Evaluation of Novel Radioiodinated C7-substituted $\Delta^{6,7}$–estradiol Derivatives for Molecular Recognition of ER-Positive Breast Tumours

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Abstract: The estrogen receptor is a striking target for molecular imaging and therapy of certain malignancies. In the case of cancer, the ability of imaging the expression of the ER would offer a noninvasive means of classifying tumours guiding the optimal choice of therapy. The importance of estradiol based radioligands as potential imaging agents for estrogen receptor rich tumours and the growing interest in Auger electron emitters for potential radiotherapy of estrogen receptor-positive breast cancers lead to the synthesis and evaluation of a series of radioiodinated derivatives of estradiol.

Seeking for new probes for targeting the estrogen receptor two novel radioiodinated $\Delta^{6,7}$–estradiol derivatives containing different C7-alkyl chains were prepared and labelled with ^{125}I. To evaluate the potential use of their ^{123}I-analogues for imaging breast tumours the biological behaviour of the new radioligands was studied in immature female rats to determine their uptake in and selectivity for tissues containing estrogen receptors. The different alkyl chains induced major modifications in the uptake efficiency, selectivity and in the metabolization degree of the compounds. It is not very clear, however, whether the alterations in uptake efficiency and selectivity are the result of differences in lipophilicity or altered patterns of metabolism. Even so, these radioligands exhibit different biological patterns that may be helpful in optimizing the imaging of ER positive tumours.

Key Words: Estradiol, estrogen receptor, radioiodination, SPECT imaging, breast cancer.

INTRODUCTION

Breast cancer is the most malignant form of diagnosed cancer among women and still remains a major cause of death in the western world. Early detection is essential for breast cancer survival and patient's welfare owing to its propensity to metastize even before the disease can be detected clinically or by screening mammography. A majority of breast cancers express estrogen receptors (ER) in the primary tumour and in many cases respond successfully to antiestrogens, such as tamoxifen and raloxifene. Thus, non-invasive imaging of breast tumours based on their hormonal receptor's content can provide an early assessment of response to hormone therapy using single photon emission computed tomography (SPECT) or positron emission tomography (PET) [1-3]. Additionally, the growing interest in Auger electron emitters for potential radiotherapy of ER-positive breast cancers brings up the requirement of a steroid carrier possessing high selectivity and good accumulation properties in the target tissue [4,5]. Such clinical approaches would require a receptor-ligand, labelled with a radionuclide suitable for imaging and/or radiotherapy, with high affinity for the estrogen receptor, low affinity for non-specific binding sites and good *in vivo* metabolic stability [6].

The search for selective agents to image receptor densities in steroid-sensitive tumours *in vivo*, as well as the need for probes to study hormone action, has lead to the synthesis and evaluation of a series of halogenated derivatives of estradiol over the last 20 years [1,7]. These include estradiol derivatives labelled with bromine [8,9], fluorine [10-14] and iodine [15-24]. However, few of these agents have reached the clinical stage [12,25-27]. The development of radioiodinated imaging agents for estrogen-positive breast tumours has been based on subtle modifications of the natural estrogen structure through the placement of different chemical groups around the ring system. Attachment of radioiodine to estradiol as a 16α-iodo or (17α,20*E/Z*)-iodovinyl substituent imparts minimal interference to the ER binding site and good *in vivo* stability [15,22,23]. Further substitutions at the 11β-position are well tolerated, often resulting in either slightly reduced or in some cases even in increased specific binding as compared to (17α,20*E/Z*)-iodovinyl estradiol itself. Thus, radioiodinated 16α-iodo and 17α-iodovinylestradiols with a number of different substituents at the 11β-position (methoxy, ethoxy, chloromethyl, ethyl) have been biologically evaluated [20,22,28-31]. Both the [^{123}I] radiolabelled 20*E* and 20*Z* isomers of the 11β-methoxy derivatives have been clinically assessed, with the 20*Z* isomer giving the better images of ER-positive tumours [27]. The *in vivo* stability of the 20*Z* isomer has been questioned despite its higher affinity for ER [32,33]. Interconversion to the 20*E* isomer has been reported to occur in the uterus of immature female rats [33]. However, chromatographic analysis of plasma samples of healthy volunteers and cancer patients, as well as *in vitro* plasma incubations, confirmed the *in vivo* stability of the 20*Z* isomer

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[27]. Addition of a 7α-methyl substituent in 17α-(20*E/Z*)-iodovinyl estradiols has been reported to enhance receptor affinity as well as uterus uptake [20,29]. Long alkyl chains can also be accommodated in the 7α-position of the estradiol molecule without a substantial reduction of affinity for the estrogen receptor as found for fulvestrant, a new pure ER antagonist recently approved by the FDA for the treatment of advanced or metastic breast cancer [34,35].

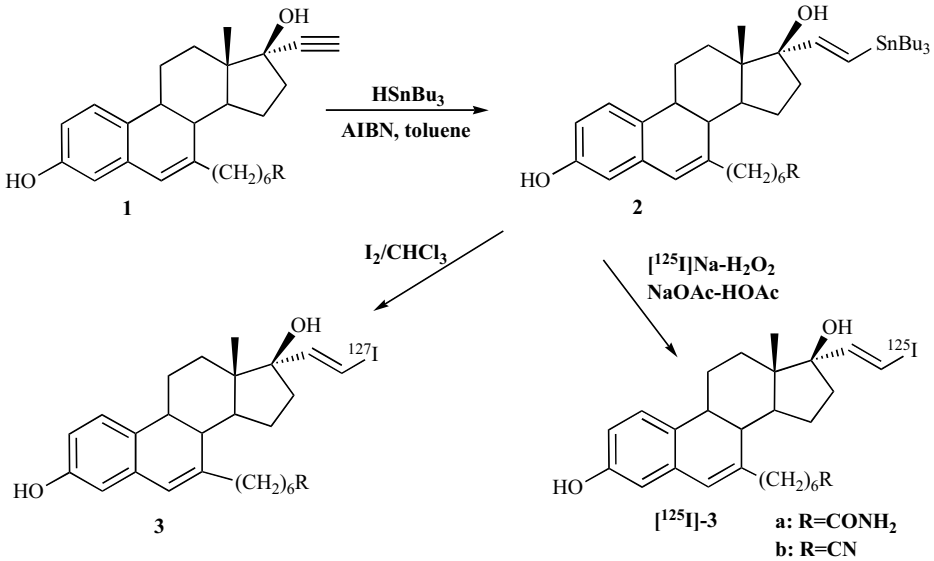
In light of these findings our strategy involves the introduction of a hexyl chain with either a cyano or an amide terminal group at the C7 position of a 6,7-dehydroestradiol as a means to enhance and/or alter the receptor response. The introduction of a C6-C7 double bond induces a conformational change in the B ring which is reflected in the biological characteristics of the molecules [36]. Herein, we report the radiosynthesis and biological evaluation of two novel radioiodinated C7-substituted 17α-iodovinylestra-1,3,5(10), 6-tetraene-3,17β-diols (Scheme **1**). The purpose of this study is to understand further how the effect of altering the chemical structure of the chain in the more flexible $\Delta^{6,7}$–estradiol derivatives may influence the ability of these compounds for molecular recognition of ER-positive breast tumours.

MATERIALS AND METHODS

All commercial reagents and solvents were of analytical grade and were used without further purification. HPLC-grade solvents were used for HPLC purification and analysis. Proton and carbon nuclear magnetic resonance spectra (^{1}H and ^{13}C) were performed on a Varian Unity 300 MHz spectrometer using $CDCl_3$ as solvent. High resolution mass spectra were obtained on a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR/MS) Finnigan FT/MS 2001-DT. Electron impact mass spectra (ESI/QITMS) were acquired from a Bruker HCT Mass Spectrometer. Analytical thin-layer chromatography was run using Merck 60 F 254 silica gel plates and spots were visualized under UV light (254 nm). Purification of intermediate or final products was performed by column chromatography using silica gel (70-230 mesh) from Merck. High Performance Liquid Chromatography (HPLC) analyses were performed on a Perkin-Elmer system equipped with a biocompatible quaternary pump (series 200), a UV/vis detector (SPD-10 AV, Shimadzu, UV detection at 254 nm) and a radioactivity detector (LB 509, Berthold). Purification of radioiodinated compounds and analysis of the final products were carried out by analytical reverse-phase HPLC on an EC-Nucleosil C18 column (250 x 4 mm, 5 µm, Macherey Nagel) with a flow rate of 0.5 mL/min using methanol in water (80/20) as eluent. Purification of compound **3a** was achieved on a reverse-phase VP-Nucleosil C18 column (250 x 8 mm, 7 µm, Macherey Nagel) with a flow rate of 2 mL/min using the same binary system. [^{125}I] Sodium iodide was obtained from Perkin Elmer, USA, as a non-carrier added solution in 0.1 M aqueous NaOH. Radiochemical yields were calculated from the initial amount of radioactivity used in the radiolabelling reaction and the radioactivity of the HPLC purified products. The synthesis of the ethynyl precursors **1a** and **1b** has been described previously [37]. [2,4,6,7-^{3}H]E_2 (specific activity of 84.0 Ci/mmol) was obtained from Amersham, GE Healthcare, UK. The recombinant human ERα protein was obtained from PanVera, Invitrogen Corporation, CA, USA. Hydroxylapatite (HAP) was obtained from Calbiochem (San Diego, CA, USA).

General Procedure for the Synthesis of the Tributylstannyl Precursors

A mixture of approx. 0.1 mmol of **1a** or **1b** and tributyltin hydride (2 eq) in dry toluene (3-5 mL) was heated overnight at 80ºC under nitrogen in the presence of a catalytic amount of azobisisobutyronitrile (AIBN). The cooled mixture was poured onto crushed ice and extracted with ethyl acetate (3 x 10 ml). The organic phase was washed with water and brine, dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*.



Scheme 1. Synthesis of (*E*)-7-(5-carboxamidopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**3a**) and (*E*)-7-(6'-cyanohexyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**3b**) and respective radioiodinated analogues (**[^{125}I]-3a)** and (**[^{125}I]-3b)**.

(E)-7-(5′-Carboxamidopentyl)-17α-tributylstannylvinylestra-1,3,5(10),6-tetraene-3,17β-diol (2a)

The resulting crude was purified by column chromatography on silica gel (chloroform/methanol 6:1) to yield **2a** as a yellow oil (30 mg, 25%). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.82-0.91 (m, 12H), 1.23-2.23 (m, 45H), 6.04 (d, 1H, ^{E}*J*=19.3 Hz), 6.10 (s, 1H), 6.17 (d, 1H, ^{E}*J*=19.3 Hz), 6.34 (d, 1H, *J*=2.4 Hz), 6.61 (dd, 1H, *J*=2.4 Hz, *J*=7.8 Hz), 6.98 (d, 1H, *J*=7.8 Hz).

(E)-7-(6'-Cyanohexyl)-17α-tributylstannylvinylestra-1,3,5(10),6-tetraene-3,17β-diol (2b)

The resulting crude was purified by column chromatography on silica gel (ethyl ether/petroleum ether/chloroform 1:1:1) to yield **2b** as a yellow oil (31 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.85-0.92 (m, 12H), 1.22-2.36 (m, 43H), 6.06 (d, 1H, ^{E}*J*=19.1 Hz), 6.12 (s, 1H), 6.19 (d, 1H, ^{E}*J*=19.1 Hz), 6.50 (d, 1H, *J*=2.3 Hz), 6.60 (dd, 1H, *J*=2.3 Hz, *J*=8.5 Hz), 7.03 (d, 1H, *J*=8.5 Hz).

General Procedure for the Synthesis of Iodinated Ligands

To a solution of **2a** or **2b** (0.04-0.05 mmol) in chloroform (1.6 ml) was added a 0.1 M solution of iodine in chloroform (approx. 1 mL) until the colour of iodine persisted. This was followed by addition of potassium fluoride in methanol (160 µl of a 1 M KF solution) and an aqueous sodium bisulfite solution 5% (160 µl). Then, the product was extracted with ethyl acetate. The organic phase was washed with water, dried over $MgSO_4$ and evaporated to dryness under reduced pressure.

(E)-7-(5′-Carboxamidopentyl)-17α-iodovinylestra-1,3,5(10), 6-tetraene-3,17β-diol (3a)

The residue was purified by semi-preparative RP-HPLC using the described experimental conditions and compound **3a** was obtained as a colourless solid after evaporation to dryness (5 mg, 37%). ^{1}H NMR (300 MHz, $CDCl_3$, δ) 0.92 (s, 3H), 1.20-2.26 (m, 27H), 6.10 (s, 1H), 6.30 (d, 1H, ^{E}*J*=14.4 Hz), 6.44 (dd, 1H, *J*=2.7 Hz, *J*=8.2 Hz), 6.52 (d, 1H, *J*=2.7 Hz), 6.77 (d, 1H, ^{E}*J*=14.4 Hz), 6.99 (d, 1H, *J*=8.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$, δ) 13.68 (C18), 15.97 and 17.55 (*n*-hexyl chain), 24.34 (C15), 25.50 (C11), 27.08, 27.45, 27.83 and 29.72 (*n*-hexyl chain), 31.67 (C16), 35.88 (C12), 41.41 (C8), 43.08 (C9), 45.92 (C13), 48.66 (C14), 74.84 (C20), 86.06 (C17), 112.56 (C1), 116.25 (C4), 116.77 (C2), 124.16 (C6), 128.99 (C10), 135.54 (C5), 145.84 (C7), 150.32 (C19), 160.60 (C3), 175.36 (-C=O). HRMS (EI(+) 10eV T≃300ºC) Found:549.17250, calculated $C_{27}H_{36}NO_3I(M^+)$: 549.17344.

(E)-7-(6'-Cyanohexyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (3b)

The residue was purified by preparative thin layer chromatography (ether/petroleum ether 40-60ºC/chloroform 1:1: 1). The iodinated compound was extracted from the plate with the same mixture of solvents affording **3b** (2 mg, 10%) as a colourless solid after evaporation to dryness. ^{1}H-NMR (300 MHz, $CDCl_3$, δ) 0.90 (s, 3H), 1.20-2.36 (m, 25H), 6.14 (s, 1H), 6.32 (d, 1H, ^{E}*J*=14.4 Hz), 6.51 (d, 1H, *J*=2.7 Hz) 6.60 (dd, 1H, *J*=2.7 Hz, *J*=8.1 Hz), 6.77 (d, 1H, ^{E}*J*=14.4 Hz), 7.08 (d, 1H, *J*=8.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$, δ) 13.11 (C18), 14.87, 17.02 and 19.25 (*n*-hexyl chain), 23.82 (C15), 25.93 (C11), 26.35, 27.19 and 27.34 (*n*-hexyl chain), 31.03 (C16), 35.71 (C12), 40.79 (C8), 42.40 (C9), 45.30 (C13), 48.11 (C14), 74.50 (C20), 85.46 (C17), 111.31 (C4), 112.05 (C2), 117.23 (-C≡N), 123.04 (C1), 124.06 (C6), 132.33 (C10), 135.39 (C5), 146.42 (C7), 149.66 (C19), 153.62 (C3). ESI/MS Found: 553.9 $(M+Na)^+$, calculated: 553.5 $(M+Na)^+$.

Radioiodination

The *trans* isomers of compounds [^{125}I]-7-(5′-carboxamidopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol **[^{125}I]-3a** and [^{125}I]-7-(6'-cyanohexyl)-17α-iodovinylestra-1, 3,5(10),6-tetraene-3,17β-diol **[^{125}I]-3b** were prepared by radioiodination of the corresponding tributylstannyl precursors, **3a** and **3b** respectively, using *in situ* generated peracetic acid as oxidizing agent according to a described labelling procedure [23,29]. To a solution containing the 17α-tributylstannylvinylestra-1,3,5(10),6-tetraene-3,17β-diol (approx. 100 µg) in 100 µL of methanol and 50 µL of 5% NaOAc solution (w/v) was added 0.5-1 mCi of [^{125}I]NaI (18-37 MBq) and 50 µL of an oxidant solution consisting of 30% H_2O_2/HOAc (2:1). The mixture was vortexed and allowed to react for 10 min at room temperature. The reaction was quenched by the addition of 25 µL of an aqueous 5% $Na_2S_2O_3$ solution (w/v). Then, the mixture was diluted with 100 µL of water and the radioiodinated compound was extracted with dichloromethane (2 x 100 µL). The combined organic layers were evaporated under a stream of nitrogen. Then, the residues were dissolved in methanol and purified on an analytical RP-HPLC column using an isocratic gradient mixture of 80% methanol and 20% water with a flow rate of 0.5 mL/min. Quality control radiochromatograms were obtained under the same HPLC conditions.

Receptor Binding Affinity

The ERα competitive binding assay was performed according to the method described by Jiang W-R *et al.* [38] with minor modifications. 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 hydroxylapatite 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 (1-5x10^4 nM) of the test compound in the ERα binding buffer, 45 µl of [^{3}H]E_2 solution (23.8 nM) and 5 µl (0.25 pmol) of ERα protein solution. Nonspecific binding by the [^{3}H]E_2 was determined by addition of a 50 µM concentration of the nonradioactive E_2. The binding mixture was incubated at 4 ºC for 16-18 h. At the end of the incubation, 200µl of the HAP slurry was added and the tubes were incubated on ice and vortexed three times in 15 min. An aliquot of 1ml of washing buffer was added, mixed and centrifuged at 10,000 rpm 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 the ^{3}H radioactivity in a liquid scintillation counter (Packard Tri-CARB 3170 TR/SL). The data

obtained from duplicate measurements were expressed as the percent specific binding of [^{3}H]E_2 versus the log molar concentration of the competing compound. The IC_{50} values represent the concentration of the test compound required to reduce the [^{3}H]E_2 binding by 50%.

In Vitro Stability Studies

In vitro stability of the radioiodinated compounds was assessed in physiological saline, human serum, rat blood and rat liver homogenate by HPLC. *Physiological saline:* the radiolabelled compounds (200 μL) were incubated in physiological saline containing 1% Tween-20 at 6ºC and at 37ºC. Aliquots were taken before incubation started (for zero time point analysis) as well as at various time intervals during incubation (1 h, 4 h and 24 h). *Human serum:* The radiolabelled compounds (400 μL) in physiological saline (containing 1% Tween 20) were incubated with human serum (1.6 mL) for 1, 2, 4 and 24 hours at 37ºC. Aliquots were taken (200 μL) and treated with absolute ethanol (400 μL) at 4ºC to precipitate the proteins. Samples were centrifuged at 3,000 rpm for 10 min at 4ºC. The supernatants were separated from the precipitate and analysed by HPLC. *Rat blood:* female Sprague-Dawley rats, weighing approximately 250-300 g each, were sacrificed by excess anaesthesia and blood collected in heparine. The radiolabelled compounds (200 μL in physiological saline containing 1% Tween-20) were incubated with rat blood (900 μL) for 1, 4 and 24 hours at 37ºC. Also, one aliquot was also taken before incubation started. Samples were centrifuged at 3,000 rpm for 10 min at 4ºC. Aliquots were taken (200 μL) and treated with absolute ethanol (400 μL) at 4ºC to precipitate the proteins. Samples were centrifuged at 3,000 rpm for 10 min at 4ºC. The supernatants were separated from the precipitate and analysed by HPLC. *Liver homogenate:* The excised liver was rapidly rinsed and placed in a chilled 50 mM TRIS/0.2 M sucrose buffer, pH=7.4, wherein it was homogenised. Aliquots (in duplicate) of the homogenate (800 μL) were incubated with the radiolabelled compounds (200 μL in physiological saline containing 1% Tween-20) for 1 and 24 hours at 37ºC. Samples were treated with absolute ethanol (2 mL) at 4ºC to precipitate the proteins. Samples were centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatants were separated from the precipitate and analysed by HPLC.

Biodistribution

Studies were carried out in immature female Sprague-Dawley rats (19-20 days), obtained from IFFA, CREDO, Spain. The experiments were conducted in compliance with the National law and with the EU guidelines for Animal Care and Ethics for Animal Experiments. The animals were injected intraperitoneally with 10 μCi (370 kBq) of the radiolabelled steroid reconstituted in 100 μL of physiological saline containing 1% Tween-20. A separate group of rats was co-injected with unlabelled estradiol (50 μg) in order to block the estrogen receptors. The injected dose (ID) was assumed to be the difference between the measured radioactivity in the syringe before and after injection. Animals were maintained on normal diet *ad libitum* and were sacrificed by excess anaesthesia at 1, 2 and 24 h post injection with radiotracer and 2 h in the case of co-injected cold estradiol. The main organs were removed, weighed and the activity measured in a gamma counter. Results were expressed as percent of injected dose per gram of organ (% ID/g organ) and presented as mean values ±SD. For blood and muscle, the values were calculated assuming that these organs constitute 6% and 40% of the total weight, respectively. Whole animal body radioactivity excretion was not quantified. The *in vivo* stability of the radiotracers was also evaluated by HPLC analyses of rat urine. The urine was collected at sacrifice time and centrifuged at 2,000 rpm for 10 min before RP-HPLC.

RESULTS

A number of factors were taken into account when selecting the alkyl chain appended to the 7 position of the $\Delta^{6,7}$-dehydroestradiol frame. It is known that short chains with bulky substituents at the 7α-position of estradiols themselves attenuate the affinity of the molecules for ER, whereas long chains can detrimentally increase the lipophilicity and the molecular weight of the compounds, which can result in a decrease of the binding affinity [39]. Therefore, hexyl substituents of intermediate chain length with either a cyano or an amide terminal group were chosen for the current study. 7-Cyanoalkyl and 7-amidoalkyl substituted 17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diols were prepared from the corresponding 17α-ethynyl precursors **1a** and **1b** [37]. The iodination and radioiodination of the stannyl derivatives were carried out according to a described procedure [23,29]. The 17α-ethynylestradiol derivatives were reacted with tri-n-butyltin hydride in toluene at 80ºC, in the presence of azoisobutyronitrile as catalyst to yield the corresponding tributylstannylvinylestradiol derivatives **2a** and **2b** as major products. The high reaction temperature, the presence of the catalyst and the low polarity of the solvent used in the reaction favoured the formation of the *trans* isomers and no trace of the *cis* isomers was observed in the hydrostannylation. Electrophilic iododestannylation of the tributylstannylvinylestradiol derivatives proceeded by addition of a 0.1 M solution of iodine in chloroform to give the corresponding *trans* iodovinylestradiol derivatives, **3a** and, **3b** in moderate yield and with the expected retention of configuration. The configuration of the synthesised compounds was established by ^{1}H-NMR and ^{13}C-NMR spectroscopic analysis. The assigned stereochemistry of the iodine on C-20 was based on the coupling constants of the two vinylic protons at C-19 and C-20 in the ^{1}H-NMR spectra and the carbon signals in the ^{13}C-NMR. Thus in the ^{1}H-NMR spectrum two doublets centred at δ=6.30-6.32 ppm for the protons at C-19 and at δ=6.75-6.77 ppm for the protons at C-20 with J=14.4Hz are indicative of *trans* (*E*) stereochemistry [40]. Signals centred at δ=74.50-75.17 ppm for C-20 and at δ= 149.66-150.32 ppm for C-19 in the ^{13}C-NMR spectrum also correspond to the *E* configuration.

The stannyl derivatives **2a** and **2b** were converted to their radioiodinated analogues **[^{125}I]-3a** and **[^{125}I]-3b** respectively by treatment with [^{125}I]NaI in the presence of H_2O_2. After solvent extraction with dichloromethane, the radiolabelled compounds were purified by analytical HPLC with simultaneous radioactivity and UV detection (254 nm) using a C18 reverse column eluted with 80% aqueous methanol. The radiochemical yields obtained for **[^{125}I]-3a** and **[^{125}I]-3b** were 28% and 61%, respectively. All the radioiodinated products

were obtained in radiochemical purity higher than 98% after reformulation in physiological saline (containing 1% Tween-20). When compared to their corresponding unlabelled analogues by HPLC all the radiotracers were shown to be the expected products on the basis of their elution profile (Fig. **1**). Moreover, the experimental HPLC conditions used in the purification have permitted an efficient separation of the **[^{125}I]-**labelled compounds from other radioactive species present in the reaction mixture as well as from their non-radioiodinated precursors, leading to radioiodinated tracers with high specific activity. Since no UV detection at the most sensitive detector setting was observed for the purified labelled compounds it was then assumed that their specific activity was in the same range as that of the starting [^{125}I]NaI, 2200Ci/mmol. The low *in vivo* concentration of steroid receptors associated with the occasionally incisive pharmacological effect of extremely low doses of receptor-binding drugs always requires a radiopharmaceutical with high specific activity.

Radiochemical stability of the radioiodinated compounds was evaluated by HPLC analysis of samples at several time points at 6ºC and 37ºC after incubation in physiological saline (with 1% Tween-20) to detect any radiochemical impurities or free iodide. All the radiolabelled compounds were shown to be radiochemically stable up to 24 hours in physiological saline at both temperatures. The radiolabelled compounds were also incubated with samples of human serum at 37ºC and treated with ethanol to precipitate the proteins. HPLC analyses of the ethanolic extracts have demonstrated the relative radiochemical stability of the radioligands towards deiodination up to 24 h of incubation.

The radiochemical stability of the two novel radioligands was also determined in rat blood. In the HPLC analysis of the amide **[^{125}I]-3a** the formation of a more hydrophilic species which increased drastically over time was observed. Indeed, at 24 hours incubation, no traces of radiolabelled amide could be detected by HPLC. Although no attempt was made to characterize this degradation species, it is assumed that it most probably corresponds to the hydrolysed amide. However no traces of radioiodide were found in the chromatographic analysis. In contrast, no degradation products could be observed from the HPLC analysis of the labelled nitrile. Since liver is a major organ of metabolization samples of rat liver homogenate were incubated with the radiolabelled compounds up to 24 h and were analysed by HPLC. In the analysis of the amide no traces of radioiodide were observed. However, at 24 hours of incubation the hydrolysis of the amide bond was also detected suggesting its low resistance to metabolic degradation. Although HPLC analysis of the nitrile did not reveal any decomposition species, a fast deiodination rate (31% at 1 h incubation) was detected indicating very low radiochemical stability in rat liver homogenate.

To investigate whether the novel iodovinyl compounds would retain binding affinity for the estrogen receptor *in vitro* binding affinities of the ethynyl precursors, **1a** and **1b**, for the recombinant human ERα were determined by competitive radiometric binding assay using [^{3}H]estradiol as tracer. Incubations were done overnight at 4ºC and hydroxylapatite was used to separate bound receptor-ligand complex. 17α-Ethynyl substituted estradiols can often be used as indicators of their 17α-vinyl iodide analogs due to similar characteristics of the two moieties, *ie.*, their lipophilicity. Thus, we anticipate that values of the ER binding affinity of the iodovinyl counterparts will follow the trend of their ethynyl precursors.

The IC_{50} values for each competing estrogen were calculated according to the sigmoid inhibition curves depicted in Fig. (**2**). The IC_{50} values of compounds **1a** and **1b** for ERα were 432.10±1.3 nM and 27.28±1.24 nM, respectively. The relative binding affinity (RBA) for each compound was calculated against estradiol (E_2) by using the following equation: RBA=IC_{50} for E_2/ IC_{50} for each compound. Relative binding affinities (RBA) for **1a** and **1b** were 1.1 and 17.2 % of that of estradiol. Under our experimental conditions the IC_{50} value of estradiol (4.69 nM±1.09) is in good agreement with the IC_{50} value reported in the literature (5.90 nM±1.19) [41]. These data indicate that the addition of an alkyl chain

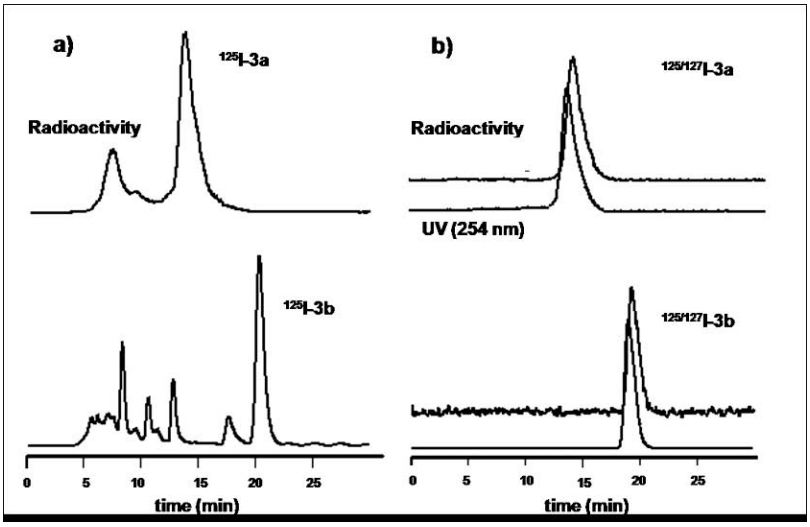


Fig. (1). a) Purification of radioiodinated mixtures by RP-HPLC. **b)** HPLC analysis of radioiodinated **[^{125}I]-3a** and **[^{125}I]-3b** after HPLC purification co-injected with reference compounds **3a** and **3b.**

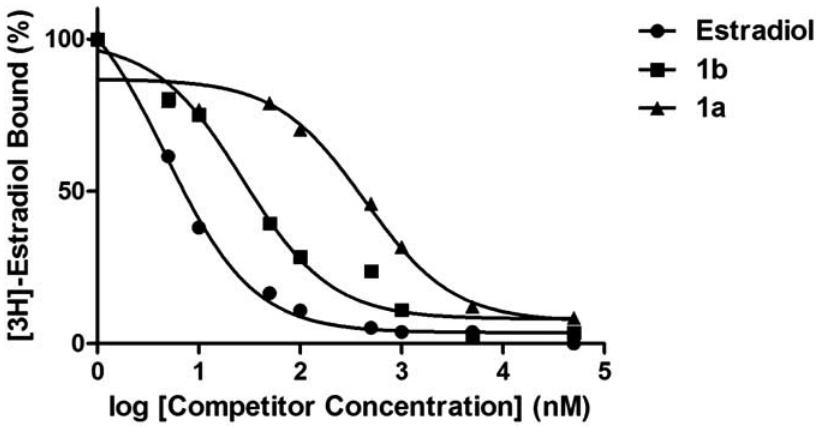


Fig. (2). Comparison of the relative ER binding affinity of compounds **1a** and **1b** with that of estradiol (E_2).

to the C7 position of the dehydroestradiol framework decreases binding affinity comparatively to that of estradiol. Moreover, the terminal group of the appended alkyl chain also affects significantly the ER binding affinity.

The *in vitro* studies can predict the *in vivo* behaviour of the radioiodinated compounds but cannot mimic the entire *in vivo* environment. Thus, to better understand how the structure of the 7-alkyl chain might affect their biological behaviour we have decided to assess the biodistribution of all the radioligands in adequate animal models regardless of the observed low RBA values for **1a** and **1b**. These studies will allow a better insight into the nature of the radiochemical species in the tissues and/or eliminated by the urinary pathway as the radioligands are exposed to metabolic pathways and have to overcome tissue membrane barriers.

Immature female rats (20-22 days) were used for *in vivo* screening of the ER radioligands, since they have low levels of endogenous estrogen and yet have ER-expressing target tissues, such as in the uterus and the ovaries. The effect of endogenous circulating estradiol can be obviated in this animal model because the estrous cycle in rats just begins 30 days after birth. Then, the biological behaviour of the radiolabelled compounds **[^{125}I]-3a** and **[^{125}I]-3b**, was evaluated through biodistribution studies in immature female Sprague-Dawley rats after intraperitoneal administration of the radiotracers at 1, 2 and 24 h. To evaluate the receptor mediated uptake in the ER over-expressing tissues, such as in the uterus and the ovaries, a separate group of animals was co-injected with unlabelled estradiol to block the receptors. The most representative tissue distribution values are shown in Tables **1** and **2**. For the two radioligands the amount of activity excreted in the urine was very small suggesting the hepatobiliary tract as the main excretory route, which is expected for this animal model. The total urinary radioactivity was excreted as a radiochemical species with the same retention time as free radioiodine as demonstrated by HPLC analysis of samples collected at sacrifice time, indicating some *in*

Table 1. Biodistribution Data in the Most Relevant Organs, Expressed as %I.D./g Organ, for the Amide [^{125}I]-3a, at 1h, 2h and 24 h After Administration in Immature Sprague-Dawley Female Rats (n=4-5)

Tissue	1h Control [^{125}I]-3a	2h Control [^{125}I]-3a	2h Block with 50µg Estradiol	24h Control [^{125}I]-3a
Blood	1.86 ± 0.20	0.87 ± 0.24	0.75 ± 0.06	0.54 ± 0.22
Liver	4.93 ± 0.93	3.49 ± 0.40	3.26 ± 0.54	0.61 ± 0.06
Small Intestine	22.36 ± 1.74	36.5 ± 1.79	45.11 ± 5.38	1.54 ± 0.21
Large Intestine	0.66 ± 0.08	1.52 ± 0.52	1.43 ± 0.30	89.77 ± 3.92
Kidney	1.94 ± 0.26	1.18 ± 0.29	0.80 ± 0.14	0.27 ± 0.03
Muscle	1.14 ± 0.51	0.55 ± 0.34	0.52 ± 0.23	0.10 ± 0.06
Thyroid	0.29 ± 0.07	0.49 ± 0.18	0.82 ± 0.13	0.03 ± 0.01
Uterus	2.86 ± 0.81	1.27 ± 0.30	1.19 ± 0.41	0.31 ± 0.06
Ovaries	1.22 ± 0.34	0.99 ± 0.49	1.29 ± 0.21	0.40 ± 0.02
Uterus/blood	1.54	1.46	2.53	0,37
Uterus/muscle	2.51	2.31	3.65	3.1

Table 2. Biodistribution Data in the Most Relevant Organs, Expressed as %I.D./g Organ, for the Nitrile [^{125}I]-3b, at 1h, 2h and 24 h After Administration in Immature Sprague-Dawley Female Rats (n=4-5)

Tissue	1h Control [^{125}I]-3b	2h Control [^{125}I]-3b	2h Block with 50µg Estradiol	24h Control [^{125}I]-3b
Blood	0.6 ± 0.3	0.6± 0.3	0.58 ± 0.05	0.11 ± 0.03
Liver	1.9 ± 0.5	1.3 ± 0.2	1.6 ± 0.3	0.4 ± 0.2
Small intestine	13.9 ± 3.0	18.2 ± 3.6	21.4 ± 1.5	1.3 ± 0.5
Large intestine	1.7 ± 0.4	1.6 ± 0.4	1.3 ± 0.4	2.2 ± 0.5
Kidney	1.7 ± 0.4	1.2 ± 0.8	1.2 ± 0.1	0.08 ± 0.02
Muscle	0.50 ± 0.09	0.4 ± 0.08	0.5 ± 0.1	0.04 ± 0.01
Thyroid	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	10.1 ± 3.1
Uterus	11.9 ± 1.6	13.3 ± 4.5	11.5 ± 1.9	0.14 ± 0.04
Ovaries	8.4 ± 2.0	5.8 ± 1.4	5.9 ± 1.5	0.11 ± 0.03
Uterus/blood	19.83	22.17	20.52	1.27
Uterus/muscle	23.8	33.25	23.0	3.5

vivo deiodination. However, the *in vivo* stability of radioiodinated steroids is mainly reflected in the amount of radioactivity in the thyroid, and it can be seen from the low thyroid levels that all the compounds are relatively stable *in vivo*.

Several observations can be made for the amide **[^{125}I]-3a** (Table **1**). First, the very low uterus uptake (1.27% ID/g at 2 h) and selectivity ratios (uterus-to-blood <2) signify non-selective distribution of this compound. Secondly, the blocking studies indicate that the target tissue uptake was not receptor mediated, since the uptake values in the blocked experiment were not significantly different from the control. The low uterus tissue uptake (1.27% ID/g at 2 h) and the large non-specific uptake in tissues such as the liver (3.49% ID/g at 2 h) can be most likely attributed to the formation, as observed by HPLC analysis, of an unidentified labelled degradation product in the rat blood and liver homogenate. The absence of an ER-mediated *in vivo* uptake mechanism in target tissues was in agreement with the low *in vitro* binding affinity values.

Next, the biodistribution studies of the nitrile were evaluated. Results of the tissue distribution studies of [^{125}I](*E*)-7-(6'-cyanohexyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**[^{125}I]-3b**) are presented in Table **2**.

The replacement of the terminal amide group by a cyano function results in a pronounced increase in uterine activity (13.3% ID/g *vs* 1.27% ID/g). This combined with low blood and non-target tissue specific activity, results in high uterus-to-blood (25:1 at 2h) and uterus-to-non-target tissue ratios (32:1). Despite its high specific uptake and high selectivity for target tissues **[^{125}I]-3b** shows no competitive binding to the receptor *in vivo*, when estradiol is coinjected. As **[^{125}I]-3b** shows a comparatively fair binding to the estrogen receptor *in vitro*, studies are underway to elucidate further its action *in vivo*.

CONCLUSION

Two novel radioiodinated C7-substituted $\Delta^{6,7}$-estradiol derivatives, (*E*)-7-carboxamidopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol and (*E*)-7-(5'-car-boxamidopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3, 17β-diol **[^{125}I]-3b**, were synthesized in high radiochemical purity. While the ethynyl precursor **1a** of steroidal amide **[^{125}I]-3a** shows a very low *in vitro* binding affinity to the estrogen receptor and **[^{125}I]-3a** exhibits an unsatisfactory biodistribution in female Sprague Dawley rats, the ethynyl precursor **1b** of steroidal cyano derivative **[^{125}I]-3b** possesses a comparatively better binding affinity to the receptor *in vitro* and **[^{125}I]-3b** shows high uterus-to-blood (25:1 at 2h) and uterus-to-non-target tissue ratios (32:1). However, despite its high specific uptake and high selectivity for target tissues, **[^{125}I]-3b** shows no competitive binding to the receptor *in vivo*, when estradiol is co-injected. It was noted that the different alkyl chains at C7 of the $\Delta^{6,7}$-estradiols cause alterations in uptake efficiency and selectivity of the compounds and in the extent of their metabolism. Thus far, it cannot be decided whether the alterations in uptake efficiency and selectivity are the result of differences in lipophilicity or altered patterns of metabolism. In spite of the reduced ability of these radioligands for *in vivo* recognition of ER their different biological behaviour may provide valuable information in designing novel probes for ER targeting.

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