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Radioiodinated ligands for the estrogen receptor: Effect of different 7-cyanoalkyl chains on the binding affinity of novel iodovinyl-6-dehydroestradiols

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ABSTRACT

Three novel 17α-ethynyl-$\Delta^{6,7}$-estra-3,17β-diols and their 17α-[^{125}I]-iodovinyl derivatives, containing different C7-cyanoalkyl chains, were studied as potential radioligands for the estrogen receptor. The influence of the chain length on the biological behaviour of the compounds was assessed through *in vitro* ER binding assays of the ethynyl derivatives and breast cancer cell uptake studies of the 17α-[^{125}I]-iodovinyl-$\Delta^{6,7}$-estra-3,17β-diols. A difference in alkyl chain induced a decrease in ER binding affinities of substances, however, the receptor-binding affinities (RBA) of all compounds were lower than that of estradiol itself. In addition, a non-specific cell binding was observed which is in accordance with the encountered ethynyl RBA values suggesting that the uptake is not ER mediated.

1. Introduction

Breast cancer is one of the most common forms of cancer and despite progress in early detection and treatment mortality is still high. Since one-third of the breast cancer patients respond to the hormonal treatment and approximately two thirds of the breast cancer tumours over express estrogen receptors, drugs based on ER ligands can be valuable tools in imaging these tumours as well as in evaluating their ER content for prognosis, treatment and follow-up of the disease (van de Wiele et al., 2000).

ER binding ligands also play a crucial role as chemotherapeutic/chemopreventive agents, and regardless of the many medicinal advances novel therapeutic strategies are still required for the treatment of ER-positive tumours. Targeted radiotherapy, using internally emitted radiation, can be an attractive option to conventional therapies such as surgery, external radiotherapy and chemotherapy. Early studies have clearly shown the extreme cellular toxicity of Auger electron-emitters and have demonstrated the ability of suitably targeted Auger electron decays within the cell nucleus to cause double strand DNA breaks and cell death with virtually no damage to the surrounding cells (Hofer, 2000). Radiopharmaceuticals based on Auger emitter radionuclides are anticipated to afford a highly selective, targeted radiotherapy, since the emitted electrons deposit their low energy within subcellular dimensions (Wheldon and O'Donoghue, 1990; Szumiel, 1994; Volkert, 1999).

One interesting approach to radionuclide therapy of ER-rich tumours is the use of ER binding ligands radiolabelled with Auger emitters. A high affinity for the ER, the selectivity of the hormone–receptor interactions and an avidity of the complex for DNA combine to provide the basis for radiotherapy with these Auger electron-emitting steroid hormone–receptor ligands (De Sombre et al., 1990). In the last decade, the synthesis and biological properties of some radiohalogenated estradiol derivatives have been reported (Katzenellenbogen, 1996; Cummins, 1993). While most of the emphasis was focused on the radioimaging potential of these radioligands, the presence of Auger electrons from the decay of $^{123/125}$I- and $^{77/80m}$Br- initiated interest

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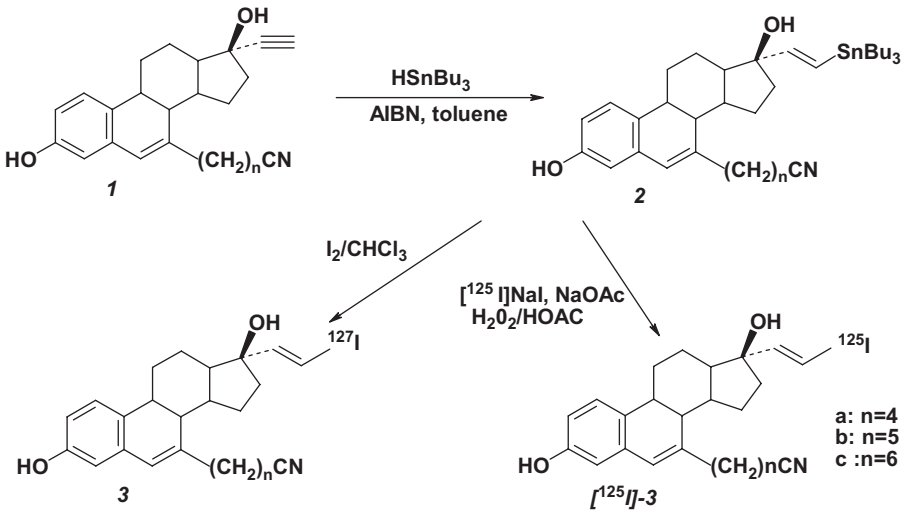

Scheme 1. Synthesis of [^{125}I](*E*)-7(4′-cyanobutyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**[^{125}I]-3a**), [^{125}I](*E*)-7-(5′-cyanopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**[^{125}I]-3b**) and [^{125}I](*E*)-7-(6′-cyanohexyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**[^{125}I]-3c**).

in their radiotherapeutic applications and several studies have shown that estrogens labelled with Auger emitters might kill ER-positive cells while sparing ER-negative cells (McLaughlin et al., 1989; De Sombre et al., 2000; Yasui et al., 2001).

As part of our investigation on new specific ligands for targeted therapy and/or nuclear imaging of ER-rich tissues, we became interested in the synthesis and evaluation of radioiodinated estradiol derivatives (Oliveira et al., 2006). Our strategy involves the introduction of a terminally functionalized long alkyl chain 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 has an effect on the conformation of the estradiol molecule due to the conformational change in the B ring and imparts a different topology to the steroid as compared to that of the saturated steroid analogue, which is reflected in the biological characteristics of the molecules (Melo e Silva et al, 2001). Herein, we report the radiosynthesis and biological evaluation of three novel radioiodinated $\Delta^{6,7}$–estradiol derivatives, substituted at the C7 position with alkyl chains of different lengths (Scheme 1). The purpose of this study is to understand further how the length of such alkyl chains, attached to the estradiol framework, may affect the receptor-binding affinity (RBA) and the uptake of such compounds in estrogen receptor (ER) positive cells. Scheme 1.

2. Materials and methods

2.1. Reagents and instruments

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 $CDCl_3$. 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 a column chromatography using silica gel (70–230 mesh) from Merck. High performance liquid chromatography (HPLC) analyses were performed on a PerkinElmer system equipped with a biocompatible quaternary pump (series 200), an 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 × 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 **3b** was achieved on a reverse-phase VP-Nucleosil C18 column (250 × 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 PerkinElmer, 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**, **1b** and **1c** has been described elsewhere (Morais et al., 2006). Tritiated estradiol, [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).

2.2. Chemistry

2.2.1. General procedure for the synthesis of the tributylstannyl precursors

A mixture of approximately 0.1 mmol of **1a, 1b** or **1c** and tributyltin hydride (2 eq) in dry toluene (3–5 mL) was heated overnight at 80 °C 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 × 10 mL). The organic phase was washed with water and brine, dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The resulting crude of **2a, 2b** and **2c** was purified by column chromatography on silica gel (ethyl ether/hexane/chloroform 1:1:1).

*2.2.1.1. (E)-7-(4′-Cyanobutyl)-17α-tributylstannylvinylestra-1,3,5(10), 6-tetraene-3,17β-diol (**2a**).* (36 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.85–0.92 (m, 12H), 1.22–2.40 (m, 39H), 6.07 (d, 1H, $^E J = 19.3$ Hz), 6.13 (s, 1H), 6.19 (d, 1H, $^E J = 19.3$ Hz), 6.50 (d, 1H,

$J = 2.5$ Hz), 6.61 (dd, 1H, $J = 2.5$, Hz $J = 8.3$ Hz), 7.08 (d, 1H, $J = 8.3$ Hz).

*2.2.1.2. (E)-7-(5′-Cyanopentyl)-17α-tributylstannylvinylestra-1,3,5(10), 6-tetraene-3,17β-diol (**2b**).* (36 mg, 30%). ^{1}H (300 MHz, $CDCl_3$, δ): 0.86–0.93 (m, 12H); 1.23–2.36 (m, 41H), 6.07 (d, 1 H, $^E J = 19.2$ Hz), 6.13 (s, 1H), 6.19 (d, 1H, $^E J = 19.2$ Hz), 6.50 (d, 1H, $J = 2.4$ Hz), 6.60 (dd, 1H, $J = 2.4$ Hz, $J = 8.4$ Hz), 7.09 (d, 1H, J = 8.4 Hz).

*2.2.1.3. (E)-7-(6′-Cyanohexyl)-17α-tributylstannylvinylestra-1,3,5(10), 6-tetraene-3,17β-diol (**2c**).* (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).

2.2.2. General procedure for the synthesis of iodinated ligands

To a solution of **2a, 2b** or **2c** (0.04–0.05 mmol) in chloroform (1.6 mL) was added a 0.1 M solution of iodine in chloroform until the colour of iodine persisted. This was followed by addition of potassium fluoride in methanol (1 M, 160 μL, 160 μmol) and an aqueous sodium bisulphite 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.

*2.2.2.1. (E)-7-(4′-Cyanobutyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-idol (**3a**).* **3a** was obtained without further purification as a colourless solid (17 mg, 87%). ^{1}H NMR (300 MHz, $CDCl_3$, δ) 0.90 (s, 3H), 1.18–2.40 (m, 21H), 6.13 (s, 1H), 6.31 (d, 1H, $^E J = 14.4$ Hz), 6.49 (d, 1H, $J = 2.4$ Hz), 6.60 (dd, 1H, $J = 2.4$ Hz, $J = 8.4$ Hz), 6.75 (d, 1H, $^E J = 14.4$ Hz), 7.06 (d, 1H, $J = 8.4$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$, δ) 13.64 (C18), 17.50 (*n*-butyl chain), 24.27 (C15), 25.32 (C11), 26.84, 27.14 and 27.54(*n*-butyl chain), 31.46 (C16), 36.12 (C12), 41.22 (C8), 42.80 (C9), 45.71 (C13), 48.60 (C14), 75.16 (C20), 85.88 (C17), 111.88 (C4), 112.74 (C2), 119.67 (–C═N), 123.94 (C6), 124.61 (C1), 131.31 (C10) 135.60 (C5), 145.76 (C7), 150.00 (C19) 154.06 (C3). HRMS (EI(+) 10 eV T∼300 °C) found:503.14168, calculated $C_{25}H_{30}NO_2I(M^+)$: 503.13158.

*2.2.2.2. (E)-7-(5′-Cyanopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**3b**).* The residue was purified by preparative RP-HPLC using methanol in water (80/20) as eluent. (10 mg, 42%). ^{1}H NMR (300 MHz, CD_3,OD, δ): 0.90 (s, 3H), 1.17–2.47 (m, 23H), 6.16 (s, 1H), 6.34 (d, 1H, $^E J = 14.4$ Hz), 6.45 (d, 1H, $J = 2.4$ Hz), 6.54 (dd, 1H, $J = 2.4$ Hz, $J = 8.4$ Hz), 6.82 (d, 1H, $^E J = 14.4$ Hz), 7.03 (d, 1H, $J = 8.4$ Hz).^{13}C NMR (75 MHz, CD_3OD, δ) 13.68 (C18), 16.15 (*n*-pentyl chain), 24.49 (C15), 25.37 (C11), 26.99, 28.24 and 18.58 (*n*-pentyl chain), 31.57 (C16), 35.61 (C12), 41.77 (C8), 43.27 (C9), 45.86 (C13), 48.67 (C14), 73.46 (C21), 85.49 (C17), 111.71 (C4), 112.05 (C6), 124.35 (C1), 130.47 (C10), 135.81 (C5), 146.38 (C7), 151.28 (C20), 155.53 (C23). ESI/MS found:539.9 $(M+Na)^+$, calculated: 539.2 $(M+Na)^+$.

*2.2.2.3. (E)-7-(6′-Cyanohexyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol (**3c**).* The residue was purified by preparative thin-layer chromatography (ether/hexane/chloroform 1:1:1). The iodinated compound was extracted from the plate with the same mixture of solvents affording **3c** (2.2 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 $(M+Na)^+$.

2.3. Radiochemistry

The *trans* isomers of compounds [^{125}I]-7-(4′-cyanobutyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol **[^{125}I]-3a**, [^{125}I]-7-(5′-cyanopentyl)-17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diol **[^{125}I]-3b** and [^{125}I]-7-(6′-cyanohexyl)-17α-iodovinylestra-1,3,5(10), 6-tetraene-3,17β-diol **[^{125}I]-3c** were prepared by radioiodination of the corresponding tributylstannyl precursors, **2a**, **2b** and **2c** respectively, using *in situ* generated peracetic acid as oxidizing agent according to a described labelling procedure (Ali et al., 1988, 1991). To a solution containing the tributylstannyl precursors, **2a**, **2b** and **2c** (approximately 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 (2×100 μL). The combined organic layers were evaporated under a stream of nitrogen. Then, the residue was dissolved in methanol and purified on an analytical RP-HPLC column using an isocratic 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.

2.4. Receptor-binding affinity

The ERα competitive binding assay was performed according to a described method (Jiang et al., 2006) 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 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 protein 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 $10{,}000 \times g$ 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, vortex 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 vs. the log molar concentration of the competing compound. The IC_{50} values (calculated using the SigmaPlot software) represent the concentration of the test compound required to reduce the [^{3}H]E_2 binding by 50%.

2.5. In vitro stability studies

The *in vitro* stability of the radioiodinated compounds was assessed in physiological saline by HPLC analysis. The radiolabelled compounds (200 μL) were incubated in physiological saline containing 1% Tween-20 at 6 °C and at 37 °C. Aliquots were taken before incubation started (for zero time point analysis), as well as at various time intervals during incubation (1, 4 and 24 h).

2.6. In vitro cellular uptake studies

An ER (+) human breast cancer cell line, MCF-7 (ATCC), was used in this assay. 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 cellular uptake studies, cells were plated at a density of 2×10^5 cells/0.5 mL per well of a 24-well plate 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 radioiodinated compounds. After 0.25, 0.5, 1, 2 and 24 h incubation, the cells were washed twice with cold PBS, lysed with 0.1 M NaOH and the cellular extracts were counted for radioactivity. Each experiment was performed in quadruplicate. To investigate whether the cellular uptake of the radioiodinated compounds **[^{125}I]-3a, [^{125}I]-3b** and **[^{125}I]-3c** occurs via an ER-mediated process similar experiments were carried out in parallel by the addition of 100 μL of estradiol (10 μM) or tamoxifen (1 μM).

3. Results and discussion

It is known that short chains with bulky substituents at the 7α-position of estradiol framework attenuate its affinity for ER, whereas long chains can detrimentally increase the lipophilicity and the molecular weight of compounds, which can result in a decrease of the binding affinity (Anstead et al., 1997). Therefore, for the current study, butyl, pentyl and hexyl substituents were chosen, *i.e.*, substituents of intermediate chain length with a cyano terminal group. 7-Cyanoalkyl substituted 17α-iodovinylestra-1,3,5(10),6-tetraene-3,17β-diols were prepared from the corresponding 17α-ethynyl precursors **1a**, **1b** and **1c** (Morais et al., 2006). The 17α-ethynylestradiol derivatives were reacted stereoselectively with tri-*n*-butyltin hydride in toluene at 80 °C, in the presence of azoisobutyronitrile as catalyst to yield the corresponding *trans* tributylstannylvinylestradiol derivatives **2a**, **2b** and **2c**. The high reaction temperature, the presence of the catalyst and the low polarity of the solvent used in the reaction favored the formation of the *trans* isomers (Ali et al., 1988, 1991). Assignment of the *trans* geometry was confirmed by ^{1}H-NMR spectroscopic analysis. The proton germinal to the tin was split into a doublet with a coupling constant of 19 Hz, a typical value for this kind of system.

The iodination and radioiodination of the stannyl derivatives were carried out according to a described procedure (Ali et al., 1988, 1991). Electrophilic iododestannylation of these compounds gave the corresponding iodovinylestradiol derivatives, **3a**, **3b** and **3c**, in a moderate yield and with the expected retention of configuration. 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 $\delta = 6.31$, 6.34 and 6.32 ppm, respectively, for the protons at C-19 and at $\delta = 6.75$, 6.82 and 6.77 ppm, respectively, for the protons at C-20 with $J = 14.4$ Hz are indicative of *trans* (*E*) stereochemistry. Signals centred at $\delta = 75.16$, 73.46 and 74.50 ppm for C-20 and at $\delta = 150.00$, 151.28 and 149.66 ppm

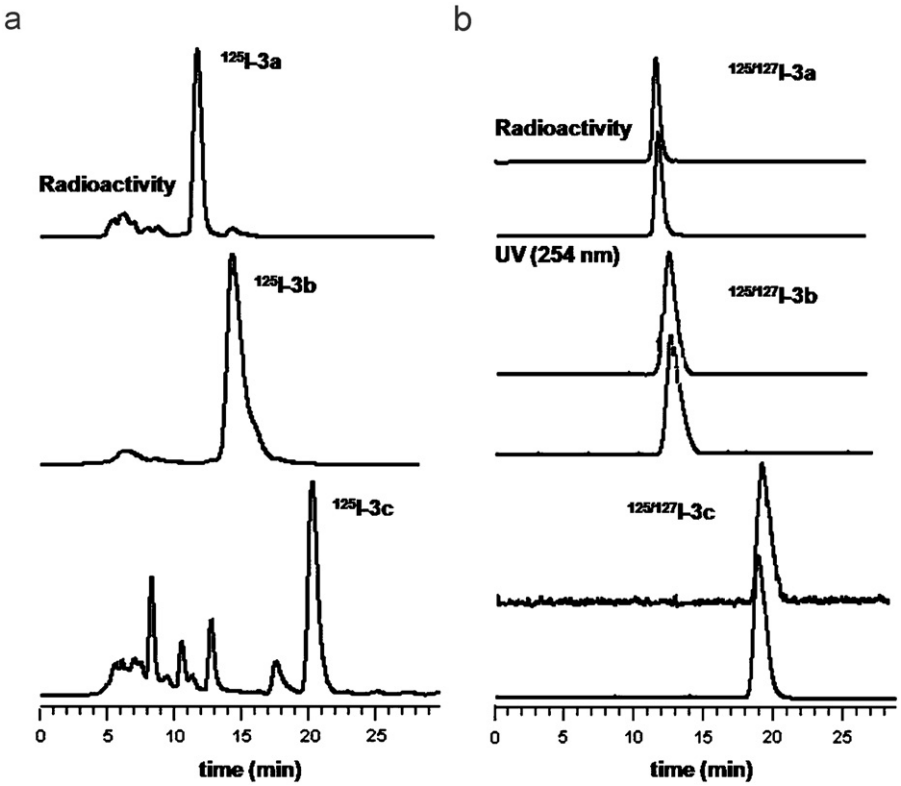

Fig. 1. (a) Purification of radioiodinated mixtures by RP-HPLC. (b) HPLC analysis of radioiodinated **[^{125}I]-3a, [^{125}I]-3b** and **[^{125}I]-3c** after HPLC purification co-injected with reference compounds **3a, 3b** and **3c**. Isolation and analysis were carried out on a Nucleosil C-18 reversed-phase column (250 × 4 mm, 5 μm) eluted with a mixture of MeOH:H_2O (80:20) at 0.5 mL/min. The eluent was simultaneously monitored by an UV detector (254 nm) and a radioactivity detector.

for C-19 in the ^{13}C-NMR spectrum also correspond to the *E* configuration (Hofmeister et al., 1986).

The stannyl derivatives **2a**, **2b** and **2c** were also converted to their radioiodinated analogues **[^{125}I]-3a, [^{125}I]-3b** and **[^{125}I]-3c**, 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**, **[^{125}I]-3b** and **[^{125}I]-3c** were 86%, 94% and 48%, 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 under the simultaneous radioactivity and UV detection, all radiotracers were shown to be the expected products on the basis of their elution profile (Fig. 1).

The experimental HPLC conditions used in the purification have permitted an efficient separation of the labelled compounds from other radioactive species present in the reaction mixture, as well as from theirs non-radioiodinated precursors, leading to radioiodinated compounds with high specific activity. The *in vitro* stability of the radioiodinated compounds under physiological conditions was evaluated by HPLC analysis of samples at several time points at 6 °C and 37 °C 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 h in physiological saline at both temperatures.

To investigate whether the novel compound would retain the binding affinity for the ER *in vitro* binding affinities of the ethynyl precursors, **1a**, **1b** and **1c**, for the recombinant human ERα were determined by competitive radiometric binding assay using tritiated estradiol as tracer. Incubations were carried out overnight at 4 °C and HAP was used to separate bound receptor–ligand complex.

Vinyl iodide moieties are often taken to be roughly equivalent to an ethynyl substituent due to their similar characteristics, *e.g.,* steric dimensions, lipophilicity, and its replacement by an iodovinyl should result in a biologically equivalent analogue. Thus, we anticipate that the ER binding affinity of the iodovinyl counterparts will follow the same trend as the ethynyl precursors. Fig. 2.

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, 1b** and **1c** for ERα were 859, 53.4 and 27.2 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, 1b** and **1c** were 1.03%, 9.16% and 17.19% of that of estradiol. Under our experimental conditions, the IC_{50} of estradiol (6.14 nM ± 1.91) is in good agreement with the literature IC_{50} (5.90 nM ± 1.19) (Pillon et al., 2005).

The data indicates that the addition of a cyanoalkyl chain to the C7 position of the dehydroestradiol framework decreases the binding affinity, comparatively, to that of the estradiol. Moreover, the length of the alkyl chain also affects the ER-binding affinity. Binding affinity values are increased by lengthening the C7-chain, and thus moving the terminal polar cyano group away from the steroidal frame. The different RBAs may also be directly related to the overall lipophilicity of ligands.

To find out whether the radiolabelled compounds are taken up by cells effectively, binding studies were conducted with the human breast MCF-7 cancer cells. In order to assess specific binding, similar experiments were performed simultaneously,

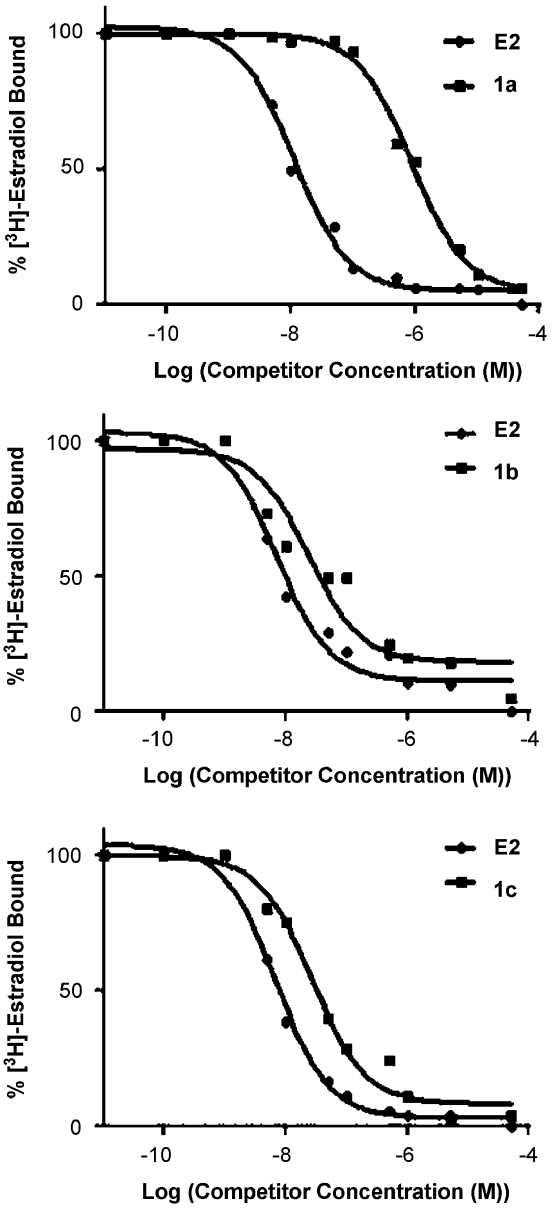

Fig. 2. Comparison of the relative ER-binding affinity of compounds **1a, 1b** and **1c** with that of estradiol (E_2). The relative binding affinity of each competing compound was determined by measuring its inhibition of the binding of [^{3}H]E_2 to the recombinant human ER**α**. The data for nine concentrations (1–5 × 10^4 nM) was taken. Each point represents the mean of duplicate measurements.

incubating the cells with the labelled compounds in the presence of estradiol or tamoxifen. The cellular uptake of labelled compounds **[^{125}I]-3a [^{125}I]-3b** and **[^{125}I]-3c** is presented in Fig. 3.

Cell uptake in MCF-7 cells was observed for the three radioligands, although no significant variation was noticed in the percentage of binding over the studied incubation time (Fig. 2). **[^{125}I]-3b** cellular uptake was significantly higher when compared to the other estradiol counterparts and even higher than the uptake observed for [^{125}I]-17α-iodovinylestradiol that was used for comparison. However, there was no evidence of a decrease in the uptake of any of the radiotracers under study, when cells were treated with estradiol, this in contrast to [^{125}I]-17α-iodovinylestradiol. These findings may indicate that the mechanism of cellular uptake is not an ER-mediated process. This is in agreement with the lower RBA values encountered for the ethynyl precursors and suggests that the uptake may be due to

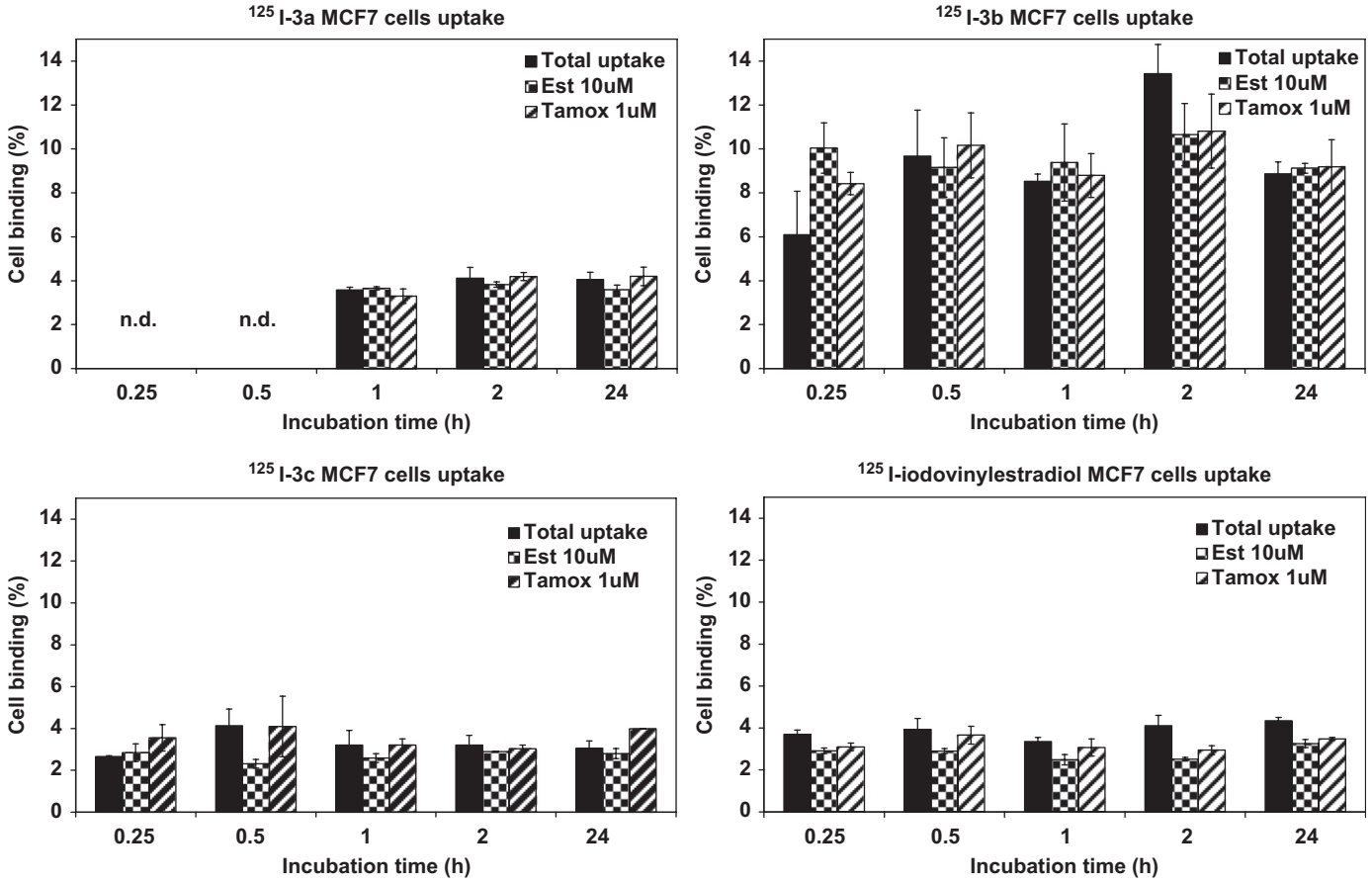

Fig. 3. Cellular uptake of **[^{125}I]-3a**, **[^{125}I]-3b** and **[^{125}I]-3c** in human MCF-7 breast cancer cells, with and without co-incubation with estradiol or tamoxifen. [^{125}I]IVE was used for comparison, (nd = not determined).

another mechanism. Detailed investigations on the cell uptake of such compounds are underway.

4. Conclusion

Three novel radioiodinated $\Delta^{6,7}$–estradiol derivatives, with C7 ω-cyanoalkyl substituents of different chain length were synthesised successfully in high radiochemical yields and in high radiochemical purity after HPLC purification. The introduction of a ω-cyanoalkyl substituent at C7 of the estradiol led to a decrease of binding affinity to ER. The length of the alkyl chain affects the ER-binding affinity, clearly enhancing with an increase of chain length, following the trend butyl < pentyl < hexyl. Cellular binding studies with MCF-7 cells suggest a non-ER-mediated uptake mechanism.

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