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Research paper

Synthesis, characterization and in vitro cytotoxicity studies of a macromolecular conjugate of paclitaxel bearing oxytocin as targeting moiety

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Abstract

The present study describes the experimental synthetic procedure and the characterization of a new polyaspartamide macromolecular prodrug of paclitaxel, bearing oxytocin residues as targeting moieties. In vitro stability studies of bioconjugate, performed in media mimicking biological fluids (buffer solutions at pH 7.4 and 5.5) and in human plasma, evidenced the high stability of the targeting portion (oxytocin)-polymer linkage and the ability of this conjugate to release linked paclitaxel in a prolonged way in plasma. Moreover, preliminary in vitro antiproliferative studies, carried out on MCF-7 cells, that are oxytocin receptor positive cells, showed that the polymeric conjugate has the same cell growing inhibition ability of free drug.

Keywords: Polyaspartamide; Oxytocin; Drug targeting; Paclitaxel; Bioconjugate

1. Introduction

In the cancer therapy the small difference in activity of the most part of conventional chemotherapic agents against tumor tissues, in comparison with non-tumor tissues, leads to a similar toxicity towards ill and healthy tissues with consequent serious damages for patients until death. Targeted drug delivery can substantially improve cancer therapy because if the drug can be addressed more effectively to the tumor, less drug reaches normal tissues resulting in fewer like-threatening side effects. For these reasons great interest is addressed by the scientific community to the research of more selective and effective tumor targeting groups that could be conjugated to naked drugs or to sophisticated colloidal drug delivery systems such as nanoparticles, polymeric micelles or macromolecular prodrugs; moreover, starting from a better knowledge of the biochemistry and molecular biology of tumors, several kinds of molecules have been proposed and tested as tumor targeting groups such as antibodies, oligosaccharides, peptides, vitamins [1,2].

Oxytocin (OT), the hypothalamic nonapeptide hormone, besides its physiological activity on target cells [3–5] has demonstrated to play a role even on tumors derived from some organs [6–9]. Many breast cancer cells including, MCF-7, in fact overexpress, both at RNA and protein levels [5,10] the specific G protein-linked cell surface receptors that bind oxytocin (OTRs) [7,11,12]. Oxytocin/OT receptors interaction causes the rapid internalization of the nonapeptide/receptor complex and its disappearance from cell surface via classic clathrin-mediated pathway [13–15]. The demonstrated wide distribution of OTRs within neoplastic tissues [9] can represent an interesting

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way to achieve cell specific drug delivery. The conjugation of an antitumoral drug to a composite system, bearing oxytocin as targeting moiety, can in fact determine the specific accumulation into receptor expressing cancer cells reducing drug interaction with healthy tissues. Among antitumoral agents used in therapy, paclitaxel (TXL) shows an exceptional activity against primary epithelial ovarian carcinoma as well against, breast, colon, head, non-small cell lung cancers and AIDS related Kaposi's sarcoma [16]. This drug promotes tubulin polymerization and formation of extraordinarily stable and dysfunctional microtubules, which disrupts the normal tubule dynamics, required for cellular division, provoking cell death [16]. In order to gain a supramolecular system able to efficiently address paclitaxel to disease sites, we planned to couple the above-mentioned drug to a polymer with pendent oxytocin groups. The α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) [17,18], a freely water-soluble, non-toxic, non-antigenic and non-immunogenic multifunctional macromolecule, proposed as plasma substitute, seems to be the ideal candidate since it has been successfully proposed as drug carrier [19–22]. In this paper to obtain a long-blood circulation polymeric system, we have chosen a PHEA copolymer, bearing attached to the backbone few PEG chains of 2 kDa, namely PHEA–PEG_{2000}, previously prepared and characterized by our group [23]. In that study we have demonstrated that the PEG grafting endows PHEA derivatives with prolonged permanence in the bloodstream [23]. Taking into account these considerations, we have introduced oxytocin residues on PHEA–PEG_{2000}macromolecules by a succinic spacer linked by amidic linkage to the α-NH_2 group of oxytocin. To the obtained conjugate, 2′-*O*-succinylpaclitaxel molecules were introduced using carbonyldiimidazole as condensant agent. The PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel conjugate so obtained was subjected to in vitro stability study mimicking biological fluids (buffer solutions at pH 7.4 and 5.5) and in plasma in order to obtain preliminary information on its use as drug delivery system.

Preliminary in vitro antiproliferative studies were carried out on MCF-7 cells, that are oxytocin receptor positive cells, in order to check the cell growing inhibition activity of the obtained conjugate in comparison with free drug.

2. Materials and methods

Paclitaxel was a kind gift of Pharmacia & Upjohn (Nerviano, Italy). Oxytocin was purchased from Bachem (Switzerland). D,L-aspartic acid, ethanolamine, *O*-(2-aminoethyl)-*O*′-methylpolyethylene glycol 2000 (PEG_{2000}-NH_2) (<0.4 mmol NH_2/g) and 1,1′-carbonyldiimidazole (CDI) were purchased from Fluka (Buchs, Switzerland). Anhydrous solvents (acetone, pyridine, ethanol, diethylether) were obtained from Sigma Chemical Co (USA); chloroform, dichloromethane and methanol were from Merck (Germany); anhydrous *N*,*N*-dimethylformamide (DMF), Sephadex G-25 for gel filtration (50–150 μ) and absolute ethanol were purchased from Aldrich (Italy). All other chemicals were of reagent grade.

PHEA–PEG_{2000} was prepared and purified according to a previously reported procedure [23]. The amount of linked PEG_{2000} chains, determined by ^{1}H NMR, was found to be about 1.24% mol/mol [23]. The weighed-average molecular weight of PHEA–PEG_{2000}, measured by SEC in aqueous solutions, was 18.0 kDa ($M_w/M_n = 1.6$). Human plasma was obtained from voluntary healthy blood donors.

Infrared spectra were obtained using a Perkin–Elmer 1720 IR Fourier Transform Spectrophotometer in potassium bromide disks.

Ultraviolet (UV) spectra were recorded by using a Jasco Instrument.

The mass spectra were obtained using a Hybrid Q-Star Pulsar-i (MSD Sciex, Applied Biosystems, Toronto, Canada) mass spectrometer.

The ^{1}H NMR spectra were obtained with a Bruker AC-250 instrument operating at 250.13 MHz. HPLC analyses were carried out on a system consisting of a Varian 9012 Liquid Chromatography equipped with a Rheodyne Injector 7125 (fitted with a 10 μL loop), a Kontron HPLC Detector 432 on line with a computerised HP workstation. In the method HPLC, a reversed phase C_{18} column (μBondapak; 10 μm of 250 mm × 4.6 mm i.d., obtained from Waters) equipped with a direct-connect guard column C_{18}(Waters) was used.

Molecular weights of conjugates were determined by SEC chromatographic system equipped with a 410 differential refractometer (DRI) from Waters (Milford, MA, USA) as concentration detector. Two SEC methods were used to determine weighted molecular weight of conjugates. First one consisted of 0.15 M NaCl as mobile phase, at the temperature of 35 °C, with a flow rate of 0.8 mL/min and two Ultrahydrogel columns from Waters (1000 and 250 Å of pore size). The second one consisted of DMF + 0.01 M LiBr as mobile phase, at the temperature of 50 °C, with a flow rate of 0.6 mL/min and two Phenogel columns from Phenomenex . The molecular weights were estimated based on PEO/PEG standards (range 318.000-4.000 Da).

2.1. Synthesis of N-succinyl–oxytocin (*1*)

To a solution of 2.5×10^{-2} g (2.5×10^{-2} mmol) of Oxytocin in 2 mL of anhydrous DMF, 66 μL (1.25×10^{-2} mmol) of triethylamine (TEA) and 0.4×10^{-2} g (4×10^{-2} mmol) of succinic anhydride were added. The reaction mixture was kept at 35 ± 0.1 °C under argon for 1 h. After this time the solvent was removed under reduced pressure and the residue was treated with 5 mL of acetone. The resulting precipitate was filtered and then washed five times with acetone. The resulting product **1** was characterized by mass spectrum, UV spectroscopy and ^{1}H NMR analysis.

The mass spectrum shows the M at m/e 1107.

UV analysis (H_2O) showed two main peaks at λ 230 nm and at 275 nm ($E^{1\%}_{275} = 10.2$).

^{1}H NMR (D_2O) showed besides all peaks related to Oxytocin [24] a peak at δ (ppm) 2.5 [m, 4H, CO–CH_2CH_2–CO].

2.2. Synthesis of PHEA–PEG_{2000}-succinyloxytocin (2)

To a solution of 4.8×10^{-2} g (4.3×10^{-2} mmol) of **1** in 2 mL of anhydrous DMF, a solution of 1.0×10^{-2} g (6.1×10^{-2} mmol) of CDI in 0.5 mL of anhydrous DMF was added dropwise at 25 ± 0.1 °C, then the reaction mixture was kept at 25 °C for 3 h. After this time a solution of 0.13 g (0.68 mmol) of PHEA–PEG_{2000} in 3 mL of anhydrous DMF was added dropwise. The reaction mixture was maintained at 25 ± 0.1 ° C for 2 h and then set outside at room temperature for 4 days under occasional shaking; subsequently the solvent was removed under reduced pressure and the residue was dissolved in 2 mL of water and purified by gel permeation chromatography on Sephadex G-25. The pure conjugate was obtained with a 90% yield (w/w) based on starting material (PHEA–PEG_{2000}) and it was characterized by UV spectroscopy, ^{1}H NMR and by SEC analyses. The absence of free drug was confirmed by HPLC analysis.

The amount of linked oxytocin was evaluated by UV spectroscopy and ^{1}H NMR analysis.

By UV spectroscopy (H_2O), comparing the value of $E^{1\%}_{275}$ of **2** ($E^{1\%}_{275} = 1.63$ in aqueous solution) with that of **1** ($E^{1\%}_{275} = 10.2$ in the same medium) the Succinyloxytocin content was found to be about 16% w/w corresponding to about 2.7% mol/mol.

^{1}H NMR (D_2O) showed besides all peaks related to Oxytocin [24], peaks at δ (ppm) 2.5 [m, 4H, CO–$\mathbf{CH_2CH_2}$–CO], 2.8 [2H, m, CO–(CH)–$\mathbf{CH_2}$–CONH of PHEA–PEG], 3.2 [2H, m, –NH–$\mathbf{CH_2}$–CH_2–OH of PHEA–PEG], 3.6 [2H, m, –NH–CH_2–$\mathbf{CH_2}$–OH of PHEA–PEG], 3.8 [178 H, s, –$\mathbf{CH_2CH_2}$– of PHEA–PEG], 4.7 [1H, m, CO–(**CH**)–CH_2–CONH of PHEA–PEG] and 6.9–7.2 [4 H, dd, of aromatic protons of tyrosine of Oxytocin].

The amount of linked Oxytocin (molar percentage) was calculated comparing the integral values of the peak at δ 6.9–7.2 assignable to *N*-succinyloxytocin with the integral of the peaks related to protons at δ 2.8 assignable to PHEA–PEG_{2000} and it was found about 2.8% mol/mol corresponding to 17% w/w.

2.3. Synthesis of 2′-O-succinyl-paclitaxel derivative (3)

2′-*O*-succinyl-paclitaxel was prepared from paclitaxel according to a previously described procedure [25]. Briefly, to a solution of paclitaxel in dry pyridine (0.4 g/mL), succinic anhydride was added in a proper amount to have 12.5:1 – succinic anhydride/paclitaxel molar ratio. The reaction mixture was kept at room temperature for 3 h. Then the solvent was evaporated under vacuum and the residue was suspended in distilled water, kept under stirring for 30 min and centrifuged. The final product was characterized by elemental analysis, melting point, FT-IR, UV spectroscopies and ^{1}H NMR analysis. The analytical and spectral data of **3** were in agreement with attributed structure [25].

2.4. Synthesis of PHEA–PEG_{2000}-succinyloxytocin–succinylpaclitaxel (4)

To a solution of 0.13 g (0.136 mmol) of **3** in 1.3 mL of anhydrous DMF, a solution of 0.27×10^{-2} g (0.163 mmol) of CDI in 1 mL of anhydrous DMF was added dropwise at 25 ± 0.1 ° C and the reaction mixture kept at the same temperature under stirring for 3 hrs. After this time, a solution of 0.1 g (0.45 mmol) of **2** in 1.5 mL of anhydrous DMF was added dropwise, then 100 μL (0.72 mmol) of TEA was added and the resulting mixture kept at 25 ± 0.1 °C for 4 h and subsequently at 35 ± 0.1 °C for 24 h. After this time further 50 μL (0.36 mmol) of TEA was added and the reaction mixture kept under inert atmosphere at 35 ± 0.1 °C for 63 h. After this time the solvent was removed under vacuum and the residue washed seven times with 15 mL of CH_2Cl_2/ether (1:1, v/v) mixture collecting each time the residue by centrifugation at 10,600 rpm for 15 min. The residue was dried under vacuum, dissolved in distilled water and dialyzed for 3 days against distilled water, then the solution lyophilized. The pure conjugate, obtained in 89% w/w yield based on the starting material (**2**), was characterized by IR, UV spectroscopy and ^{1}H NMR analysis.

The amount of free drug in the product **4** was determined by HPLC analysis.

IR spectrum (KBr) showed: a broad band in the range 3600–3000 cm^{-1} (–OH, NH), 1730–1718 cm^{-1} (stretching C═O of succinic ester bonds), 1645 cm^{-1} (amide I of PHEA), 1542 cm^{-1}(amide II of PHEA).

UV analysis (H_2O) of conjugate **4** reveals absorption in the range between 240 and 290 nm, in that PHEA–PEG_{2000} did not absorb, attributable to the linked drug and oxytocin molecules.

^{1}H NMR (D_2O) showed all peaks assignable to oxytocin and paclitaxel. In particular at δ 2.4 [m,protons of succinic groups], 2.8 [2H, m, CO–(CH)–$\mathbf{CH_2}$–CONH of PHEA–PEG], 3.0 [m, –NH–$\mathbf{CH_2}CH_2$–OH of PHEA–PEG], 3.6 [m, NH–CH_2–$\mathbf{CH_2}$–OH of PHEA–PEG], 3.8 [178 H, s, –$\mathbf{CH_2}$–$\mathbf{CH_2}$– of PHEA–PEG], 5.3–5.9 [m, 3H, $^{2'}$**CH**; ^{2}**CH**; $^{3'}$**CH** of Paclitaxel], 6.3 [m, 2 H, ^{13}**CH;** ^{10}**CH** of Paclitaxel], 7.2–7.4 [4 H, m, of aromatic protons of tyrosine of Oxytocin], 7.5–8.1 [m, **3′-Ph; 3′-NBz** of Paclitaxel], 8.2 [m, 2H, **2′OBz** of Paclitaxel].

The amount of paclitaxel linked to the carrier was evaluated by ^{1}H NMR comparing the integral of peaks related to protons at δ 5.3–5.9, 6.3 and 8.2 assignable to protons that belong to linked drug with the integral of the peaks related to protons at δ 3.0 (assignable to –NH–$\mathbf{CH_2}$–CH_2–OH of PHEA–PEG). This value was found to be about 7.3% mol/mol. The amount of linked Oxytocin was calculated comparing the integral values of the peaks at δ 7.2–7.4 assignable to *N*-succinyloxytocin with the integral

of peaks related to protons at δ 3.0 assignable to PHEA–PEG (assignable to –NH–**CH**$_2$–CH$_2$–OH of PHEA) and it was found to be about 2.8% mol/mol.

The weighed average molecular weight and the polydispersity index of conjugate **4** were determined by SEC analysis.

2.5. Synthesis of PHEA–PEG$_{2000}$-succinylpaclitaxel (5)

To a solution of 0.1 g (0.105 mmol) of **3** in 1 mL of anhydrous DMF, a solution of 2.0×10^{-2} g (0.126 mmol) of CDI in 1 mL of anhydrous DMF was added dropwise at 25 ± 0.1 °C and then the reaction mixture was kept at the same temperature for 3 h. After this time, a solution of 0.1 g (0.622 mmol) of PHEA–PEG$_{2000}$ in 1.5 mL of anhydrous DMF and then 100 μL (0.72 mmol) of TEA was added and the reaction mixture was kept at 25 ± 0.1 °C for 4 h and subsequently at 35 ± 0.1 °C for 24 h. After this time further 50 μL (0.36 mmol) of TEA were added and the reaction solution was kept under inert atmosphere at 35 °C for 63 h. The solvent was removed under vacuum and the residue washed seven times with 15 mL of CH_2Cl_2/ether (1:1, v/v) mixture, collecting each time the residue by centrifugation at 10,600 rpm for 15 min. The residue was dried under vacuum, dissolved in distilled water and dialyzed for 3 days against distilled water, then the solution lyophilized. The pure conjugate, obtained in 89% w/w yield based on the starting material (PHEA–PEG$_{2000}$), was characterized by IR, UV spectroscopies and ^{1}H NMR analysis. The amount of free drug in the product **5** was determined by HPLC analysis.

IR spectrum (KBr) showed: a broad band in the range 3600–3000 cm^{-1} (–OH, NH), 1730–1718 cm^{-1} (stretching C=O of succinic ester bonds) 1645 cm^{-1} (amide I of PHEA), 1542 cm^{-1}(amide II of PHEA).

UV analysis (H_2O) of conjugate **5** reveals bands in the range between 240 and 290 nm, in that starting PHEA–PEG$_{2000}$ did not absorb, with two maximum absorption at 273 nm, attributable to the linked paclitaxel.

^{1}H NMR (D_2O/ CD_3OD, 7:3) showed peaks at 1.0 [m, ^{17}**CH**$_3$; ^{16}**CH**$_3$], 1.6 [s, ^{19}**CH**$_3$], 1.8 [s, ^{18}**CH**$_3$], 2.1 [m, **OAc**], 2.2 [m, **OAc**], 2.5–2.8 [m, 4 H , –CO–**CH**$_2$–**CH**$_2$–CO– and 2H, m, CO–(CH)–**CH**$_2$–CONH of PHEA–PEG], 3.1 [m, –NH–**CH**$_2$CH$_2$–OH of PHEA–PEG], 3.6 [m, ^{3}**CH**, –NH–CH$_2$–**CH**$_2$–OH of PHEA–PEG], 4.1 [m, ^{20}**CH**$_2$], 4.4–4.7 [m, ^{7}**CH**; NH–**CH**(CO)NH of PHEA–PEG], 4.9 [d, ^{5}**CH**], 5.3 [d, $^{2'}$**CH**], 5.5 [d, ^{2}**CH**], 5.8 [dd, $^{3'}$**CH**], 6.3 [m , ^{13}**CH**; ^{10}**CH**], 7.1-8.1 [m, **3′**-Ph; **3′-NBz**], 8.2 [m, 2H, **2-OBz**].

The amount of linked drug was evaluated by UV spectroscopy and ^{1}H NMR analysis.

By the UV spectroscopy, comparing $E^{1\%}_{273}$ of **5** ($E^{1\%}_{273} =$ 19.8 in a mixture water/ethanol, 1/1) with that of paclitaxel ($E^{1\%}_{273} =$ 180.7 in the same medium), the amount of linked paclitaxel in **5** was found to be about 10.9% w/w.

By ^{1}H NMR analysis, comparing the integral of the peaks related to protons at δ 1.0, 1.6, 1.8, assignable to methyl groups at C_{16}, C_{17}, C_{18}, C_{19} positions and that at δ 8.2 assignable to protons of benzyl group, that belong to linked drug with the integral of the peaks related to protons at δ 3.1 (assignable to –NH–**CH**$_2$–CH$_2$–OH of PHEA–PEG), the average value of linked drug was found to be about 2.9% mol/mol, calculated as molar percentage of polymer repeating units containing linked drug, with respect to total repeating units and corresponding to about 11.7% w/w.

2.6. Dynamic light scattering (DLS) measurements

The hydrodynamic diameter of conjugate **4** was evaluated by dynamic light scattering (DLS) using a Malvern Zetasizer nano-ZS instrument (Malvern Instruments Ltd, Malvern, UK) during 180 s at 25 °C, with a scattering angle of 173°. The experimental correlation was measured and analysed by Dispersion Technology Software v 3.31 which allows to obtain distribution of hydrodynamic diameter. All measurements were performed in triplicate.

2.7. Stability studies of oxytocin and N-succinyloxytocin derivative

The stability of Oxytocin and *N*-succinyloxytocin derivative was investigated in buffer solution at pH 7.4 (10 mM phosphate buffer, 0.15 M NaCl) and in human plasma. Samples of the two compounds (10 mg/mL) were incubated at 37 ± 0.1 °C in phosphate buffer at pH 7.4. At scheduled time intervals, samples in buffer solution were withdrawn and assayed by HPLC to evaluate intact oxytocin amount. Experiments were carried out for 24 h. In plasma stability studies were carried out by adding 100 μL of an aqueous solution of oxytocin or *N*-succinyloxytocin (10 mg/mL) to samples of preheated plasma in such a way as to have a starting concentration of 1 mg/mL. Samples were kept at 37 ± 0.1 °C by a water bath, under continuous stirring and at suitable intervals, 1 mL of trifluoroacetic acid (10% v/v) was added in order to deproteinize plasma. After mixing and centrifugation for 10 min at 11,800 rpm at 4 °C, 10 μL of clear surnatant was analysed by HPLC monitoring the released species. Preliminary experiments showed that no interfering peaks were in blank plasma chromatogram and that the method allowed a plasma recovery for oxytocin and its succinylderivative derivative of about 99.5%.

The following HPLC conditions were used for these studies: a reversed phase C_{18} column eluted at room temperature with CH_3COO^- NH_4^+ (0.025 M)/CH_3OH (70:30, v/v); the flow rate was 1 mL/min and the eluate was monitored at wavelength of 275 nm. Each experiment was carried out in triplicate.

2.8. Hydrolysis studies of 4 in buffer solutions at 7.4 and 5.5

Equimolar solutions (10^{-3} mM paclitaxel equivalent concentration) of adduct **4**, 2′-*O*-succinyl-paclitaxel or free

paclitaxel in phosphate buffer solution at pH 7.4 and 5.5 were incubated at 37 ± 0.1 °C. At scheduled times solutions were sampled and analysed by HPLC to determine the free paclitaxel and 2′-*O*-succinylpaclitaxel content. The used HPLC condition was $CH_3CN/CH_3COO^-\ NH_4^+$, 35 mM, pH 5.5, 45:55 (v/v), flow 1 mL/min, $\lambda = 229$ nm. Each experiment was repeated in triplicate.

Oxytocin release from conjugate **4** was detected as follows: 100 μL aliquots of a solution of **4** conjugate (10 mg/mL) were added to samples (1 mL) of preheated buffer solutions at pH 7.4 and 5.5 maintained at 37 ± 0.1 °C. At prefixed time intervals 10 μL of solutions was withdrawn and analysed by HPLC monitoring the amount of released oxytocin and succinyl derivative. Each experiment was repeated in triplicate.

2.9. Release studies of 4 in human plasma

One hundred microlitres of equimolar paclitaxel solutions (10^{-2} mM paclitaxel equivalent concentration) of **4**, 2′-*O*-succinylpaclitaxel or free paclitaxel in 10 mM phosphate buffer, 0.15 M NaCl at pH 7.4 was added to 1 mL of plasma. Samples were kept at 37 ± 0.1 °C, under mild stirring. At scheduled times, 100 μL aliquots were taken and added to 50 μL of 0.04 mg/mL *N*-octylbenzamide solution in acetonitrile (internal standard). The samples were added to 1 mL of acetonitrile and centrifuged at 12,000 rpm for 3 min at 4 °C. The obtained precipitate was washed with 0.5 mL of acetonitrile and centrifuged as above reported. The acetonitrile aliquots were pooled and the organic solvent was removed under vacuum. The residue was dissolved in 200 μL of acetonitrile/35 mM ammonium acetate pH 5 mixture (45:55, v/v) and analysed by HPLC. The previously verified reliability and accuracy of the method demonstrated a paclitaxel recovery of 98%. Each experiment was repeated in triplicate. In plasma release of oxytocin and *N*-succinyloxytocin from **4** was also evaluated. The reactions were initiated by adding 100 μL of aqueous solution of conjugate **4** (10 mg/mL) to samples of preheated plasma (900 μL) to have an initial concentration of conjugate of 1 mg/mL. Samples were kept at 37 ± 0.1 °C by a water bath, under continuously stirring and at suitable intervals, 1 mL of trifluoroacetic acid (10% v/v) was added in order to deproteinize plasma. After immediate mixing and centrifugation for 10 min at 11,800 rpm at 4 °C, 10 μL of clear surnatant was analysed by HPLC monitoring the released species. Each experiment was carried out in triplicate.

2.10. Cell culture assay

The in vitro antiproliferative activity of prepared compounds was assayed on MCF-7 cell line (human breast cancer cells). Cytotoxicity of **2**, **4** and **5** conjugates was evaluated by MTT method. The experiment was carried out as follows: 50 μL of cells (2×10^5 cells/mL), that were in the logarithmic phase growth, was plated in 96-well microtitre plates and incubated with 50 μL/well of cell culture medium RPMI 1640 (without red phenol, added of 10% foetal bovine serum and of antibiotics) containing paclitaxel, 2′-*O*-succinylpaclitaxel, PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel (**4**) and PHEA–PEG_{2000}-2′-*O*-succinyl-paclitaxel (**5**) at concentration ranging from 0.1 to 5 μM, prepared by dilution of stock solutions, in ethanol for paclitaxel and 2′-*O*-succinylpaclitaxel and in double distilled water for **4** and **5** conjugates. Controls were obtained by incubation of fifty μL of cells (2×10^5 cells/mL), with the same volume of water or ethanol used in experiments with tested samples (i.e. 1 μL). After 72 h of incubation at 37 ± 0.1 °C and 5% CO_2, the wells were added of 10 μL/well of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) solution (5 mg/mL in 0.02 M phosphate buffer, 0.15 NaCl, pH 7.2) and the plate was incubated under the same conditions for further 4 h. After this time formazane, produced by mitochondria enzymes in the viable cells, was solubilized adding to each well 100 μL of a mixture of 2-propanol, Triton X-100 (10%) and HCl 0.1 N. The amount of formazane was measured by a multi-well plate reader ELX-800 BIO-TEK, through the analysis of optical density at 570 nm after background correction at 690 nm. The cell growth inhibition was expressed as percentage of the untreated controls, using the following equation: A_{570}-A_{690} of treated sample/A_{570}-A_{690} of untreated sample.

The same procedure was followed after incubating PHEA–PEG_{2000} and PHEA–PEG_{2000}-Succinyloxytocin derivatives at the same range polymer concentrations used in previous experiment. Each experiment was carried out in triplicate.

3. Results and discussion

Breast cancer is the most common malignant tumor of endocrine organs. Many receptors are present in breast cancer tissues as those for estrogens, glucocorticoids, insulin-like growth factor, etc. [26–29]. An interesting receptor expressed on over 90% breast cancer cells, including MCF-7, is the Oxytocin receptor [7]. The conjugation of oxytocin to a macromolecular drug carrier could allow addressing whole system to tumor site reducing the distribution into healthy tissues. Following this rationale, we prepared a macromolecular conjugate bearing oxytocin residues as targeting moieties.

3.1. Synthesis of PHEA–PEG_{2000}-succinyloxytocin

Aimed to obtain a system with a long term circulation time, we employed a macromolecular carrier bearing PEG chains at molecular weight of 2 kDa on PHEA backbone since, as previously demonstrated [23,30], the insertion of PEG chains into PHEA structure improves pharmacokinetic and biodistribution properties of macromolecular systems prolonging blood circulation time. In particular the distribution half life of pegylated PHEA

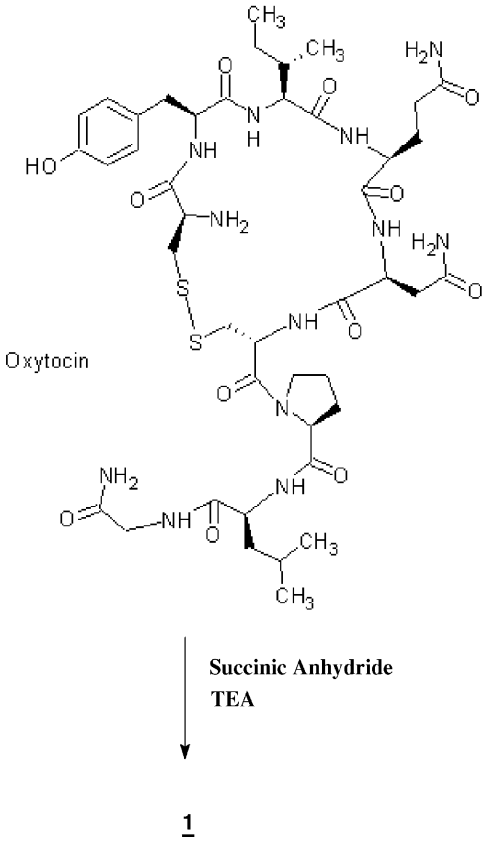
copolymers is about twice than that naked PHEA indicating PEG chains prolong and increase the distribution phase of PHEA [23].

The introduction of chosen targeting moiety (oxytocin) into polymeric chain was performed by two-step reaction:

1. Synthesis of *N*-succinyloxytocin derivative,
2. Conjugation of *N*-Succinyloxytocin to PHEA–PEG_{2000} in order to obtain PHEA–PEG_{2000}-Succinyloxytocin conjugate.

Scheme 1 reports the synthesis of *N*-Succinyloxytocin derivative. ^{1}H NMR analysis demonstrated that succinylation takes place and by nynhidrin determination that it was preferentially at the level of α-NH_2. The chemical structure of *N*-succinyloxytocin was confirmed by mass analysis, UV spectroscopy and ^{1}H NMR analysis.

Scheme 2 reports the PHEA–PEG_{2000}-Succinyloxytocin conjugate synthesis. *N*-succinyloxytocin derivative reacted with PHEA–PEG_{2000} in the presence of carbonyldiimidazole (CDI) as coupling agent. After purification by gel permeation chromatography on Sephadex G-25 and lyophilization the recovery yield was of 90% w/w based on the starting PHEA–PEG_{2000}. The amount of unconjugated targeting moiety (free succinyloxytocin) was about 0.3% w/w.



Scheme 1. Synthesis of *N*-succinyloxytocin (**1**).

The chemical structure of PHEA–PEG_{2000}-succinyloxytocin was determined by UV and ^{1}H NMR spectroscopy.

The conjugation of Oxytocin to PHEA–PEG_{2000} did not alter its UV absorption profile in the range of 230–350 nm with an absorption maximum at 275 nm.

The ^{1}H NMR spectrum of PHEA–PEG_{2000}-succinyloxytocin shows signals attributable to protons of oxytocin linked by succinic spacer (see Section 2).

The amount of oxytocin linked to the polymeric carrier was estimated by UV and ^{1}H NMR (See Section 2).

The results obtained with the two analytical procedures were in fair agreement and indicated that the oxytocin content in the conjugate was about 16.5% w/w.

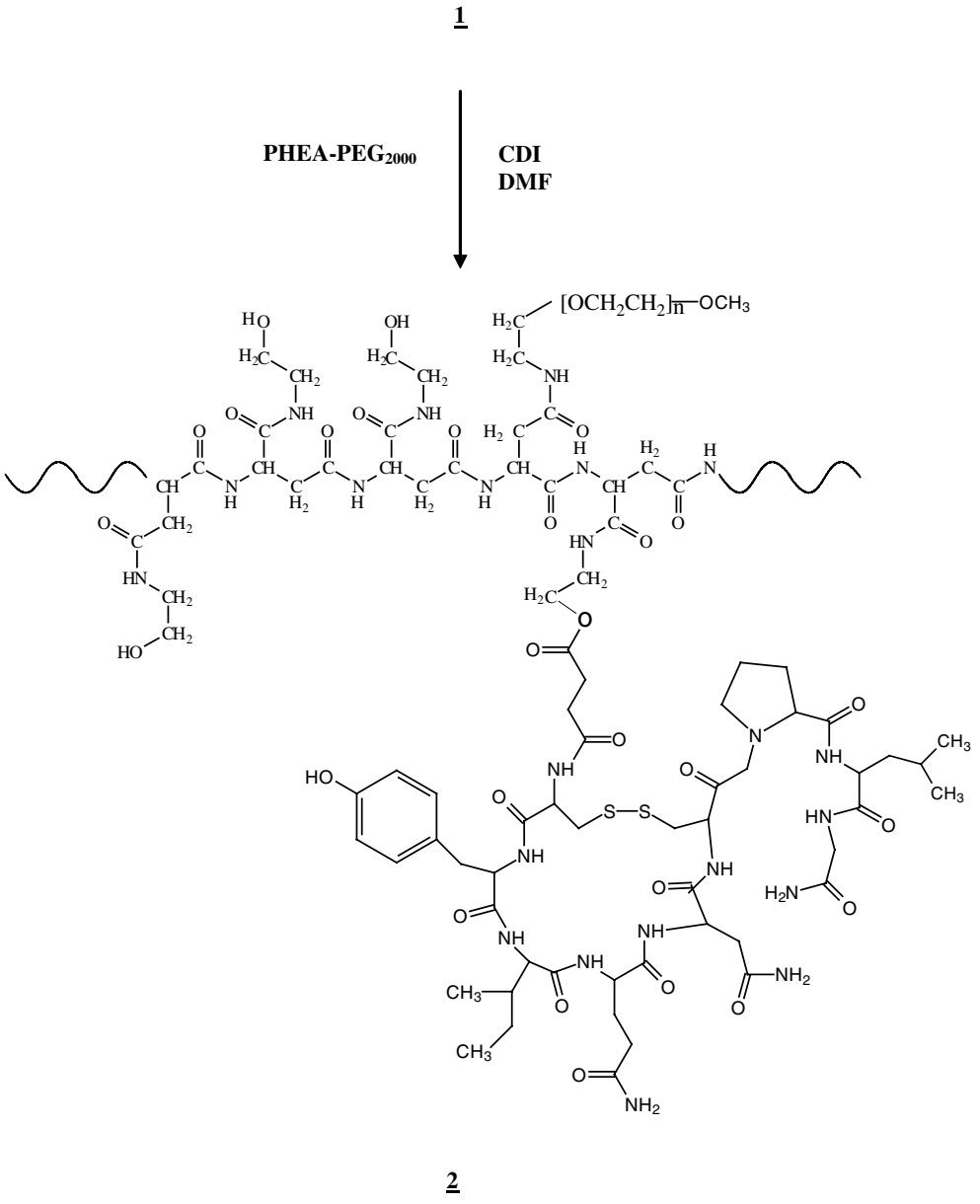
3.2. Synthesis of PHEA–PEG_{2000}-succinyloxytocin–succinylpaclitaxel

Paclitaxel is a well-known antitumor agent, effective in breast and ovarian cancers ([16 and notes therein]); however despite its common use in many clinical protocols, its poor physico-chemical properties, such as low water solubility and chemical stability, involve the use in marketing formulations of vehicles not well tolerated for i.v. administration, such as ethanol. A way to improve water solubility and chemical stability of this drug is that to link it to a water soluble polymer, as reported in our previous study [25]. Now in order to conjugate paclitaxel to the macromolecular carrier targeted to breast tumor cells, with the aim to combine an improvement of its water solubility and chemical stability, with the targeting to specific tumor cells, a 2′-*O*-succinyl- derivative of drug was synthesized, following a previously reported procedure [25], and then this derivative attached to PHEA–PEG_{2000} copolymer by means of carbonyldiimidazole (CDI) obtaining PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel derivative (Scheme 3). The absence of free drug in purified adduct was confirmed by HPLC analysis. The occurred ester bond of 2′-*O*-succinylpaclitaxel to PHEA–PEG_{2000} bearing oxytocin molecules was proved by IR and ^{1}H NMR. The analytical and spectral data were in agreement with the attributed structure.

The PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel IR spectrum shows besides the typical bands of PHEA at 1654 cm^{-1} (amide I) and 1542 cm^{-1} (amide II), the additional bands at 1735–1718 cm^{-1} corresponding to the ester carbonyl group of the succinic residue present both in linked oxytocin and paclitaxel derivatives.

The ^{1}H NMR spectrum shows signals attributable to protons of both conjugated paclitaxel and oxytocin (see Section 2).

The amount of linked paclitaxel, evaluated by ^{1}H NMR (see Section 2), was found to be about 7.3% mol/mol corresponding to 22% w/w in paclitaxel considering the weight of the whole conjugate. Likely the oxytocin content, evaluated after drug conjugation by ^{1}H NMR (see Section 2), was found equal to about 2.8% mol/mol. The obtained



Scheme 2. Coupling reaction of *N*-succinyloxytocin derivative (**1**) to PHEA–PEG_{2000} ($n = 44$).

value indicates that no hydrolysis occurred between oxytocin and polymeric backbone during drug coupling.

The synthesis of PHEA–PEG_{2000}-Succinylpaclitaxel was also performed to obtain a conjugate without targeting moieties. The pure conjugate was characterized by IR, UV and ^{1}H NMR analysis.

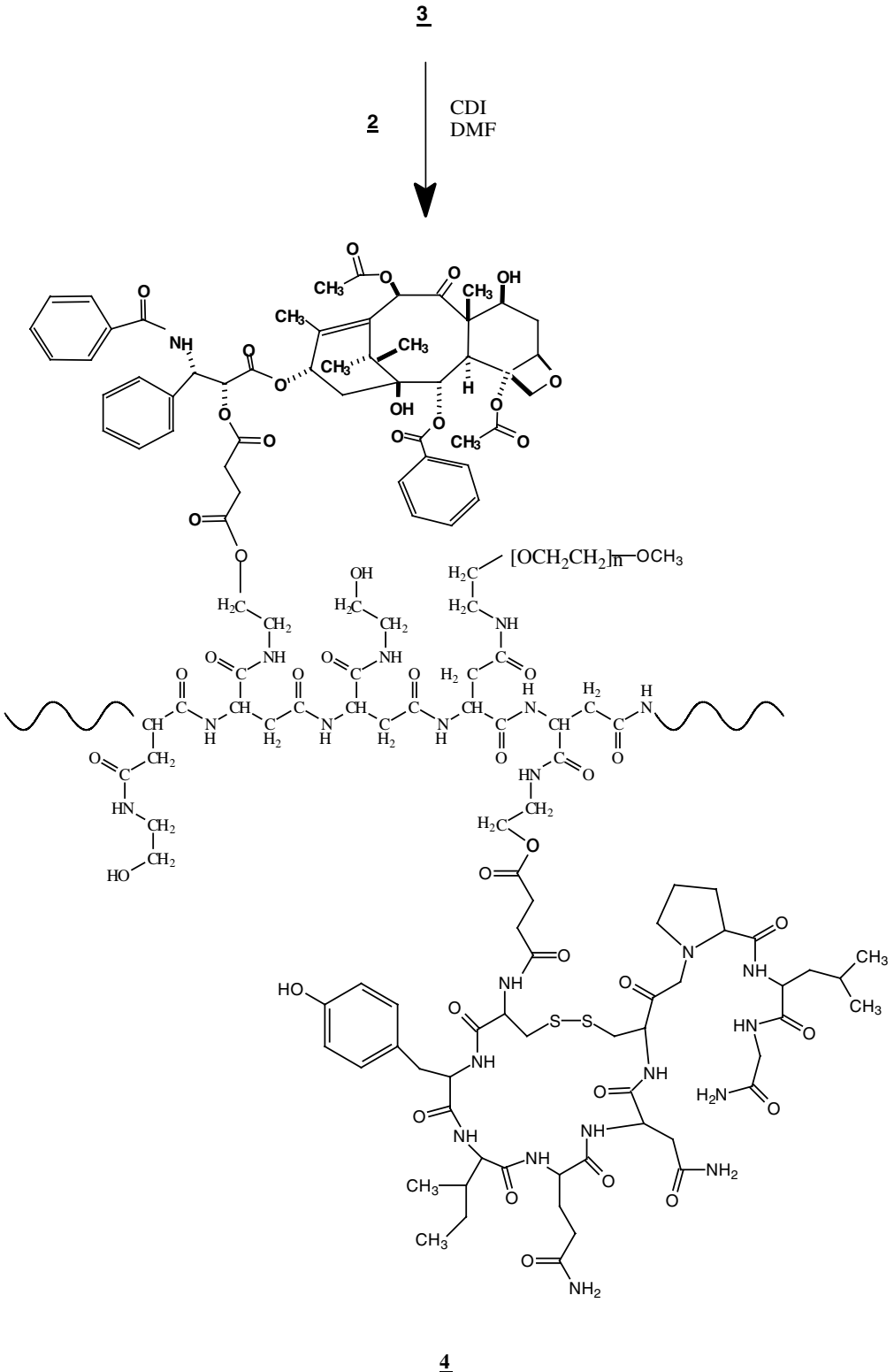
IR spectrum shows the typical bands of PHEA at 1654 cm^{-1} (amide I) and 1542 cm^{-1} (amide II). The additional band at 1735–1718 cm^{-1} corresponds to the carbonyl ester group of the succinic residue of the linked paclitaxel.

The ^{1}H NMR spectra show signals attributable to protons of both conjugated paclitaxel and succinic spacer (see Section 2).

The amount of paclitaxel linked to the carrier was evaluated by UV and ^{1}H NMR analysis.

By UV analysis (H_2O/EtOH, 1/1, v/v), comparing the value of $E^{1\%}_{273}$ of PHEA–PEG_{2000}-Succinylpaclitaxel ($E^{1\%}_{273} = 20.2$) with that of paclitaxel ($E^{1\%}273 = 180.7$) the drug content was found to be about 10.9% w/w.

By ^{1}H NMR (D_2O/CD_3OD 7:3, v/v) comparing the integral of peaks related to protons at δ 1.0, 1.6, 1.8 and 8.2 assignable to linked drug with the integral of peaks related to protons at δ 3.1 assignable to PHEA–PEG, the drug content was found to be about 2.9% mol/mol corresponding to 11.7 w/w in paclitaxel considering the whole weight of the conjugate.



Scheme 3. Coupling reaction of Succinylpaclitaxel derivative (**3**) to PHEA–PEG_{2000}-Succinyloxytocin ($n = 44$) (**2**).

3.3. Molecular characterization of conjugates

The weight average molecular weights (M_w) and polydispersity index (P. I.) of PHEA–PEG_{2000} and conjugates **2** and **4**, obtained by SEC analysis (see Section 2), are reported in Table 1. Analyses were performed both in aqueous and organic media.

As evidenced by the weighted average molecular weight of **4** in aqueous medium, some phenomena of aggregation of macromolecules occurred, probably as a consequence of

Table 1
Weight average molecular weights (M_w) and polydispersity index (P.I.) of PHEA–PEG_{2000} and of 2 and 4 conjugates

	M_w (KDa) Organic phase	P.I.	M_w (KDa) Aqueous phase	P.I.
PHEA–PEG_{2000}	16.5	1.7	18.0	1.6
Conjugate 2	23.1	2.0	22.8	2.2
Conjugate 4	24.7	1.7	245.0	1.2

the amphiphilic properties of obtained conjugate, due to the presence in the same conjugate of hydrophobic paclitaxel molecules and hydrophilic PEG chains. The presence of polyethyleneglycol chains and paclitaxel molecules, in fact, suggests an eventual micelle-like structure of conjugate in aqueous medium [31,32].

Moreover, in order to determine the hydrodynamic diameter of conjugate **4** in aqueous medium, DLS measurements were also performed.

Obtained data showed a size multimodal distribution, in particular three peaks were evidenced by size distribution report by intensity; however the elaboration of data by volume % or number % showed the highest percentage of small diameter aggregates (7 nm ± 0.1) and the quite total absence of the other peaks, indicating that a very little amount of aggregates with larger diameter were present.

The DLS results allowed to hypothesize a micelle-like structure of conjugate **4** in water as a consequence of hydrophilic–hydrophobic ratio of the prepared conjugate.

3.4. In vitro release studies

In order to gain preliminary information about the potential use of **4** as drug delivery system, this conjugate was subjected to in vitro hydrolysis studies in buffer solution at pH 7.4 (mimicking extra cellular fluids), at pH 5.5 (mimicking intratumoral compartment) and in human plasma. After 24 h of incubation at pH 7.4 a release of about 5% w/w of linked paclitaxel was observed from conjugate **4** (Fig. 1), whereas no trace of its succinyl derivative was found; at pH 5.5 about 12% of the linked paclitaxel was released after 24 h (data not shown) and also in this case no succinyl derivative was detected. These results evidenced, either the good stability of this polymeric conjugate at pH 7.4 as well as its ability to give a prolonged release of the drug, in the intact form, at pH 5.5.

Release studies in human plasma showed the hydrolysis of about 18% w/w of linked drug (in the intact form) after 24 h (Fig. 2), indicating that also in plasma a prolonged release can be obtained. But to evaluate the ability of the synthesized system to obtain an efficient drug targeting toward tumours expressing oxytocin receptor, the stability of ester bond between *N*-succinyloxytocin and polymeric chains was evaluated. The experimental results showed the very good stability of prepared conjugate in the used conditions since no release of oxytocin or its succinyl derivative was observed after 24 h either in phosphate buffer solution at pH 7.4 , 5.5 or in human plasma.

The same experiments (hydrolysis at pH 7.4, 5.5 and in plasma) were performed on conjugate **5**; in this case about 4%, 10% and 16.5% of the linked drug were released from the conjugate after 24 h of incubation, respectively, at pH 7.4, 5.5 and in plasma, indicating that the drug release profiles of drug (in the intact form) from conjugate **5** (without oxytocin) were quite similar to that obtained with conjugate 4 (bearing oxytocin groups). Moreover this result evidenced that the oxytocin conjugation did not change the drug release rate.

3.5. In vitro preliminary cytotoxic studies

In order to have some information on the pharmacological behaviour of macromolecular conjugate of paclitaxel bearing oxytocin in side chain, some preliminary in vitro cytotoxicity studies were carried out using breast cancer MCF-7 cells. Fig. 3 shows the viability of MCF-7 cells incubated with free paclitaxel, 2′-*O*-succinylpaclitaxel derivative, conjugate **4**, **5** at 1 μM paclitaxel equivalent concentration and naked PHEA–PEG_{2000}. Control tests were also performed using known amounts of distilled water used for the dissolution of PHEA–PEG_{2000}, PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel and

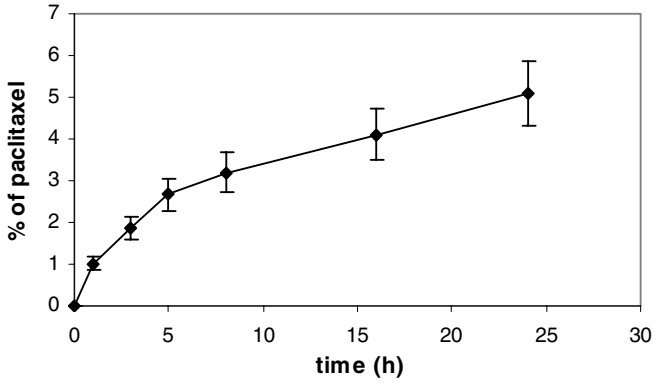


Fig. 1. Release profile of paclitaxel from conjugate **4** in buffer solution at pH 7.4.

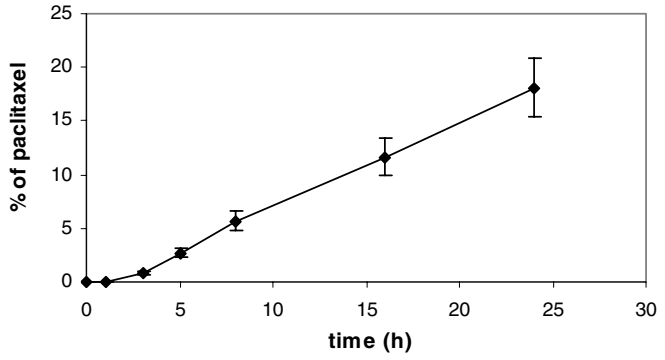


Fig. 2. Release profile of paclitaxel from conjugate **4** in human plasma.

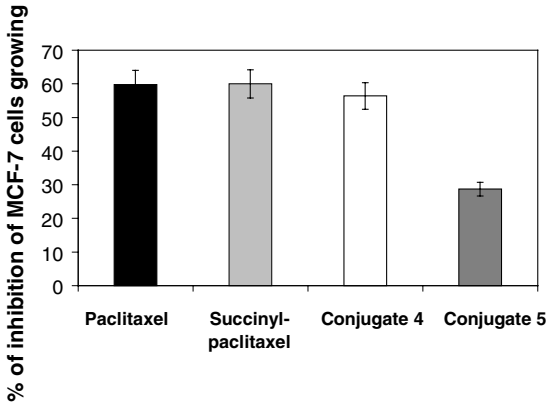


Fig. 3. Inhibition of MCF-7 cell growing after 4 days incubation with paclitaxel, 2′-*O*-succinylpaclitaxel, PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel (conjugate 4) and PHEA–PEG_{2000}- Succinylpaclitaxel (conjugate 5) at 1 μM paclitaxel equivalent concentration. Each value is the mean of five experiments.

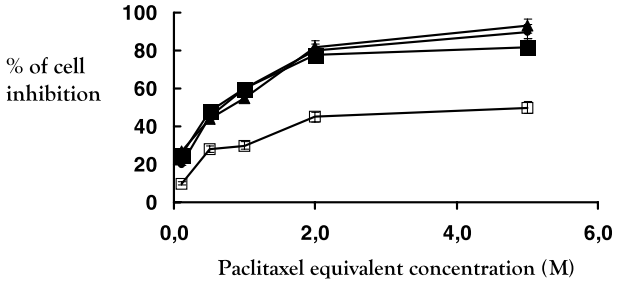


Fig. 4. Profiles of inhibition of MCF-7 cell growing with paclitaxel (●), 2′-*O*-succinylpaclitaxel (■), PHEA–PEG_{2000}-Succinyloxytocin–Succinylpaclitaxel (conjugate 4, ▲) and PHEA–PEG_{2000}-Succinylpaclitaxel (conjugate 5, □)

PHEA–PEG_{2000}-2′-*O*-succinyl-paclitaxel and ethanol used for the drug dissolution.

As can be observed free drug, its succinyl derivative and conjugate **4** showed similar activity against MCF-7 cells growing (about 60% of inhibition at drug concentration equal to 1 μM of paclitaxel equivalent concentration), whereas conjugate **5** was twice less active. Fig. 4 shows the profiles of inhibition of cell growth of MCF-7 in the concentration range 0.1–5 μM of drug. As can be seen within the whole range of drug equivalent concentration, conjugate **4** maintains cell inhibition activity quite similar to paclitaxel and its succinyl derivative, whereas conjugate **5** has always cell inhibition lower than conjugate 4. Considering that in vitro hydrolysis experiments evidenced that the drug release profile at pH 7.4, 5.5 and in plasma of two macromolecular conjugates is quite similar, such results could be attributed to the higher internalisation of conjugate **4** into MCF-7 cells due to the presence of oxytocin residues linked to the polymeric backbone, lacking in conjugate **5**.

Cell viability profiles were also checked in the presence of PHEA–PEG_{2000}-Succinyloxytocin and distilled water solutions (control) evidencing no cytotoxic effect, under the used experimental conditions.

4. Conclusions

A new macromolecular conjugate for antitumor therapy was synthesized by using Paclitaxel as drug, a PHEA copolymer bearing PEG chains as carrier, and Oxytocin as targeting group. In vitro hydrolysis studies evidenced that targeting group is stably linked to the polymeric backbone and that the conjugate is quite stable in plasma and able to give a prolonged release of drug molecules at pH 5.5 mimicking intratumoral compartment. Finally preliminary in vitro cell activity evidenced that conjugate with targeting group is more active than conjugate without targeting portion evidencing reasonably the ability of targeting group to improve cellular internalisation.

Acknowledgement

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