with trypsin-EDTA. Finally, the activity in the supernatant and the cells was measured in a scintillation counter. Results were expressed as percentage of the activity bound to cells (Xiaotian et al., 2016).

Tritiated estradiol (estradiol $[6,7-^{3}H(N)]$ was used as reference. In brief, 0.8 MBq of tritiated estradiol was incubated with cells for 1 h in the above described conditions. A sample of 300 uL of either supernatant or cell was mixed with 3000 uL of scintillation cocktail (LSC PerkinElmer Ultima Gold-XR cocktails) in a liquid scintillation counter for and the activity determined using the internal standard method.

2.5.1.2. Blocking assay. Cells were incubated with C (0.8 MBq, 4×10^{-6} M per flask) in the presence of increasing concentrations of unlabeled ligand L (4×10^{-5} -4x10⁻⁴M) for 1 h at 37 °C and 5% CO₂. After the incubation the culture milieu was removed and cells were

washed twice with PBS and treated with Trypsin-EDTA. The activity in the supernatant and the activity in the cells were measured in a solid scintillation counter. Uptake was expressed as the percentage of total incubation activity in the cells (Xiaotian et al., 2016).

2.5.2. Biodistribution studies

All procedures were carried out following the national legislation and were approved by the National commission of animal experimentation (CNEA)

2.5.2.1. Biodistributions in normal animals. All animal studies were performed in compliance with the national laws of animal experimentation and approved by the Ethics Committee of the Faculty of Chemistry, Universidad de la República (Uruguay). Wistar rats (3 months old, female, 200–250 g, 3 animals per group) were injected via a lateral tail vein with C (0.8 MBq in 0.1 mL per rat). After the established biodistribution time (30 min, 1 h, and 2 h) the animals were sacrificed by cervical dislocation under anesthesia (thiopental 0.4 mL per rat, 10 min before the sacrifice). Whole organs and samples of blood and muscle, bone and urine were collected, weighed, and assayed for radioactivity. Correction by different sample geometry were applied when necessary. Results were expressed as percentage dose per gram of tissue (Tejería et al., 2017).

2.5.2.2. Biodistribution in animals bearing induced breast tumors. A suspension of MCF-7 breast tumor cells (3×10^6) in Geltrex (Gibco):phosphate-buffered saline (50:50) was injected subcutaneously into left flank of nude mice (4–6 week old). Tumor growth was stimulated by the daily application of dermal gel containing estradiol (Oestrogel Servimedic), until the week prior to biodistribution (Behzadi et al., 2015; Lee and Choi, 2013; Kim et al., 2016).

After 30–45 days tumors were of adequate size and animals were injected via a lateral tail vein with C (0.1 ml, 1.5 MBq). Biodistribution was performed according to the above described procedure. Results were expressed as percentage dose per gram of tissue. Relationship tumor/muscle and tumor/blood were calculated.

3. Results and discussion

3.1. Synthesis of L

The synthesis of the estradiol derivative L was performed by a three step procedure shown in Fig. 2. Ethinylestradiol was selected as starting material because it is a commercially available agonist of the ER and bears a terminal alkyne that can be easily derivatized by a Huisgen 3 + 2 cycloaddition catalyzed by Cu(I). The terminal alkyne of ethinylestradiol reacts with an azide (N-BOC-azidoalanine) to form a triazole. Cu(I) is generated in situ by the action of ascorbic acid in the presence of Cu(II). (Fig. 2). This so-called "click chemistry" reaction has been extensively applied to the preparation of ligands for ^{99m}Tc labeling by our group (Tejería et al., 2017; Fernández et al., 2012a; Fernández et al., 2012b) and other researchers (Mindt et al., 2006; Kluba and Mindt, 2013; Struthers et al., 2010; Worrell et al., 2012; Singh et al., 2016; Wanga et al., 2016) due to the selectivity that simplifies purification procedures. The product was characterized by proton NMR. Results were consistent with the expected structure. The second step in the synthetic sequence was the deprotection of the amino group that was performed according to a previously described procedure (Wuts and Greene, 2006). The final step in the preparation of L was developed following the technique described by Goubert (Goubert et al., 2007) and involves the incorporation of the dithiocarbamate function by reacting the amino group with carbon disulfide and sodium hydride. The product was characterized by NMR, mass spectrometry and infrared spectroscopy. Results were in accordance with the proposed structure. The ligand L combines the active part of estradiol responsible for the interaction with the estrogen receptor (Jerevaa et al., 2017) and the dithiocarbamate group that acts as electron donor suitable for the coordination of Tc through the formation of a symmetric ^{99m}Tc(V) nitrido complex.

3.2. Synthesis of ^{99m}Tc- complex (C)

Labeling of L with ^{99m}Tc was performed through the formation of a symmetric nitrido complex. This type of complexes is characterized by the presence of a technetium-nitrogen triple bond that leaves 4 coordination positions available for ligand binding. They can be filled by 2 identical or different bidentate ligands, giving rise to symmetric or asymmetric complexes, respectively. The symmetric complexes complete the coordination sphere with 2 identical molecules of a bidentate donor π ligand, usually dithiocarbamates. The resulting arrangement of atoms exhibits a very high chemical stability towards both oxidation and reduction reactions involving the technetium ion and pH variations (Dehnickc and Strahie, 1992; Marchi et al., 1990; Bolzati et al., 2004a,b; Pasqualini et al., 1994; Rey, 2009). Due to these reasons the Tc-nitrido core is considered an excellent alternative for the preparation of potential radiopharmaceuticals (Giglio et al., 2011; Liu, 2008; Fernández et al., 2011).

The preparation of the 99mTc complex was performed by



Fig. 2. Synthesis of estradiol derivative (L).



Fig. 3. Labeling with ^{99m}Tc.



Fig. 4. High-performance liquid chromatography profile of the nitrido precursor and the 99m Tc complex C.

substitution starting from a suitable precursor containing the metal in the required oxidation state. This precursor was obtained in aqueous medium (NaCl 0.9%) using SnCl₂ as reducing agent and succinic dihydrazide (SDH = H₂NNHCOCH₂CH₂CONHNH₂) as nitrido source. The reaction was developed at room temperature in 20 min (Fig. 3). The RCP of the precursor was determined by thin layer chromatography and was higher than 95% in all cases. The substitution of the nitrido precursor with L was optimized to obtain a final complex with adequate RCP.²¹. The reaction yielded a single species with a RCP > 90% and a retention time of 11.7 min determined by HPLC. Substitution was also followed stepwise by HPLC since the precursor has a retention time of aprox. 3 min (Fig. 4).

3.3. Physicochemical evaluation

3.3.1. Stability studies

Stability of C was studied in labeling milieu and in human serum The RCP at different incubation times was evaluated by HPLC using the method described in 2.1. C showed high stability in both conditions for at least 3 h after labeling with a RCP higher than 95% over the studied period. These results are consistent with our hypothesis and very promising for a potential radiopharmaceutical.

3.3.2. Lipophilicity

The lipophilicity was studied at pH 7.4. A log D of 0.8 ± 0.1 was obtained for C. This value is consistent with a neutral and lipophilic compound, correlating with the proposed structure (Fig. 2). According to the lipophilicity it is expected that C could penetrate biological membranes (log D between -0.5 and 2 at pH = 7.4) according to the literature (Xiangli et al., 2011). The lipophilicity of C is lower than the lipophilicity of natural estradiol (Loftsson, 2015).

3.3.3. Protein-binding studies

Protein binding was determined by size exclusion chromatography. A low protein binding is desirable to achieve a favorable pharmacokinetics of the potential radiopharmaceutical (Schmidt et al., 2010). It also correlates with the stability, since weakly bound ligands tend to be substituted by donor groups from the blood proteins increasing protein binding results (Fernández et al., 2012a,b). However, many endogenous substances like lipids, vitamins and hormones are physiologically transported by plasma proteins. Moreover, according to the literature estradiol and related hormones are bound to testosteroneestradiol binding globulin (Te-BG) and to albumin in more than 90% (Schmidt et al., 2010; [datasheet]. Estradiol. Catalog No.S1709; De Vries et al., 2010; Wu et al., 1976; Pacifici et al., 1989). The ethinylestradiol our staring material of synthesis has a protein binding of 98.3 \pm 0.6 according to the literature (Kuhnz et al., 1990). ^{99m}Tc-derivative C exhibited a moderate protein binding $(41 \pm 9)\%$ during all the studied period, significantly lower than that of ethinylestradiol.

3.4. Biological evaluation

3.4.1. Studies in MCF7 cells

In vitro cell studies were performed in MCF-7 human mammary adenocarcinoma cells This is an adherent cell line that express the estrogen receptors ([(datasheet MCF7 ATCC[®] HTB-22[™]]) and consequently is an adequate model to study the ER binding ability of the potential radiopharmaceutical.

The uptake of C was determined at 3 incubation times 1, 2 and 4 h. Results expressed as percentage of incubation activity found in the cells are shown in Fig. 5 and Table 1. The percentage of uptake was stable throughout the studied period. The mean value for the period was



Table 1

In vitro cell uptake.

Complex	Time incubation (hours)	% Uptake
С	1	6.0 ± 1.3
С	2	6.7 ± 0.5
С	4	7.8 ± 2.3
Tritiated Estradiol	1	$6.6~\pm~1.4$

Values are expressed as mean \pm S.D (n = 3).

6.8 \pm 0.9%. In order to validate our experimental procedure we reproduced the uptake assay (1 h of incubation) using tritiated estradiol (Estradiol [6.7-³H (N)]), American Radiolabeled Chemicals, Inc, Siracusa, USA.

Estradiol is the natural molecule binding to the ER and consequently its uptake by breast cancer cells is considered as reference. The uptake at 1 h of incubation was (6.6 \pm 1.4)% (Table 1), This result is comparable to that obtained for C in exactly the same experimental conditions and much higher than that of other ^{99m}Tc-labeled estradiol derivative already published by our group (2.0 \pm 0.2)% [20] and of other Tc labeled estradiol derivatives from the literature (6.0%, 0.55% and 1.55%) (Xiaotian et al., 2016; Takahashi et al., 2007). This high uptake is very promising and could be explained taking into consideration that C is a symmetric Tc(V)-nitrido complex bearing two units of the pharmacophoric group. Our findings are in agreement with other researchers that have reported the positive effect in binding of dimerization or multimerization, in particular in potential radiopharmaceuticals for targeting RGD peptides, hypoxia, among others (Huang et al., 2012; Wang et al., 2015; Debordeaux et al., 2015).

A blocking study was also performed to study the inhibition of the uptake of the radiotracer by competition with increasing concentrations of cold ligand (from 10 to 100 fold higher to that present in the radiolabeled C) to asses specificity of the interaction with the tumor cells (Bigott-Hennkens et al., 2008). Unlabeled estradiol derivative L was used as blocking agent since estradiol in highly insoluble in water and other biologically compatible solvents. Results are shown in Fig. 6. The uptake without blocking agent was significantly reduced by the competition with cold ligand resulting in a gradual decrease in uptake from 50% to 70% of the initial value. This result indicates that an specific interaction could be responsible of the uptake of C within the cell.

3.4.2. Biodistribution in normal animals

The in vivo behavior of C was evaluated by biodistribution studies in Wistar rats between 30 min and 2 h after the intravenous injection of the radiotracer. Results expressed as % dose/g in the principal organs are shown in Table 2.

C showed low thyroid activity, indicating negligible reoxidation $(0.17 \pm 0.11)\%/g$ at 2 h post injection. Despite the intermediate values of lipophilicity and protein binding, initial blood activity was relatively low $(0.53 \pm 0.19\%/g)$ at 30 min post-injection and remains



Fig. 6. Blocking study.

Table 2

% Injected activity/g in the most significant organs as a function time in normal animals.

Organ	% Injected Activity. g ⁻¹			
	30 min	60 min	120 min	
Blood	0.53 ± 0.19	0.51 ± 0.15	0.55 ± 0.02	
Liver	1.52 ± 0.28	1.16 ± 0.02	1.14 ± 0.25	
Heart	0.18 ± 0.13	0.19 ± 0.08	0.17 ± 0.04	
Spleen	0.60 ± 0.29	3.00 ± 0.69	4.95 ± 1.67	
Kydneys	0.76 ± 0.30	1.40 ± 0.38	2.57 ± 0.86	
Thyroid	0.97 ± 1.54	0.07 ± 0.06	0.17 ± 0.11	
Muscle	$0.16~\pm~0.18$	$0.11~\pm~0.12$	$0.31~\pm~0.48$	

Values are expressed as mean \pm S.D (n = 3).

constant during the 2 h of study. Liver uptake was moderate showing a % dose/g of 1.52 ± 0.28 , 1.16 ± 0.02 and $1.14 \pm 0.25\%$ at 30 min, 1 h and 2 h post-injection, respectively. Uptake in liver is significantly lower than that of a^{99m}Tc-labeled estradiol tricarbonyl derivative previously obtained by our group (Tejería et al., 2017). Comparison with [¹⁸F]FES, the gold standard for estrogen receptors imaging showed that blood uptake follows a similar clearance profile, although activity is slightly higher for C (0.16 ± 0.03 compared to 0.53 ± 0.19 at 30 min Post inj.) (Klesewetter et al., 1984). However, liver uptake is significantly lower for C (1.52 ± 0.28 compared to 2.10 ± 0.67 at 30 min).

The major excretion pathway of C is the hepatobiliary system (36.4 \pm 5.6% in intestines at 2-h post-injection) with only a small percentage of activity in the urine.

3.4.3. Biodistribution in animals with breast tumor

In order to assess the potentiality of our approach for the design of potential radiopharmaceuticals for nuclear oncology, evaluation of C in nude mice bearing tumors induced by inoculation of MCF-7 breast tumor cell was performed. The main results and the relationship between tumor/muscle and tumor/blood are shown in Table 3. The % injected acivity/g in the tumor (0.59 \pm 0.12) is five times higher than

Table 3					
Uptake in	ER	+	induced	breast	tumors.

Organ	% Injected Activity. g^{-1}		
	60 min		
Blood	2.12 ± 0.05		
Muscle	0.16 ± 0.01		
Tumor	$0.59~\pm~0.12$		
Tumor/Muscle	3.59 ± 0.63		
Tumor/Blood	0.35 ± 0.19		

Values are expressed as mean \pm S.D (n = 3).

the percentage in the muscle (0.16 \pm 0.01). This results show that the radiotracer has a high affinity for the tumor. The relationship tumor/ muscle is 3.59 \pm 0.63. This value is comparable with others tracers previously reported (3.94 \pm 0.7) (Xiaotian et al., 2016).

4. Conclusions

A novel estradiol derivative bearing a dithiocarbamate group has been synthesized by a sequence of simple reactions on the terminal triple bond of the ER agonist ethynil estradiol. Dithiocarbamate acts as bidentate ligand for complexation Tc yielding a symmetrical Tc(V)-nitride complex bearing two units of the pharmacophore. The product was obtained in adequate yield and purity and the structure was corroborated by spectroscopic methods. Labeling was performed successfully and a single product with high RCP and adequate stability was obtained. Physicochemical evaluation showed moderate lipophilicity and protein binding, properties that are considered adequate to ensure penetration through biological membranes. Studies of cellular uptake in ER positive breast cancer cells showed high binding, in the same range as native estradiol and higher that previous 99mTc-labeled estradiol derivatives published by our and by other research groups. Part of the internalized activity was released from the cells upon incubation but the rate of efflux was slow and thus is not considered a major drawback for the use of this tracer as potential Competition studies demonstrated that the binding could be attributed to specific interaction with ER receptors. Biodistribution in normal rats showed and adequate profile, similar to that of the gold standard [18F]FES and compatible with the proposed use as potential imaging agent. Biodistribution in animals with tumors induced by inoculation of MCF-7 breast cells gave a good relationship between tumor and muscle.

The important influence of the chelating system in technetium-labeled small biomolecules is well documented in the literature. Pharmacokinetic properties are significantly affected by the overall chemical structure of the complex but also the specific receptor binding could be affected. We have demonstrated that the dithiocarbamate group coordinates strongly to the metallic radionuclide generating a complex with adequate physicochemical and biological properties, superior to other previously reported technetium labeled ER receptor agonist.

Declaration of interest

None.

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