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New estradiol based ^{111}In complex towards the estrogen receptor

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Abstract: The oestrogen receptor (ER) is an important tumour target for molecular imaging and radionuclide therapy due to its overexpression in many malignant cells as compared to normal cells. Aiming to find new functional molecular imaging/therapeutic agents for ER positive tumours, we have synthesized a new estradiol derivative substituted at the 16-α position with a diethylene triamine tetraacetic acid (DTTA)-like chelating ligand through a four-carbon spacer. The new bioconjugate ($\mathbf{H_4L}$), was used to synthesize the corresponding indium complexes ($\mathbf{InL}$/[^{111}**In**]**L**). The radioactive complex [^{111}**In**]**L** was prepared in high yield (>98%) at final concentrations of 1×10^{-4} M and its chemical identity was ascertained by comparing its HPLC gamma-chromatogram to the HPLC UV-vis-chromatogram of the $\mathbf{InL}$ analogue. [^{111}**In**]**L** is hydrophilic and kinetically stable in the presence of an excess of apo-transferrin and in human blood serum. Cellular studies in breast cancer cells (MCF-7 and MDA-MB-431) suggest that [^{111}**In**]**L** uptake may be mediated by an ER dependent mechanism. Biodistribution studies were performed in mice indicating a rapid clearance from most organs and a slow total excretion that occurs mainly by hepatobiliar pathway. High *in vivo* stability of [^{111}**In**]**L** was confirmed by HPLC analysis of urine and blood samples. Nevertheless, the hydrophilicity, the low ER affinity and the biodistribution of [^{111}**In**]**L** indicate that structural modifications are required to improve its behaviour for ER targeting *in vivo*.

Keywords: Estrogen receptor, DTTA-estradiol conjugate, cancer imaging, ^{111}In complex.

1 Introduction

Cancer is a leading cause of death worldwide and an increasing number of new malignant tumours are diagnosed every year. Despite the scientific advances in its early detection and treatment, an effective cure for most types of cancers still remains to be found [1, 2]. Chemotherapy and endocrine therapy are valuable therapeutic alternatives however, many patients become resistant to the pharmacologic treatments and develop metastatic tumours [3, 4]. Several mechanisms have been proposed to explain the tumour progression and drug resistance [5–7] making clear that an accurate tumour staging is imperative for adequate treatment planning and to improve patients' outcome [1, 8]. The increasing knowledge of the cellular, molecular and genetic background of tumours led to the identification of several biomarkers which have been considered as potential targets for *in vivo* molecular imaging and therapeutic purposes [9]. The oestrogen receptor (ER), a ligand-inducible transcription factor that belongs to the nuclear receptor super family, is an important biomarker in breast cancer since this receptor is up-regulated in many malignant breast tumours. Therefore, it is an attractive target for molecular imaging/systemic radiotherapy and its role in carcinogenesis has been extensively investigated [10–14]. Moreover, ER status can predict the disease prognosis or response to hormonal therapy [15]. For these reasons, the search for novel ligands to specifically target ER in tumours is an important but demanding task.

Estradiol is a hormone with important roles in physiological and pathological conditions including proliferation and survival of cancer cells. Until recently its biological effects have been attributed to regulation of gene transcription of two well-known ERs, ERα and ERβ. Additionally, ER also stimulates non-genomic proliferative and anti-apoptotic signalling pathways medi-

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ated by ligand binding and growth factors [16–18]. Taking advantage of the increased ER expression in many tumours, radioligands based on gamma- and positron emitting estradiol derivatives have been developed for non-invasive functional ER imaging in cancer by single photon emission computerized tomography (SPECT) or positron emission tomography (PET) [19–24]. However few of them have reached the clinical stage [25, 26]. SPECT imaging of ER in breast cancer has been successfully achieved with radioiodinated 16α-[^{123}I]iodoestradiol and 17α-[^{123}I]iodovinylestradiols, while the most promising radiofluorinated estradiol derivative in PET imaging has been the 16α-[^{18}F]-estradiol (^{18}F-FES) which has been clinically evaluated for its predictive value on the responsiveness of breast cancer to endocrine therapy with tamoxifen [27–30]. Several attempts to design and prepare steroidal and nonsteroidal estrogen derivatives labeled with ^{99m}Tc, the most commonly available radionuclide in nuclear medicine, have also been reported. Nevertheless, none of them have revealed suitable ER targeting selectivity *in vivo* to be clinically useful [31–35].

The radiometal ^{111}In is the second most widely used gamma emitter for SPECT imaging. It has a half-life of 67.9 h and decays by electron capture with two γ-photon emissions at 173 and 247 keV (89 and 95% abundance, respectively). Although most of the emphasis has been focused on the imaging potential of ^{111}In radiolabelled compounds, the simultaneous emission of low-energy Auger electrons by this radionuclide offers the possibility for target specific radionuclide therapy, by inducing DNA damage and cell death. Due to the short range of Auger electrons, this application will be only possible when the decay of the ^{111}In radiolabelled compounds occurs in the nucleus, in close proximity of DNA, to promote single-strand or double-strand breaks [36]. Recently, radiometals like ^{99m}Tc and ^{111}In have started to be explored for this purpose with encouraging results [37–41]. Estradiol derivatives have already been successfully described as effective delivery vectors for metals into ER(+) cells [42]. Therefore, one promising approach for radionuclide therapy of ER(+) tumours would be the use of estradiol derivatives labelled with ^{111}In which, to the best of our knowledge, has only been explored by our group [43]. The potential of ^{111}In for therapeutic use was already demonstrated with the administration of high doses of ^{111}In-DTPA-octreotide (Octreoscan) to patients with neuroendocrine tumors. Data from these treatments indicated that Auger electrons can be safely and effectively used however the survival rates appeared to be less than those with ^{90}Y and ^{177}Lu therapies [44, 45].

The most frequently applied method of linking the metal ion $^{111}In^{3+}$ to a biomolecule is by means of a bifunctional chelating agent, usually a polyamino polycarboxylic ligand that efficiently coordinates the radiometal. Among the chelating agents, the cyclic 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) has been widely used since it forms complexes with very high thermodynamic stability and kinetic inertness [46–48]. However, the acyclic ligand diethylenetriaminepentaacetic acid (DTPA) is also used to stabilize ^{111}In, providing very stable complexes [49].

Aiming to contribute for the design of estradiol based ^{111}In-complexes for functional molecular imaging and/or therapy of ER positive tumours, we have synthesized a new DTTA-like chelator bearing a 16α-substituted estradiol derivative as well as the corresponding indium complex. Herein, we describe the synthesis and characterization of these compounds and the biological behaviour of the radioactive complex.

2 Results and discussion

2.1 Synthesis and characterization of H_4L

To synthesize the DTTA-like chelating agent, we have followed the strategy first described by Williams and Rapoport [50], which involved the bis *N*-alkylation of an amine with the *tert*-butyl protected bis-carboxymethylated amino ethyl bromide **1**. Compound **1** was obtained from ethanolamine by a two step synthesis. As despicted in Scheme 1A, **1** reacts with benzylamine in CH_3CN yielding the bis *N*-alkylated compound **2** in good yield. Reaction conditions were optimized in order to avoid overalkylation of the primary amine. The benzyl group in **2** was then removed by catalytic hydrogenation yielding the intermediate **3**, *tert*-butyl protected DTTA, a tetracarboxylic analogue of the diethylenetriaminepentaacetic acid (DTPA). Compound **3** bears a central secondary amine that can act as a useful functional group for the attachment of the DTTA chelating moiety to a variety of biomolecules in order to readily obtain target specific metal conjugates.

The 16α-halo-17-keto steroid **4** was prepared according to the synthetic procedure described by Katzenellenbogen and Fevig [51]. Briefly, the lithium enolate of the *tert*-butyldimethylsilyl protected estrone is stereospecifically alkylated at the 16-α position with the activated electrophile *trans*-1,4-dibromo-2-butene affording **4**. The alkyl bromide chain on the steroid provided the linker unit

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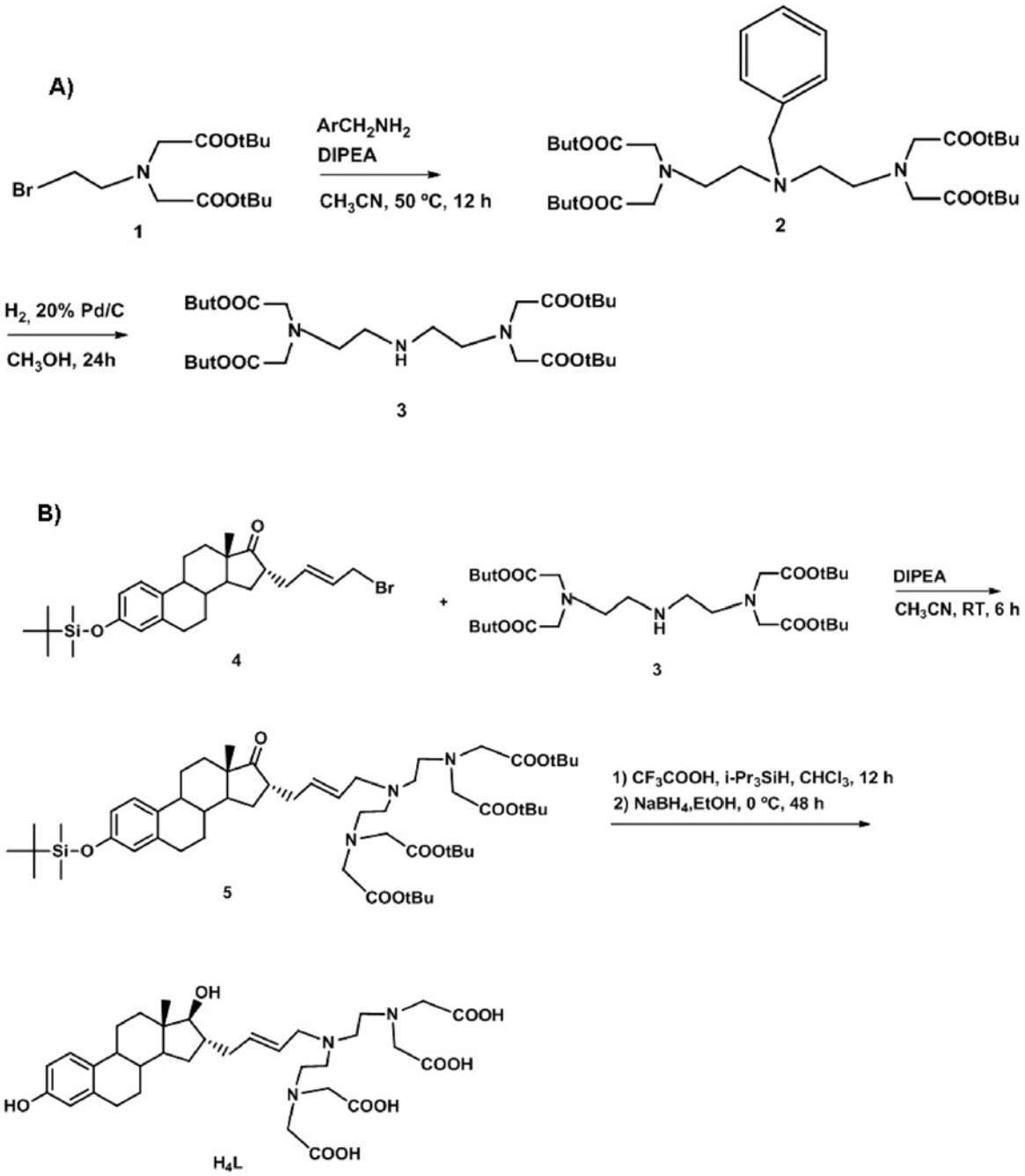

Scheme 1: (A) Synthetic scheme for the preparation of the DTTA based chelating ligand. (B) Coupling of estradiol derivative **4** with the chelating moiety **3** and synthesis of $\mathbf{H_4L}$.

to couple the biomolecule (estradiol) to the bifunctional chelating agent (DTTA).

Therefore, compound **4** reacted with the protected DTTA (**3**), using DIPEA as base, yielding **5** after purification by column chromatography (Scheme 1B). The formulation of **5** was based on NMR and ESI-MS. In fact, the chemical shifts of the CH_2N protons of the DTTA backbone and the absence of the multiplet due to the steroid side chain CH_2Br protons provided evidence for the *N*-alkylation reaction, further supported by mass spectrometry analysis.

In compound **5**, the *tert*-butyl and the *tert*-butyldimethylsilyl groups were simultaneously removed with trifluoracetic acid, using *i*-Pr$_3$SiH as scavenger. The progress of the reaction was followed by HPLC.

Then, reduction of the C17-ketone was performed with $NaBH_4$, at 0 °C, for approximately 48 h. The pure ligand ($\mathbf{H_4L}$) was obtained after purification in a C-18 Sep-Pak

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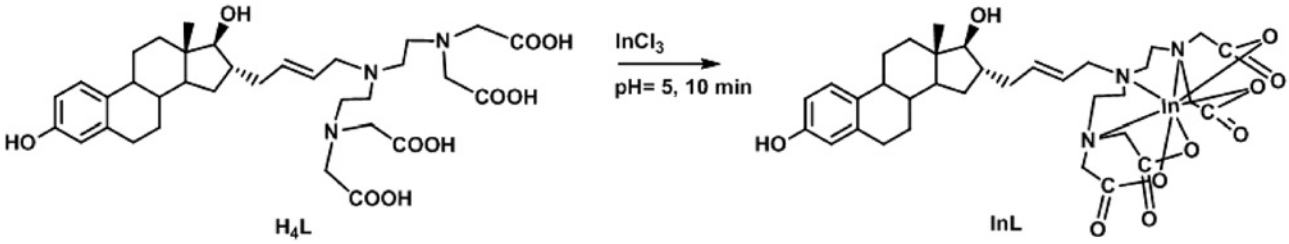

Scheme 2: Synthetic scheme for the preparation of the inactive complex **InL**.

cartridge. Due to the complexity of the ^{1}H-NMR spectra, the ^{13}C-NMR revealed to be crucial for the characterization of $\mathbf{H_4L}$. The appearance of a signal at 87.6 ppm corresponding to the C17 hydroxyl group and the absence of the ketone carbon signal at 220.7 ppm confirmed the reduction reaction. Goto *et al.* [52] described that the coupling constant of the C17-proton (J_{16-17}) is typically between 6 and 8 ppm when the stereochemistry of the protons is 16β and 17α. This stereochemistry was supported by the presence of the 17α-proton NMR signal as a doublet at 3.25 ppm with J = 6.0 Hz, confirming that the "linker" is at the 16α position of the steroid and that the alcohol has formed at the 17β position as it appears in the endogenous estradiol. The presence of a unique 13-methyl proton signal corroborated the formation of a single product.

2.2 Synthesis and characterization of InL

The inactive Indium complex (**InL**) was obtained by reacting $InCl_3$ with an aqueous solution of $\mathbf{H_4L}$, at pH = 5 (Scheme 2). The quantitative formation of **InL** was reliably observed by HPLC after 10 min (rt ($\mathbf{H_4L}$) = 10.82 min; rt (**InL**) = 9.53 min). After appropriate work-up of the reaction mixture the white solid obtained was analysed by IR, NMR and ESI/MS.

In the IR spectra a sharp and strong band at 1627 cm^{-1}, significantly shifted relatively to the same stretching band in the free ligand $\mathbf{H_4L}$ ($\Delta\nu$co = 36 cm^{-1}), was assigned to the coordinated carboxylates. The ^{1}H and ^{13}C NMR spectra were in agreement with the proposed formulation. In fact, in the **InL** ^{13}C NMR spectra, the four equivalent COO^{-} carbons of the chelating unit in the free ligand, exhibited signal splitting upon complexation with In^{3+}. A similar effect was also observed for the carboxylate CH_2-carbons. The negative-ion mass spectra showed the expected molecular-ion peak ([M]$^{-}$, m/z: 700.4) which confirmed the formulation proposed for **InL**.

2.3 Radiosynthesis and characterization of [^{111}In]L

Radiolabelling of $\mathbf{H_4L}$ was achieved by reacting the ligand with $^{111}InCl_3$ in an ammonium acetate buffer solution at pH = 5 and at room temperature [53, 54]. Complex [^{111}**In**]**L** was obtained in high radiochemical yield and with high radiochemical purity (>98%), using a final ligand concentration of 1.0×10^{-4} M after 10 min reaction time. The chemical identity of the ^{111}InL was ascertained by comparing its HPLC profile to that of the inactive indium complex (**InL**), analysed as described above (Figure 1).

Hydrolysis of $^{111}In^{3+}$ with the formation of colloidal radiochemical species can be a noteworthy challenge during the radiolabelling reaction. The eventual presence of such hydrolysed species has been checked by paper chromatography, using Whatmann no. 1 cellulose strips as support and 55% methanol as mobile phase, since these radiochemical species cannot be identified by HPLC analysis. The radiochromatograms confirmed the absence of such species (Rf = 0) and only the complex ^{111}InL could be detected (Rf = 1). The presence of free [^{111}**In**]**L** was also evaluated, both by HPLC and by ITLC. However, no free metal was detected in the two systems (ITLC: $^{111}InCl_3$ Rf = 0, HPLC: $^{111}InCl_3$ rt = 3.19 min).

The lipophilicity was assessed through the calculation of the partition coefficient between 1-octanol and PBS us-

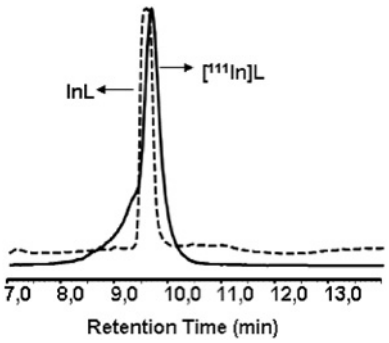

Fig. 1: HPLC chromatograms of **InL** (rt = 9.53 min, UV detection, λ = 220 nm) and [^{111}**In**]**L** (rt = 9.66 min, γ detection).

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ing the "shake-flask" method [55]. The log $P_{o/w}$ value for [^{111}In]L was found to be −0.99 ± 0.02 which means an hydrophilic character for the radiocomplex, certainly due to the nature of the metal moiety and to the overall negative charge, since estradiol itself has a lipophilic nature (log $P_{o/w}$ = 3.26) [7, 17, 56].

2.4 *In vitro* stability, transferrin exchange and protein binding studies

The *in vitro* radiochemical stability of the compound was assessed by HPLC analysis of aliquots taken at several time points after incubation at 37 °C in order to predict its *in vivo* behaviour. [^{111}In]L has demonstrated to be radiochemically stable up to 5 days at 37 °C in physiological saline buffer (pH = 7.4). HPLC analysis of the ethanolic extracts proved that [^{111}In]L was also stable in human blood serum for 48 h, with no detectable formation of other radiochemical species. The percentage of serum protein binding estimated from the radioactivity retained in the protein precipitate is relatively low (<10%) at all time points, corroborating that in the serum there was mainly the unbound [^{111}In]L as radiochemical species.

Another challenge for *in vivo* application of ^{111}In complexes is the ligand exchange with transferrin, the iron-transport protein. Our studies have shown that [^{111}In]L is stable in a solution of apo-transferrin (3 mg/mL) with no transchelation up to 48 h. Figure 2 presents the HPLC chromatograms of [^{111}In]L in serum, PBS and apo-transferrin after 24 h of incubation.

2.5 Cellular uptake studies

Cellular uptake kinetics of [^{111}In]L was assessed in suitable human breast cancer cells, such as MCF-7 (ER+) and MDA-MB-231 (ER-) to find out whether the complex is effectively taken up by cells. In order to assess specific binding, similar experiments were performed simultaneously, incubating the MCF-7 cells with [^{111}In]L in the presence of estradiol. The cellular uptake of [^{111}In]L in both cell lines is presented in Figure 3.

A low uptake of [^{111}In]L was found in both cell lines, 0.95%±0.04/million cells and 0.38%±0.10/million cells in MCF-7 and MDA-MB-231, respectively. These low values probably result from the hydrophilic character and negative charge of the radiocomplex that prevent high cell membrane penetration by passive diffusion. The percentage of [^{111}In]L taken by MCF-7 cells increases over incubation time while no relevant increase was noticed in

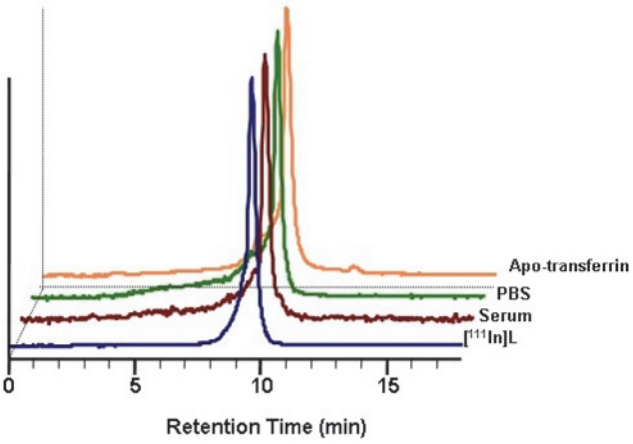

Fig. 2: HPLC chromatograms (γ detection) presenting the *in vitro* stability of [^{111}In]L in serum, physiological saline buffer (PBS), and apo-transferrin solution, 24 h after incubation. The chromatogram of the [^{111}In]L used in these studies is also presented for comparison.

MDA-MB-231 cells. Despite the low absolute values found, a decrease in the uptake (approximately 40%) was observed when ER+ cells were treated with estradiol. These findings, in addition to the lowest uptake obtained in the ER- cells, suggest that the cellular uptake mechanism may occur via an ER-mediated process. Nevertheless the disappointing overall uptake selectivity does not suggest [^{111}In]L as a promising candidate for *in vivo* imaging.

2.6 Receptor binding affinity

In spite of the low cellular uptake, the relative binding affinity of H_4L and InL for the isolated recombinant human ERα was checked trying to understand the role of this receptor in the cellular uptake mechanism as these two assays measure different properties and give complementary information. While the receptor binding assay with

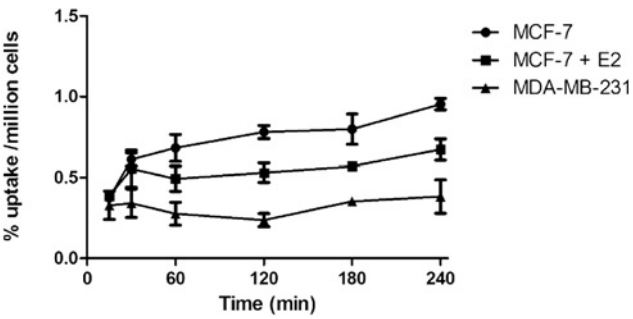

Fig. 3: Cellular uptake of [^{111}In]L in MCF-7 breast cancer cells, with and without co-incubation with molar excess of estradiol and in MDA-MB-231 breast cancer cells.

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Table 1: Biodistribution data of [^{111}In]L, in female CD-1 mice, at 1 and 2 h after i.v. administration ($n = 3$).

Organ	% I.A./g	
	1 h	2 h
Blood	0.4 ± 0.2	0.11 ± 0.02
Liver	9.3 ± 1.2	0.8 ± 0.5
Intestines	37.1 ± 3.2	40.4 ± 1.1
Spleen	0.12 ± 0.03	0.03 ± 0.01
Heart	0.11 ± 0.02	0.05 ± 0.01
Lung	0.2 ± 0.1	0.10 ± 0.07
Kidney	1.3 ± 0.3	0.4 ± 0.1
Muscle	0.13 ± 0.01	0.04 ± 0.01
Bone	0.14 ± 0.05	0.06 ± 0.01
Stomach	7.1 ± 0.5	0.6 ± 0.4
Uterus	0.09 ± 0.01	0.10 ± 0.04
Ovaries	0.5 ± 0.3	0.15 ± 0.06
Excretion (%I.A.)	10.6 ± 1.1	11.5 ± 0.8

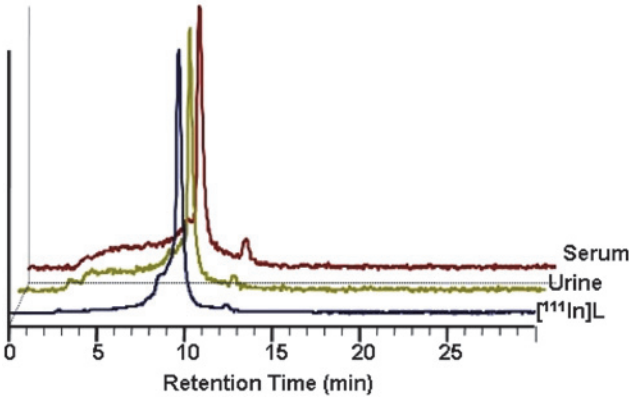

Fig. 4: HPLC chromatograms (γ detection) presenting the *in vivo* stability testing of [^{111}In]L in mice urine and blood serum, 2 h p.i. The initial chromatogram of the [^{111}In]L is also presented for comparison purposes.

the free ER just assesses the receptor binding, the cellular binding assays evaluate not only the receptor binding but also the ability of the complexes to penetrate into the cell.

The binding affinities were determined by *in vitro* competitive radiometric binding assays using [^{3}H]-estradiol as tracer. Thus, affinities correspond to the inhibition of the binding of [^{3}H]-estradiol to the recombinant human ERα and are expressed as IC_{50} and relative binding affinity (RBA) against estradiol. The IC_{50} values for each competing estrogen are calculated according to the sigmoid inhibition curves. However data from these assays indicate that none of the compounds exhibit relevant binding affinity for ERα since low displacement of the [^{3}H]-estradiol was found probably due to very low solubility of the compounds in the assay solvents. Under our experimental conditions the IC_{50} of estradiol (1.69 ± 6.08 nM) is in good agreement with the values previously reported [20, 22].

2.7 Biodistribution

Biodistribution studies of [^{111}In]L were carried out in adult healthy female CD-1 mice to get a first insight on the *in vivo* stability and pharmacokinetics profile of the complex and to understand how the molecular structure affected the biological behaviour despite the poor ER affinity. The tissue distribution of [^{111}In]L for the most relevant organs at 1 and 2 h after intravenous injection is presented in Figure 4. Total radioactivity excretion at both time points was also determined.

The biodistribution profile of the compound showed a rapid clearance from the blood stream and fast liver uptake that rapidly clears into the intestines indicating the hepatobiliary tract as the major excretory pathway, which is in agreement with the usual excretion pattern of estradiol derivatives in rodents [21, 57]. Indeed, hepatobiliary excretion of radioligands towards ER in rats was described in early studies from Katzenellenbogen group. In these studies they observed that the largest amounts of the labelled estrogen were found in the liver and intestines, organs involved in steroids metabolism and clearance [58]. Nevertheless, there was a small contribution of the urinary excretion route (1.3 ± 0.3% IA/g kidney and 10.6 ± 1.1% IA excreted in urine at 1 h post injection (p.i.)). In fact, after 1 h there was no remarkable radioactivity accumulation in any organ except those related with excretory routes (liver, kidney and mainly intestines). The rapid clearance from blood and highly irrigated organs like liver, heart and lungs reflects the expected biological behaviour from the low protein binding and kinetic inertness towards transchelation with transferrin found by *in vitro* studies. Additionally, the low uptake in liver (0.8 ± 0.5% IA/g) and lungs (0.10 ± 0.07% IA/g at 2 h p.i.) indicated that no free ^{111}In was released from the complex, since the ion $^{111}In^{3+}$ would localize in these organs due to the strong binding ability to transferrin [46]. Otherwise the low spleen uptake (0.12 ± 0.03% IA/g and 0.03 ± 0.01% IA/g at 1 and 2 h p.i., respectively) confirmed the absence of *in vivo* hydrolysis of ^{111}In, as the hydrolysed radiochemical species of colloidal nature would tend to accumulate in the reticulo endotelial cells. The uptake in the ER expressing tissues, such as uterus and ovaries, was very low (0.09 ± 0.01% IA/g and 0.5 ± 0.3% IA/g at 1 h p.i. in uterus and ovaries, respectively) which is in agreement with the low RBA found by *in vitro* assay.

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The whole animal body radioactivity excretion was relatively slow (10.6 ± 1.1% IA and 11.5 ± 0.8% IA at 1 and 2 h p.i., respectively) as expected from the predominant hepatobiliar excretory route.

The high *in vivo* stability of the compound was further demonstrated by HPLC analysis of urine and blood serum samples collected at sacrifice time (Figure 4). Blood serum chromatogram indicated that most of the radiochemical species circulating in blood stream presented the same retention time of the injected [^{111}**In**]**L** complex. Moreover, analysis of the activity excreted in urine at 1 h and 2 h after injection revealed that [^{111}**In**]**L** remained stable and was excreted as intact radiochemical species.

3 Conclusions

A new DTTA-estradiol bifunctional chelator containing a four-carbon spacer (**H_4L**) was successfully synthesized through conjugation of DTTA to a 16α-halo-17-keto steroid and fully characterized by common analytical techniques. Using this estradiol derivative, **InL**/[^{111}**In**]**L** complexes were synthesized. The radioactive complex was prepared in high radiochemical yield and high radiochemical purity and its characterization performed by HPLC comparison with the inactive **InL** complex. [^{111}**In**]**L** is hydrophilic, presents low serum protein binding, high *in vitro* stability in physiological media and kinetic inertness to transchelation in the presence of an excess of apo-transferrin. In two breast cancer cell lines, a low cellular uptake, which decreases when MCF-7 cells were treated with estradiol, was obtained suggesting an ER-mediated process. However, the low values of RBA found for the ligand do not corroborate these findings. *In vivo*, [^{111}**In**]**L** is highly stable with no release of In^{3+} from the complex or formation of other radiochemical species, as confirmed by the biodistribution profile and HPLC analysis of blood serum and urine samples collected over p.i. time. Preliminary animal studies indicated rapid clearance from main organs, a long residence time into intestines and a slow total radioactivity excretion that occurs predominantly *via* hepatobiliar tract. In spite of its favourable radiochemical and stability behaviour, the high hydrophilicity and the low binding affinity to ER hampered further biological evaluation of the [^{111}**In**]**L** in adequate animal models. Therefore, work will proceed in order to introduce structural modifications in both the bifunctional chelator and in the spacer attempting to improve the cell membrane penetration and the receptor binding affinity to get *in vivo* ER targeting.

4 Experimental

4.1 General procedures

All chemicals were reagent grade purchased from Sigma-Aldrich Co. and were used without further purification unless otherwise stated. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a Varian Unity 300 MHz spectrometer. ^{1}H NMR and ^{13}C NMR chemical shifts are reported relatively to residual solvent signals or tetramethylsilane (TMS) as reference. IR spectra were recorded as KBr pellets using a Jasco FT/IR 4100 spectrometer. C, H, N analyses were performed using a CE Instruments EA 110 automatic analyzer. Electrospray ionization/quadropole ion trap mass spectrometry (ESI/QITMS) was acquired using a Bruker HCT Mass Spectrometer. Chemical reactions were monitored by thin-layer chromatography (TLC) on Merck plates pre-coated with silica gel 60F254. Column chromatography was performed on silica gel 60 (Merck). Sep-Pak purification was performed with a Waters (USA) Sep-Pak C18 6cc Vac Cartridge, 500 mg (particle size: 37–55 μm). $^{111}InCl_3$ (370 MBq/mL in HCl) was obtained from Mallinckrodt Medical B.V., Netherlands.

4-[3′-(tert-butyldimethylsilyloxy)-17′-oxoestra-1′,3′,5′(10′)-trien-16′α-yl]bromobut-2-ene (compound **4**) was synthetized according to a published procedure [51].

High-performance liquid chromatography (HPLC) analysis was performed on a Perkin Elmer system, equipped with a biocompatible quaternary pump (LC 200), an UV/Vis detector (LC 290, Perkin Elmer) and a radioactivity detector (LB 509, Berthold).

The ligand and the inactive Indium complex were detected by UV (λ = 220 nm) and the ^{111}In containing compound was identified by gamma detection. HPLC analysis of the inactive and radioactive compounds was achieved on a reverse phase (RP) Nucleosil Column (C18, 100-5, 250 × 4 mm) eluted with a binary gradient system with a flow rate of 1 mL/min – eluents: A – aqueous 0.1% CF_3COOH; B – CH_3CN with 0.1% CF_3COOH; method: 0–1 min 15% B, 1–16 min 15%–100% B, 16–28 min 100% B, 28–30 min 100%–15% B.

Labelling efficiency and radiochemical purity was assessed by ascending instant thin layer chromatography on silica gel (ITLC-SG, Varian, Agilent Technologies) and paper strips (Whatman no. 1). Radioactive distribution on the ITLC-SG and paper strips was detected using a Berthold LB 505 (Germany) detector coupled to a radioactive scanner. Radioactivity measurements were done using a dose calibrator (Aloka Curiemeter, IGC-3, Japan) or a gamma-counter (Berthold, LB 2111, Germany).

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4.2 Synthesis of tert-butyl-N-benzyl-diethylenetriamine-N′,N′,N″,N″-tetraacetate (2)

To a solution of **1** (384 mg, 1.1 mmol) in dry CH_3CN (10 mL), benzylamine (56.5 µL, 0.52 mmol) and N,N-diisoproylethylamine (225 µL, 1.29 mmol) were added. The reaction mixture was heated at 50 °C for 12 h, and then concentrated in vacuo. The crude product was purified by column chromatography on silica gel (*n*-hexane/EtOAc 3 : 2–0 : 1) yielding compound **2** as a yellow oil. Yield 323 mg, 59%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 7.19–7.27 (m, 5H, Ar), 3.57 (s, 2H, CH_2Ar), 3.36 (s, 8H, CH_2COOtBu), 2.79 (t, 4H, CH_2N, J = 7.1 Hz), 2.57 (t, 4H, CH_2N, J = 7.1 Hz), 1.38 (s, 36H, t-Bu). ^{13}C NMR ($CDCl_3$, 300 MHz) δ (ppm) 170.7 (C,*C*O), 139.5 (C, Ar), 128.8, 128.1, 126.8 (CH_2, Ar), 80.74 (C, *t*-Bu), 59.0 (CH_2, *C*H_2Ar), 56.1 (CH_2, *C*H_2COOtBu), 52.7 (CH_2, *C*H_2N), 51.9 (CH_2, *C*H_2N), 28.1 (CH_3, *t*-Bu). ESI-MS m/z calcd for $C_{35}H_{60}N_3O_8$ ([M+H]$^+$): 650.4, found 650.4

4.3 Synthesis of tert-butyl-diethyl-enetriamine-N,N,N′,N′-tetraacetate (3)

A mixture of **2** (225 mg, 0.35 mmol) in CH_3OH (10 mL) and Pd/C (20 wt %, 45 mg) was stirred at room temperature for 24 h under hydrogen atmosphere. The reaction mixture was filtered through Celite and the filtrate was concentrated using a rotary evaporator providing **3** as a yellow oil. Yield 178 mg, 91%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 3.44 (s, 8H, CH_2COOtBu), 2.96–2.80 (m, 8H, CH_2N), 1.44 (s, 36H, t-Bu). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ (ppm) 170.8 (C,*C*O), 80.9 (C, *t*-Bu), 56.3 (CH_2, N*C*H_2COOtBu), 52.9 (CH_2, *C*H_2N), 46.8 (CH_2, *C*H_2N), 27.9 (CH_3, *t*-Bu).

4.4 Synthesis of tert-butyl-N-{4-[3′-(tert-butyldimethylsilyloxy)-17′-oxoestra-1′,3′,5′(10′)-trien-16′α-yl]bromobut-2-enyl}-diethylenetriamine-N′,N′,N″,N″-tetraacetate (5)

Compound **3** (120 mg, 0.21 mmol) was dissolved in dry CH_3CN (5 mL) and N,N-diisoproylethylamine (58 µL, 0.44 mmol) was added. To this solution, **4** in dry CH_3CN (57 mg, 0.11 mmol) was added drop-wise at 0 °C and the reaction mixture was stirred for 6 h at room temperature. After concentration in vacuo, the crude product was purified by column chromatography on silica gel with EtOAc providing **5**, as a yellow oil. Yield 186 mg, 89%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 7.07 (d, 1H, 1-C*H*, J = 7.1 Hz), 6.59 (dd, 1H, 2-C*H*, J_1 = 2.4 Hz, J_2 = 10.8 Hz), 6.54 (d, 1H, 4-C*H*, J = 2.1 Hz), 6.23–6.18, 5.78–5.73 (m, 2H, C*H* =C*H* side chain), 3.42 (s, 8H, CH_2COOtBu), 3.21 (t, 4H, CH_2N, J = 10.2 Hz), 3.03 (t, 4H, CH_2N, J = 10.8 Hz) 2.82–2.80 (m, 2*H*, 6-CH_2), 1.49–2.56 (m, unassigned CH and CH_2), 1.42 (s, 36H, COOtBu), 0.95 (s, 9H, Si-tBu), 0.92 (s, 3H, 18-CH_3), 0.16 (s, 6H, Si-CH_3). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ (ppm) 220.7 (CO, *C*17), 170.4 (CO, *C*O*t*Bu), 153.5 (COH, *C*3), 143.4 (CH, *C*H=CH), 137.6 (CH, *C*5), 132.4 (CH, *C*10), 126.1 (CH, *C*1), 120.0 (CH, *C*4), 118.4 (CH, CH=*C*H), 117.3 (CH, *C*2), 81.7 (C, *t*-Bu), 77.2, 61.8, 56.3, 55.9 (CH_2, N*C*H_2COOtBu), 51.4, 48.8, 48.7, 48.0, 44.0, 43.6, 38.2, 29.4, 28.1 (CH_3, *t*-Bu), 27.6, 27.1, 26.5, 25.7 (CH_3, SiC(*C*H_3)$_3$), 18.1 (C, Si*C*(CH_3)$_3$), 14.5 (CH_3, *C*18), – 4.41 (CH_3, Si(*C*H_3)$_2$). ESI-MS m/z calcd for $C_{56}H_{94}N_3O_{10}Si$ ([M+H]$^+$): 996.7, found 996.8.

4.5 Synthesis of N-{4-[3′,17′β-dihydroxy-estra-1′,3′,5′(10′)-trien-16′α-yl]bromo-but-2-enyl}-diethylenetriamine-N′,N′,N″,N″-tetraacetic acid (H_4L)

CF_3COOH (300 µL) and *i*-Pr_3SiH (4 µL) were added to a solution of **5** (74 mg, 0.074 mmol) in $CHCl_3$ (0.2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and then concentrated in vacuo. The solid obtained was then redissolved in EtOH (2 mL) and solid $NaBH_4$ (22 mg, 0.59 mmol) added. The suspension was stirred at 0 °C for 48 h and after this time the reaction was quenched by the addition of acetone. After concentration under reduced pressure, the crude product was redissolved in water/methanol (1:3) and eluted through a Sep-Pak C18 cartridge with water and increasing concentration of methanol (30%–100%). The purity of the aliquots was analyzed by HPLC and the fractions containing the product (eluted with 30% methanol) were combined and evaporated under N_2 to remove the organic solvent. After lyophilization, $\mathbf{H_4L}$ was obtained as a white powder. Yield 22 mg, 44%. IR ν_{max} (KBr)/cm^{-1} 3405, 2925, 2863, 1591 (C=O), 1409 ^{1}H NMR (CD_3OD, 300 MHz) δ (ppm) 7.06 (d, 1H, 1-C*H*, J = 8.4 Hz), 6.52 (dd, 1H, 2-C*H*, J_1 = 2.4 Hz, J_2 = 10.8 Hz), 6.46 (d, 1H, 4-C*H*, J = 2.1 Hz), 5.51-5.82 (m, 2H, C*H* =CH side chain), 3.25 (d, 1 H, 17-C*H*α, J = 6.0 Hz), 3.19 (s, 8H, CH_2COOH), 2.76 (m, 8H, CH_2N), 2.13 – 1.26 (m, unassigned CH and CH_2), 0.80 (s, 3H, 18-CH_3). ^{13}C NMR (CD_3OD, 67.8 MHz) δ (ppm) 179.6 (CO, *C*OOH), 155.9 (COH, *C*3), 138.8 (CH, *C*5), 136.0 (CH, *C*H=CH side chain), 132.6 (CH, *C*10), 127.2

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(CH, *C*1), 125.6 (CH, CH= *C*H), 116.1 (CH, *C*4), 113.7 (CH, *C*2), 87.6 (CHOH, *C*17), 61.3 (CH_2, NCH_2COOH), 53.8, 52.4, 44.3, 44.0, 42.7, 39.2, 37.4, 37.0, 29.5, 29.0, 27.4, 26.3, 11.4 (CH_3, *C*18). ESI-MS m/z calcd for $C_{34}H_{50}N_3O_{10}$ ($[M+H]^+$): 660.3, found 660.5. Elemental Analysis (%): calculated for $C_{34}H_{49}N_3O_{10}^{\cdot}(CF_3COOH)_4^{\cdot}(H_2O)_2$; C 43.8, H 5.0, N 3.7. Found C 43.7, H 5.0, N 3.8.

4.6 Synthesis of InL

Compound $\mathbf{H_4L}$ (0.02 mmol, 15 mg) was dissolved in water (3 mL) and the pH of the solution was adjusted to 5 by the addition of HCl 0.1 N. An equimolar amount of $InCl_3$ (anhydrous, 99.9%, Alfa Aesar, Germany) was added and the mixture was stirred for 30 min. After centrifugation, the precipitate was washed with water and lyophilized to give the desired complex **InL**. Yield 11 mg, 71%. IR ν_{max} (KBr)/cm^{-1} 3418, 2925, 1627 (C=O), 1383 ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm) 7.02 (d, 1H, J = 8.4 Hz, 1-CH), 6.48 (m, 1H, 2-CH), 6.41 (m, 1H, 4-CH), 5.77-5.52 (m, 2H, CH=CH side chain), 3.41–1.12 (m, unassigned CH and CH_2), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR (DMSO-d6, 67.8 MHz) δ (ppm) 172.7, 171.9, 171.5 (CO, *C*OOH), 155.0 (COH, *C*3), 138.3 (CH, *C*5), 137.3 (CH, *C*H=CH side chain), 130.6 (CH, *C*10), 126.1 (CH, *C*1), 120.4 (CH, *C*H=CH), 114.9 (CH, *C*4), 112.8 (CH, *C*2), 85.5 (CHOH, *C*17), 63.5, 62.9, 61.1 (CH_2, NCH_2COOH), 55.0, 52.1, 49.8, 47.9, 43.7, 42.3, 37.8, 36.2, 29.5, 28.8, 27.1, 26.1, 12.1 (CH_3, *C*18). ESI-MS m/z calcd for $C_{34}H_{45}InN_3O_{10}$ ($[M]^-$): 770.2, found 770.4

4.7 Synthesis of [^{111}In]L

Radiolabelling of $\mathbf{H_4L}$ with ^{111}In was achieved through addition of 5–30 μL (2.4–5.0 MBq/64–136 μCi) of $^{111}InCl_3$ to 170–195 μL of an 0.4 M ammonium acetate buffer (pH = 5) solution of $\mathbf{H_4L}$ (1.0×10^{-4} M) in a plastic vial. The reaction mixture was incubated for 10 min, at room temperature, for complete radiolabelling. Radiochemical purity was determined by HPLC (γ detection, rt = 9.66 min) and by planar chromatography. Paper chromatography was performed in Whatmann no. 1 cellulose strips with 55% methanol as mobile phase (free $^{111}InCl_3$ Rf = 1, colloids Rf = 0, ^{111}InL Rf = 1) and instant thin layer chromatography was performed on silica gel strips with a mixture of NaCl 0.9%: HCl 0.1 M (50 : 1) as mobile phase (free $^{111}InCl_3$ Rf = 0, colloids Rf = 0, ^{111}InL Rf = 1).

4.8 *In vitro* stability studies and protein binding estimation

The *in vitro* stability of [^{111}In]**L** was assessed in PBS pH 7.4 and human serum by HPLC analysis at several time points. *PBS:* the radioactive complex (50 μL) was incubated in PBS at 37 °C. Aliquots were taken before incubation started (for zero time point analysis) as well as at various time intervals during incubation (1 h, 3 h, 24 h and 48 h). *Human serum:* [^{111}In]**L** (50 μL) was incubated with human serum (0.5 mL) for 48 h at 37 °C. Aliquots were taken (200 μL) and treated with absolute ethanol (500 μL) at 4 °C to precipitate the proteins. Samples were centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatants were separated from the pellets and analysed by HPLC at 1 h, 3 h, 24 h and 48 h.

The pellets were washed twice with ethanol followed by centrifugation and measurement of the remaining radioactivity in the sediment to estimate the percentage of human serum protein binding.

4.9 Transchelation with apo-transferrin

The stability of [^{111}In]**L** was assessed in the presence of an excess of apo-transferrin. Aliquots of [^{111}In]**L** (50 μL) were added to an apo-transferrin solution (3 mg/mL, 10 mM sodium bicarbonate buffer, pH 7.4), incubated at 37 °C, and analyzed by HPLC at different time points (1 h, 3 h, 24 h and 48 h).

4.10 Lipophilicity

The octanol-water partition coefficient ($P_{o/w}$) of the radioactive complex was determined by the "shakeflask" method using the multiple back extraction technique. The radioactive complex (100 μL) was added to a mixture of 1-octanol (1 mL) and phosphate-buffered saline, PBS, pH 7.4 (1 mL) previously saturated in each other by vigorous stirring. The mixture was vortexed and centrifuged (3000 rpm, 10 min, r.t.) to allow phase separation. After phase separation an aliquot of the aqueous layer was transferred to another tube and further extracted with 1-octanol. Aliquots of both octanol and PBS aqueous phases were counted in a gamma counter and the concentration ratio of the complex between the phases ($P_{o/w}$) was then calculated and results expressed as log $P_{o/w}$.

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4.11 *In vitro* cellular uptake studies

Two human breast cancer cell lines from *American Type Culture Collection* (ATCC, Spain) were used in these studies, namely MCF-7 cell line that expresses the ER (ER+) and MDA-MB-231 cell line without ER expression (ER-). Cells were grown in DMEM medium (Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under a humidified 5% CO_2 atmosphere. For the studies, cells were plated at a density of approximately 2×10^5 cells per well in 24-well tissue culture plates in DMEM medium without phenol red and supplemented with 10% charcoal-stripped fetal bovine serum and 1% penicillin/streptomycin. After 48 h, the medium was removed and replaced by fresh medium containing approximately 5×10^5 cpm/0.5 mL of the [^{111}**In**]**L**. After 15 min to 4 h incubation in humidified 5% CO_2 atmosphere at 37 °C, the cells were washed twice with cold PBS, lysed with 0.1 M NaOH and the cellular extracts were counted for radioactivity in a gamma-counter. Each experiment was performed in quadruplicate. Radioactivity associated to the cell extracts at each time point was expressed as the percentage of the total activity added to the cells, normalized per million of cells and reported as an average plus the standard deviation.

To investigate whether the cellular uptake of the [^{111}**In**]**L** occurs via an ER- mediated process similar experiments were carried out in parallel by the previous addition of 100 mL of estradiol (10 mM) to the MCF-7 cells.

4.12 Receptor-binding affinity

The ERα competitive binding assay was performed according to a described method by us [22]. The ERα binding buffer used for dilution of the receptor preparations consisted of 10% glycerol, 2 mM dithiothreitol, 1 mg/mL BSA and 10 mM Tris-HCl (pH 7.5). The ERα washing buffer contained 40 mM Tris-HCl and 100 mM KCl (pH 7.4). The hydoxyapatite (HAP) slurry was adjusted to a final concentration of 50% (v/v) by using a 50 mM Tris-HCl solution (pH 7.4). The reaction mixture contained 50 µL of varying concentrations of the test compound in the ERα binding buffer, 45 µL of a solution of tritiated estradiol (23.8 nM) and 5 µL (0.25 pmol) of ERα proteins solution. Non specific binding by the tritiated estradiol was determined by the addition of a 50 µM concentration of the nonradioactive E_2. The binding mixture was incubated at 4 °C for 16–18 h. At the end of the incubation, 200 µL of the HAP slurry was added and tubes were incubated on ice and vortexed three times for 15 min. An aliquot of 1 mL of washing buffer was added, mixed and centrifuged at **10000** × g for 10 min, and the supernatants were discarded. This wash step was repeated twice. The HAP pellets were then resuspended in 750 µL cold ethanol, vortexed three times in 20 min, centrifuged and the supernatants were transferred to scintillation vials for measurement of ^{3}H radioactivity in a liquid scintillation counter (Packard Tri-CARB 3170 TR/SL). The data obtained from triplicate measurements were expressed as the percent specific binding of [^{3}H]E_2 *vs*. the log molar concentration of the competing compound. The IC_{50} values (calculated using GraphPad Prism software) represent the concentration of the test compound required to reduce the [^{3}H]E_2 binding by 50%.

4.13 Biodistribution studies

The *in vivo* behavior of [^{111}**In**]**L** complex was evaluated in groups of 3 female CD-1 mice (randomly bred, Charles River) weighting approximately 25 g each. Animals were injected intravenously with 100 µL (0.74–1.59 MBq/20–43 µCi) of each preparation via the tail vein and were maintained on normal diet *ad libitum*. All animal studies were conducted in accordance with the highest standards of care, as outlined in the European Law. Mice were sacrificed by cervical dislocation at 1 h and 2 h post-injection. The injected radioactive dose and the radioactivity remaining in the animal after sacrifice were measured in a dose calibrator. The difference between the radioactivity in the injected and sacrificed animal was assumed to be due to total excretion from whole animal body. Blood samples were taken by cardiac puncture at sacrifice. Tissue samples of the main organs were then dissected, weighted and counted in a gamma counter (Berthold). Biodistribution results were expressed as percentage of the injected dose per gram tissue (%ID/g). Blood samples, collected at sacrifice time, were centrifuged, the serum separated and treated with ethanol to precipitate the proteins and the supernatant was analysed by HPLC for stability evaluation. The urine samples were centrifuged at 3000 rpm for 10 min. and the supernatant was also analyzed by HPLC.

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