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Research paper

Synthesis, physico-chemical and biological characterization of a paclitaxel macromolecular prodrug

G. Cavallaro[a,*], M. Licciardi[a], P. Caliceti[b], S. Salmaso[b], G. Giammona[a]

[a]*Dipartimento di Chimica e Tecnologie Farmaceutiche, Palermo, Italy*
[b]*Dipartimento di Scienze Farmaceutiche, Padova, Italy*

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Abstract

Paclitaxel was attached to poly(hydroxyethylaspartamide) via a succinic spacer arm by a two-step protocol: (1) synthesis of 2′-*O*-succinyl-paclitaxel; (2) synthesis of PHEA-2′-*O*-succinyl-paclitaxel. The 2′-*O*-succinyl-paclitaxel derivative and the macromolecular conjugate were characterized by UV, IR, NMR and mass spectrometry analysis. The reaction yields were over 95% and the purity of products over 98%. Paclitaxel release and degradation from 2′-*O*-succinyl-paclitaxel occurred at a faster rate at pH 5.5 than 7.4. After 30 h of incubation at pH 5.5 and 7.4 the released free paclitaxel was about 40 and 20%, respectively. In plasma both drug release and degradation were found to occur at a higher rate than in buffer at pH 7.4 suggesting that an enzymatic mechanism could be involved. The paclitaxel release and degradation from PHEA-2′-*O*-succinyl-paclitaxel were negligible at pH 5.5 and 7.4 and very slow in plasma. Investigation carried out using murine myeloid cell line showed that the polymeric prodrug maintains partial pharmacological activity of paclitaxel. The DL50 of the conjugate (over 40 ng/ml) as compared to free paclitaxel (about 1 ng/ml) was correlated to the slow drug release. Finally a pharmacokinetic study carried out by intravenous inoculation of the macromolecular prodrug to mice demonstrated that the polymer conjugation modify dramatically the in vivo fate of the drug. The conjugate disappeared from the bloodstream much more quickly as compared to both free drug and naked polymer. Massive accumulation of bioconjugate in the liver (80% of the dose) was found to persist throughout 1 week.

Keywords: Polymeric prodrug; Polymer therapeutics; Conjugation; α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide; Paclitaxel

1. Introduction

Paclitaxel (TXL) is a diterpenoid taxane derivative of vegetable origin hailed by National Cancer Institute as the most significant advance in chemotherapy of the past 20 years [1]. This drug promotes tubulin polymerization and formation of extraordinarily stable and dysfunctional microtubules, which disrupt the normal tubule dynamics required for cellular division thus provoking cell death [2].

Paclitaxel showed an exceptional activity against primary epithelial ovarian carcinoma, breast, colon, head, non-small cell lung cancer and AIDS-related Kaposi's sarcoma [3]. However, despite being currently used in many countries for treatment of ovarian and breast cancers [4,5], its poor physico-chemical properties preclude proper formulation and clinical application. In particular, the exploitation of suitable paclitaxel dosage forms is limited by the inconvenient pK_a [6], low solubility [7], pH-dependent degradation [8], incompatibility with many common drugs and, excipients [9].

Formulations based on Cremophor EL® and ethanol vehicles for slow intravenous infusion have been developed to overcome paclitaxel's very low water-solubility, though the use of these vehicles entails incompatibility with some infusion set materials and the risk of toxicological effects and requiring therapeutic premedication [10].

Recently, new technological approaches have been proposed to overcome the physico-chemical restrictions of paclitaxel: co-solvency [11], emulsification [12], micellisation [13], liposome formation [14], use of micro-particles [15], cyclodextrins [16] and production of low molecular weight prodrugs [17].

Polymer conjugation, largely investigated with both protein and low molecular weight drugs [18,19], represents

* Corresponding author. Address: Department of Pharmaceutical Chemistry and Technology, Via Archirafi 32, 90123 Palermo, Italy. Tel.: +39-091-6236131; fax: +39-091-6236150.
E-mail address: gennacav@unipa.it (G. Cavallaro).

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a further technique to improve the physico-chemical and biopharmaceutical properties of paclitaxel. Polymer conjugation can endow derivatives with increased water-solubility and chemical stability, improved pharmacokinetic and distribution profile, reduced side effects and, sometimes, targeted properties to disease site either by active or passive mechanisms [20,21]. Hydroxypropylmethacrylamide copolymers [22], polyglutammate [23] and albumin [24] have been proposed as paclitaxel carriers. However, the difficulty of obtaining derivatives with suitable physico-chemical and biopharmaceutical properties and the multi-step reactions often necessary to obtain the conjugates are obstacles in the development of suitable polymer therapeutics [24].

α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) [25], a freely water-soluble, non-toxic, non-antigenic and non-immunogenic multi-functional macromolecule proposed as a plasma substitute, has been largely investigated as a drug carrier [26–29]. As an example, conjugation of cytarabine to PHEA via D-Val-Leu-Lys spacer allowed for enhanced drug stability and selective drug release in presence of plasmin, a protease produced at high levels in many malignant tumour tissues [30].

In order to take advantage of the excellent properties of PHEA as a drug carrier the development of a paclitaxel–PHEA polymeric prodrug was investigated. The drug was derivatized by succinic anhydride and linked to the polymer through an ester bond. In vitro and in vivo studies were carried out to evaluate the effects of chemical linkage on drug solubility, chemical stability and biological behaviour. Cytotoxicity and biodistribution were investigated and results were compared to those obtained with free paclitaxel and succinyl-paclitaxel derivative.

2. Experimental procedures

2.1. Materials

Paclitaxel was a kind gift of Pharmacia & Upjohn (Nerviano, Italy). D,L-aspartic acid, ethanolamine, 1, 1′-carbonyldiimidazole (CDI) and ethyl acetate were purchased from Fluka (Buchs, Switzerland). Anhydrous solvents (acetone, pyridine, ethanol, diethylether), colloidal silica for column chromatography (60, 70–230 mesh), *N,N*-dimethylformamide (DMF) and trifluoroacetic acid were obtained from Aldrich (Steinheim, Germany). *N,N*-dimethylformamide (DMF) was anhydrified by P_2O_5 and distilled under reduced pressure. Methanol, acetonitrile and chloroform were purchased from by Merck (Germany).

PHEA was prepared and purified according to a procedure reported previously [25]. The weight average molecular weight of PHEA, measured by light scattering, was 51,800 (Mw/Mn = 1.79). Human plasma was obtained from healthy voluntary blood donors.

The male NCL and Balb/c mice weight 24 ± 2 g, fed ad libitum, used for the in vivo studies were from the Department of Pharmaceutical Sciences, University of Padua. The animal experiments were performed in accordance with Italian law (DL n. 116/92) and European guidelines (EEC n. 86/609).

HPLC analyses were performed by using a Varian 9012 Liquid Chromatograph equipped with a Rheodyne Injector 7125 (fitted with a 10 μl loop) and a Kontron HPLC Detector 432 on line with a computerized workstation. Column: RP-C18 (μBondapak; 10 μm of 250 mm × 4.6 mm i.d.) (obtained by Waters) equipped with a direct-connect guard column C_{18}. Mobile phase: $CH_3CN/CH_3COO\ NH_4$, 35 mM, pH 5.5, 45:55, flow 1 ml/min, $\lambda = 229$ nm.

2.2. Methods

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

Succinic anhydride (0.15 g) was added to 2.5 ml of paclitaxel solution in dry pyridine (0.4 g/ml) to reach 12.5:1-succinic anhydride/paclitaxel molar ratio. The reaction mixture was kept at room temperature for 3 h and the progress of reaction was followed by TLC (CH_3COCH_3/ $CHCl_3$ 1:1, v/v). Then the solvent was evaporated under vacuum and the residue was suspended in 8 ml of distilled water, kept under stirring for 30 min and centrifuged. The residue was dissolved in acetone and precipitation was carried out by water addition. The absence of unreacted drug was verified by TLC. The white crystals collected by filtration with a 95% yield (w/w) were analysed by HPLC, isocratically eluted with acetonitrile/35 mM ammonium acetate, pH 5 (45:55, v/v) using a UV detector set at 229 nm. The paclitaxel and succinyl paclitaxel derivative concentrations in the analysed solutions were determined on the basis of the corresponding peak areas in calibration curves obtained with the pure compounds.

The final product was characterized by elemental analysis, melting point, FT-IR, UV and ^{1}H-NMR spectroscopy. The analytical and spectral data of *2* were in agreement with the attributed structure:

Anal ($C_{51}H_{55}NO_{17}$) C, 64.05, H, 5.71; N, 1.43.

IR (KBr): 3300–3500 cm^{-1} (ν OH, –NH–), 1730 cm^{-1} (ν C=O ester group), 1710 cm^{-1} (ν C=O keton group), 1650 cm^{-1} (ν C=O amide group).

UV spectrum (methanol): λ max at 229 nm ($\varepsilon = 24,000$) and 273 ($\varepsilon = 1700$).

Melting point: 178–180 °C.

^{1}H-NMR ($CDCl_3$): δ 1.11 [s, $^{17}CH_3$], 1.20 [s,$^{16}CH_3$], 1.69 [s, $^{19}CH_3$], 1.9 [s, $^{18}CH_3$], 2.2 [m, OAc], 2.4 [m, OAc], 2.5-2.8 [m, HOOC–*CH*$_2$*CH*$_2$–COO–TXL], 3.78 [d, ^{3}CH], 4.17 [d, $^{20}CH_2$], 4.3 [d, $^{20}CH_2$], 4.46 [dd, ^{7}CH], 4.96 [d, ^{5}CH], 5.50 [d, $^{2'}$CH], 5.67 [d,^{2}CH], 5.98 [dd, $^{3'}$CH], 6.22 [t, ^{13}CH], 6.27 [s, ^{10}CH], 7.09 [d, NH], 7.25 [s, 3′-Ph], 7.4 [m, 3′-NBz], 7.5 [m, 2-OBz], 7.73 [d, 3′-NBz], 8.1 [d, 2-OBz].

MS (FAB): *m/e* 954 $(M + H)^+$, 976 $(M + Na)^+$.

2.2.2. Synthesis of PHEA-2′-O-succinyl-paclitaxel conjugate (3)

One milliliter of carbonyldiimidazole (CDI) in dry DMF (0.020 g/ml) was added dropwise to 1 ml of 2′-*O*-succinyl-paclitaxel in dry DMF (0.100 g/ml) to reach 1.17:1 CDI/paclitaxel molar ratio. The mixture was kept at 25 °C for 3 h and then 1 ml of PHEA in dry DMF (0.085 g/ml) was added dropwise. The reaction mixture was maintained for 1 h at 25 °C and for a further 5 h at 40 °C. After this time, 100 μl of triethylamine was added and after 24 h at 40 °C, a further 50 μl of triethylamine were added. The reaction solution was kept under inert atmosphere at 40 °C for 63 h and then the solvent was removed under an vacuum. The residue was washed four times with 15 ml of a CH_2Cl_2/acetone (8:2) 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. After lyophilization the conjugate was obtained in 85% yield based on the starting material (PHEA). The amount of free drug in product *3* was determined by RP-HPLC analysis. The chemical structure of the conjugate was characterized by IR, UV and ^{1}H-NMR spectroscopy. The amount of paclitaxel linked to the carrier was evaluated by UV and ^{1}H-NMR.

The IR spectrum (KBr) showed: a broad band centred at 3300–3500 cm^{-1} (OH;–NH) and bands at 1732 cm^{-1} (ν C=O ester group), 1654 cm^{-1} (amide I, PHEA) and 1542 cm^{-1} (amide II, PHEA).

By UV analysis, comparing $E^{1\%}_{273}$ of *3* ($E^{1\%}_{273} = 4 \pm 0.2$ in a mixture water/ethanol, 1/1) with that of *2* ($E^{1\%}_{273} = 18 \pm 0.5$ in the same medium), the 2′-*O*-succinyl-paclitaxel content in *3* was found to be about 22% w/w (corresponding to a content of TXL of 19.8% w/w).

^{1}HNMR (DMSO): δ 1.10 [m, $^{17}CH_3$; $^{16}CH_3$], 1.49 [s, $^{19}CH_3$], 1.76 [s, $^{18}CH_3$], 2.10 [m, OAc], 2.24 [m, OAc], 2.5-2.8 [m, CO–*CH_2*–*CH_2*–COO–PHEA], 3.13 [m, –NH–*CH_2*CH_2–OH (PHEA)], 3.57 [m, ^{3}CH, –NH–CH_2–*CH_2*–OH (PHEA)], 4.09 [m, $^{20}CH_2$], 4.4–4.73 [m, ^{7}CH; NH–*CH*–(CH_2CONH–CH_2CH_2–OR)–CONH–(PHEA); NH–*CH*(CONH–CH_2CH_2–OR)–CONH–(PHEA)], 4.91 [d, ^{5}CH], 5.33 [d, $^{2'}$CH], 5.55 [d, ^{2}CH], 5.82 [dd, $^{3'}$CH], 6.28 [m, ^{13}CH; ^{10}CH], 7.09–8.25 [m, 3′-Ph; 3′-NBz; 2-OBz].

The amount of linked drug was verified by ^{1}H-NMR analysis, by comparing the integral of the peaks related to protons at δ 1.10, 1.49 and 1.76 assignable to protons of methylic groups at C16, C17, C18 and C19 positions that belong to linked drug with the integral of the peaks related to protons at δ 3.13 (assignable to –NH–CH_2–*CH_2*–OH of PHEA). The average ratio of linked drug, determined by ^{1}H-NMR, as molar percentage of polymer repeating units containing linked drug with respect to total repeating units, was found to be about 4.7 mol%.

2.2.3. Hydrolysis studies in buffer solutions at pH 5.5 and 7.4

Equimolar solutions of paclitaxel (10^{-3} mM drug equivalent concentration): PHEA-2′-*O*-succinyl-paclitaxel adduct, 2′-*O*-succinyl-paclitaxel or free paclitaxel in 10 mM phosphate buffer, 0.15 M NaCl, pH 7.4 or 5.5 were incubated at 37 ± 0.1 °C. At scheduled times the solutions were sampled and analysed by HPLC according to the protocol reported above to determine the free paclitaxel and 2′-*O*-succinylpaclitaxel content. Each experiment was repeated in triplicate.

2.2.4. Hydrolysis in plasma

One hundred microliters of equimolar paclitaxel solutions (10^{-2} mM paclitaxel equivalent concentration): PHEA-2′-*O*-succinyl-paclitaxel, 2′-*O*-succinyl-paclitaxel or free paclitaxel in 10 mM phosphate buffer, 0.15 M NaCl pH 7.4 were added to 1 ml of plasma. The samples were kept at 37 ± 0.1 °C, under mild stirring. At scheduled times, 100 μl volumes 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 precipitate was washed with 0.5 ml of acetonitrile and centrifuged as reported above. The acetonitrile volumes 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 reliability and accuracy of the method previously verified demonstrated that the method allows for paclitaxel recovery of 98%. Each experiment was repeated in triplicate.

2.2.5. Cell culture assay

Murine myeloid leukaemia NFS-60 cells in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 6000 UI of granulocyte colony stimulating factor (G-CSF) were incubated in an atmosphere of 95% air and 5% CO_2. Fifty microliters of cells (10^5 cells/ml), which were in the logarithmic phase of growth, were plated in 96-well microtitre plates and incubated with 50 μl/well of cell culture medium containing different concentrations of paclitaxel or PHEA-2′-*O*-succinyl-paclitaxel. Paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel solutions were prepared by dilution of stock solutions in methanol or 10 mM phosphate buffer, 0.15 M NaCl pH 7.2, respectively, with cell culture medium. Controls were obtained by incubation of 50 μl/well of cell culture medium containing different volumes of phosphate buffer or methanol in culture medium. After 72 h incubation at 37 °C and 5% CO_2, the wells were filled with 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. After 4 h, 100 μl/well of DMSO were added and

the incubation was prolonged overnight. The absorbance of the wells was measured at 550 and 620 nm by a multi-well plate reader. The cell growth inhibition was expressed as percentage of the untreated controls, using the following equation: A_{550}-A_{620} of treated sample/A_{550}-A_{620} of untreated sample. The IC50 was defined as the drug concentration inhibiting the cell growth of 50% as compared with untreated controls.

2.2.6. Pharmacokinetics and biodistribution

One cubic millimeter Erlich solid tumor fragments were implanted subcutaneously on to 35 NCL mice. The tumor was left to develop for 8 days to reach a weight of 0.1–0.2 g. Two hundred microliters of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2 containing 1 mg of PHEA-2′-*O*-succinyl-paclitaxel were injected into the tail vein. At scheduled times the animals were bled and sacrificed. Heart, lungs, kidneys, spleen, liver and tumor were removed.

Ten microliters of a *N*-octylbenzamide (standard) solution in methanol (30 μg/ml) were added to 50 μl of blood samples. The samples were centrifuged in heparinized vials and 20 μl of plasma were treated three times with 100 μl of acetonitrile. The acetonitrile solutions separated by centrifugation were pooled, lyophilized and dissolved again in 50 μl of acetonitrile/35 mM ammonium acetate pH 5 mixture (45:55, v/v). The paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel content in the solutions were determined by HPLC according to the procedure reported above. The amounts of paclitaxel and paclitaxel conjugate were determined from standard titration curves previously obtained by analysis of blood samples containing known amounts of free paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel; 2′-succinyl-paclitaxel derivative species was not found in any sample. The pharmacokinetic parameters were determined by computer elaboration of plasma concentrations of paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel at different times from the equation:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

The removed organs were carefully washed in saline, dried on paper, weighed; subsequently they were combined with *N*-octylbenzamide solution (1 μl/mg of tissue) and homogenized by potter and sonication. Each homogenized sample was combined with acetonitrile (250 μl/g of tissue) and the suspensions were stirred for 10 min, sonicated for 5 min and then centrifuged for 10 min at 12 rpm. The extraction procedure was repeated three times and the acetonitrile volumes were pooled. The samples were lyophilized and the residue was dissolved in 50 μl of acetonitrile/35 mM ammonium acetate, pH 5. The paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel content in the samples was determined by HPLC analysis. The peak areas corresponding to paclitaxel and paclitaxel conjugate were elaborated according to standard curves previously obtained by analysis of tissues containing known amounts of free and conjugated paclitaxel. The residual plasma volume in each tissue was estimated by treatment of 10 mice with [^{3}H-]mouse serum albumin (MSA) using a published method [31]. The plasma volumes obtained were used to correct the protein concentrations in the organs.

3. Results and discussion

3.1. PHEA-2′-O-succinyl-paclitaxel synthesis and structural characterization

Aimed at obtaining a macromolecular prodrug with appropriate drug release properties, paclitaxel was conjugated to PHEA via an ester bond which in vivo can be hydrolyzed by both chemical and enzymatic pathways.

PHEA–paclitaxel prodrug was synthesized by a two-step reaction: (1) synthesis of 2′-*O*-succinyl-paclitaxel derivative; (2) 2′-*O*-succinyl-paclitaxel conjugation to PHEA. The chemical structure of both 2′-*O*-succinyl-paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel were determined by UV, IR, ^{1}HNMR and mass spectrum.

Scheme 1 describes the synthesis of 2′-*O*-Succinyl-paclitaxel derivative carried out according to a reported method [32]. After 3 h of reaction no free paclitaxel was found by TLC analysis, indicating that the reaction was complete. ^{1}H-NMR analysis demonstrated that succinylation takes place preferentially at the level of position 2′-OH. This result is in accordance with the literature which reports that under mild conditions complete reaction of the hydroxyl group occurs in position 2′-instead of 7′-OH [32].

Scheme 2 describes the PHEA-2′-*O*-succinyl-paclitaxel conjugate synthesis. 2′-*O*-succinyl-paclitaxel was reacted with PHEA in presence of triethylamine as a catalyst and carbonyldiimidazole (CDI) as a condensing agent. After extensive purification of PHEA-2′-*O*-succinyl-paclitaxel by CH_2Cl_2/CH_3COCH_3 washing and dialysis, the recovery yield was 95% w/w based on the starting PHEA. The amount of unconjugated drug (free paclitaxel) was about 0.2% w/w.

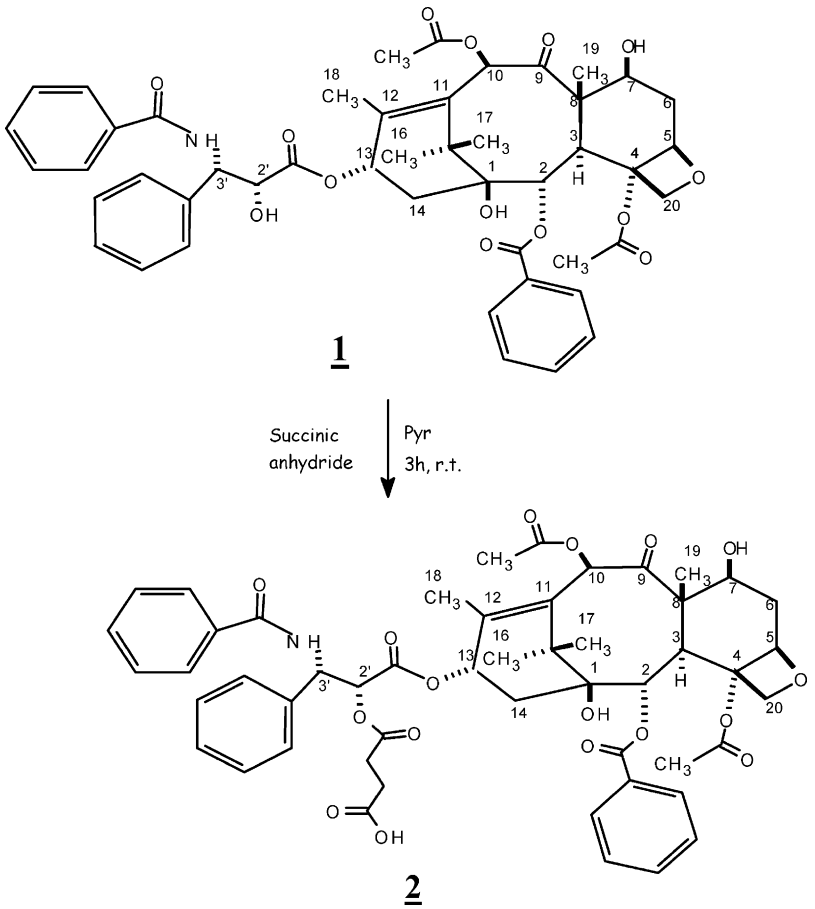
PHEA conjugation was not found to alter the paclitaxel UV absorption profile in the range of 230–350 nm and maximal absorption at 273 nm.

The PHEA-2′-*O*-succinyl-paclitaxel IR spectrum shows the typical bands of PHEA at 1654 cm^{-1} (amide I) and 1542 cm^{-1} (amide II). The additional band at 1732 cm^{-1} corresponds to the ester carbonyl group of the succinic residue.

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

The amount of drug linked to the polymeric carrier was estimated by UV and ^{1}H-NMR.

^{1}H-NMR analysis was carried out by comparing the integral values of the peak at δ 1.10, 1.49 and 1.76 attributable to methyl groups in C16, C17, C18 and C19



Scheme 1. Schematic representation of synthesis of 2′-*O*-Succinylpaclitaxel derivative (*2*).

positions of paclitaxel with the integral values of the peak at δ 3.13, attributable to $-NH-CH_2-CH_2-OH$ of the polymeric carrier. The results obtained with the two analytical procedures were in fair agreement and indicated that the paclitaxel content in the conjugate was about 19.8% (paclitaxel/conjugate, w/w).

3.2. Physico-chemical characterisations

Solubility studies demonstrated that PHEA conjugation increases paclitaxel solubility by about 100 fold providing for drug dissolution in aqueous medium at concentrations useful for therapeutic application.

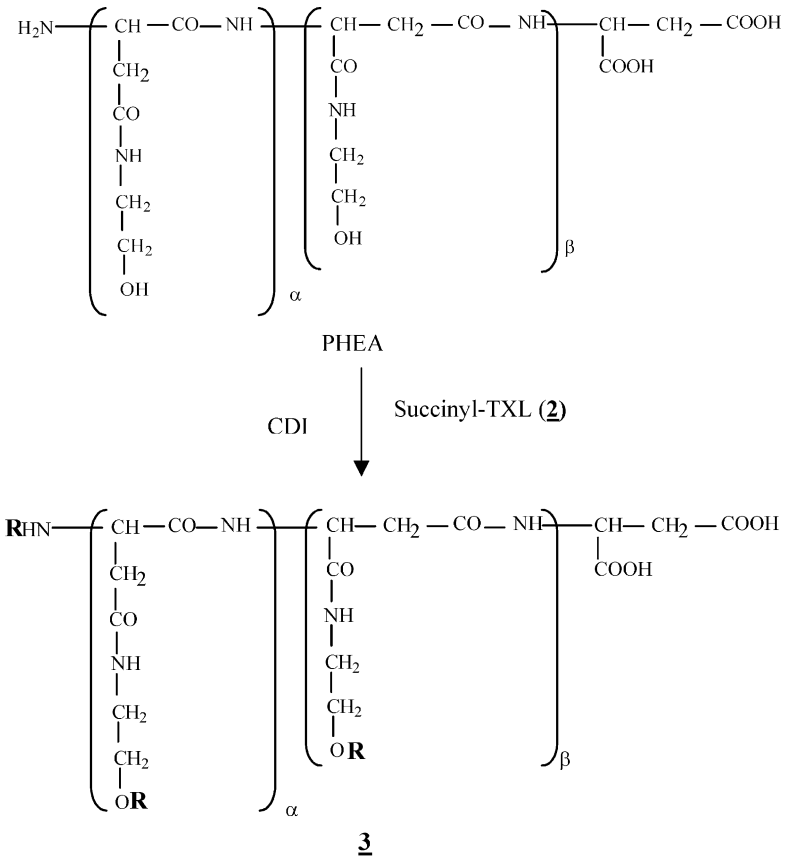
Stability and drug studies were carried out by incubation of PHEA-2′-*O*-succinyl-paclitaxel, 2′-*O*-succinyl-paclitaxel and free paclitaxel at different pH and plasma. Phosphate buffer at pH 5.5 and at pH 7.4 was selected to mimic intra tumoral and extracellular pH compartments, respectively.

The free drug degradation profiles at pH 5.5 and 7.4 depicted in Fig. 1 confirms that paclitaxel undergoes pH dependent degradation as reported in the literature [33]. Furthermore, the similar degradation profiles obtained in buffer at pH 7.4 and plasma suggest that paclitaxel degradation occurs by chemical pathways rather than by enzymatic mechanisms.

Fig. 2A–C shows the simultaneous disappearance of the 2′-*O*-succinyl-paclitaxel derivative and the appearance of paclitaxel at pH 5.5, 7.4 and in plasma.

Fig. 2 shows that paclitaxel release and degradation take place simultaneously, although these events occur at pH and incubation medium dependent rates.

Fig. 2A shows that at pH 5.5 the succinyl derivative undergoes rapid disappearance indicating that under these conditions the ester bond is quickly hydrolysed (about 60% of the 2′-*O*-succinyl-paclitaxel disappears during a 30-h incubation). On the other hand, the low amount of free paclitaxel detected in the solution (about 40% of drug in the intact form in 30 h) indicates that the drug undergoes degradation. The profiles depicted in Fig. 2B, show that at pH 7.4 the hydrolysis of the ester bond between succinic spacer and drug and the paclitaxel degradation are slower as compared to the previous case. After 30 h of incubation, succinyl derivatized paclitaxel and free paclitaxel were about 70 and 20% of the total initial drug, respectively. The lower paclitaxel degradation observed at pH 7.4 as compared to the one determined at pH 5.5 seems in contrast with the slower drug degradation at pH 5.5 than 7.4 reported in literature. However, the highest paclitaxel stability obtained by 2′-*O*-succinyl-paclitaxel incubation at pH 7.4 can be



Scheme 2. Schematic representation of synthesis of PHEA-2′-*O*-Succinylpaclitaxel conjugate (*3*).

ascribed to the protective effect of succinyl derivatization, which prevents the rapid degradation of the free drug. Therefore, it is possible to conclude that paclitaxel derivatization with a succinic group modifies the drug susceptibility to chemical degradation.

Fig. 2C shows that in plasma the 2′-*O*-succinyl-paclitaxel undergoes rapid ester bond hydrolysis and degradation which are reflected in the release of about 50% of undegraded in 30 h. The faster disappearance of the 2′-*O*-succinyl-paclitaxel derivative in plasma as compared to pH 7.4 seems to indicate that in plasma the hydrolysis of the succinic ester bond takes place by both chemical and enzymatic mechanisms.

In Fig. 3 the paclitaxel release profiles obtained by PHEA-2′succinyl derivative incubation at pH 5.5, 7.4 and in plasma are reported. The results point out that the macromolecular carrier affects the ester bond cleavage preventing the hydrolysis and rapid drug release.

At pH 5.5 neither paclitaxel nor its degradation products were detected within 30 h whereas at pH 7.4 small amounts of intact paclitaxel (less than 10%) were found. In plasma continuous paclitaxel release was observed and about 35% of drug was found within 30 hours. No succinyl-paclitaxel derivative release was found.

The analysis of the conjugate incubated in plasma for 30 h demonstrated that the linked drug maintains its structural integrity underlining the protective effect of the carrier on the drug degradation.

3.3. Biological studies

In vitro studies were carried out to evaluate the pharmacological activity of the PHEA-paclitaxel conjugate in comparison to the free drug.

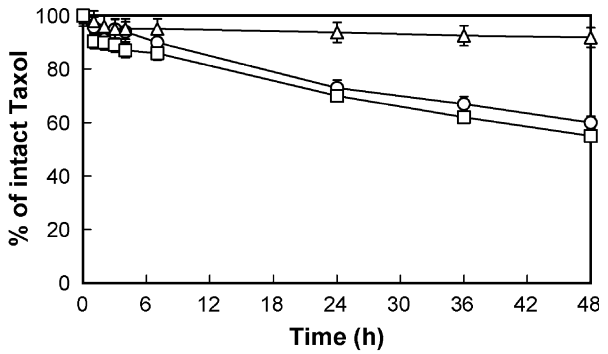


Fig. 1. Hydrolysis rate of paclitaxel in buffer solution at pH 5.5 (△), 7.4 (○) and in plasma (□).

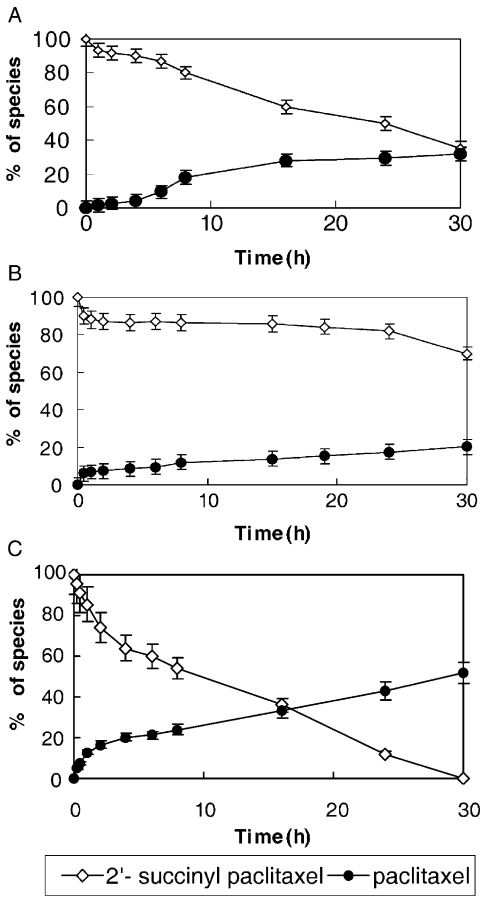


Fig. 2. Hydrolysis rate of 2′-*O*-succinyl-paclitaxel derivative at pH 5.5 (A), at pH 7.4 (B) and in plasma (C): % of remaining 2′-*O*-succinyl-paclitaxel derivative (-◇-◇-◇-) and % of released paclitaxel (-●-●-●-).

Fig. 4 shows the viability of murine myeloid leukaemia NFS-60 cells incubated with free paclitaxel, PHEA-2′-*O*-succinyl-paclitaxel, naked PHEA and amounts of methanol used for the drug dissolution. A control test was also performed using cell culture medium containing amounts of distilled water used for dissolution of PHEA and PHEA-2′-*O*-succinyl-paclitaxel.

Similar cell viability profiles were obtained with PHEA and distilled water solutions (control) indicating that PHEA does not display a cytotoxic effect per se. In contrast, both free paclitaxel and PHEA-2′-*O*-succinyl-paclitaxel showed cytotoxic activity. In particular, the IC50 of free and conjugated paclitaxel were 1.1 ng/ml and about 40 ng/ml paclitaxel equivalent, respectively, indicating that the conjugation reduces significantly the drug cytotoxicity. This result is due to the slow paclitaxel release from the carrier, which is reflected in a lower drug availability in the short time (96 h of experiment) as compared to the free paclitaxel. However, because of the high stability of the conjugate, the therapeutic system can be reasonably exploited to provide for a prolonged drug release after accumulation in the disease site.

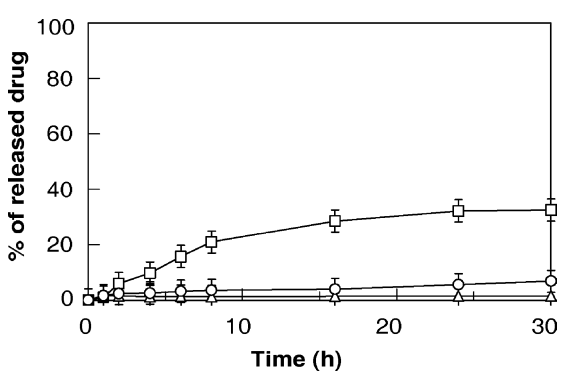


Fig. 3. Release of paclitaxel from PHEA-2′-*O*-succinyl-paclitaxel conjugate at pH 5.5 (△), pH 7.4 (○) and plasma (□) at 37 ± 0.1 °C.

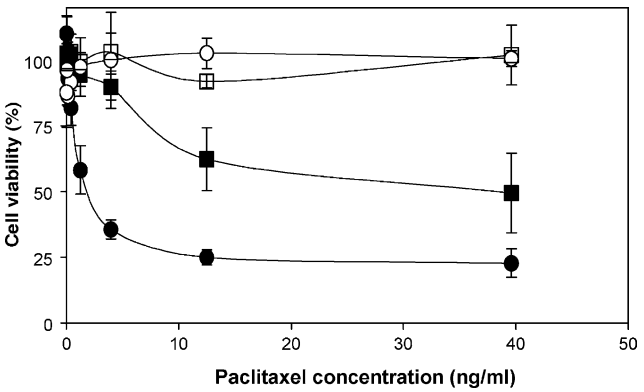


Fig. 4. NFS-60 cell viability, after 72 h incubation with methanol (○), PHEA (□), paclitaxel (●) and PHEA-2′-*O*-succinyl-paclitaxel (■). The cell viability was determined by MTT colorimetric assay and the expressed as optical density values. The standard deviation values (±SD) were calculated on the basis of five experiments.

A pharmacokinetic study was undertaken by intravenous injection of the conjugate to mice bearing a subcutaneous solid tumour. This animal model was chosen to evaluate the ability of the conjugate to distribute into permeable tissues as well as in solid tumour. The free and conjugated paclitaxel content at different time points in blood, tumour and main organs were assessed.

The time course profiles in plasma of PHEA-2′-*O*-succinyl-paclitaxel and free paclitaxel depicted in Fig. 5

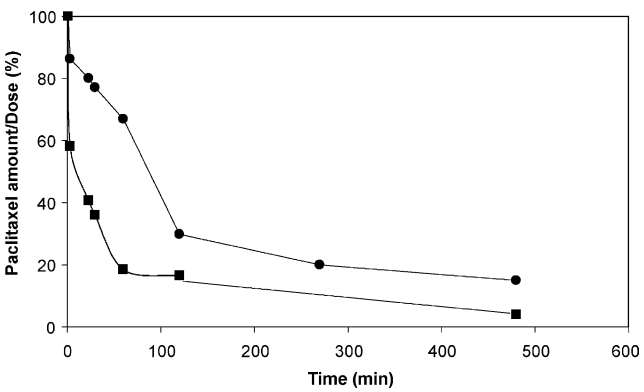


Fig. 5. Time course profiles of PHEA-2′-*O*-succinyl-paclitaxel conjugate (■) and free taxol (●) in plasma. The standard deviation (±SD) was calculated on the basis of 10/animals point.

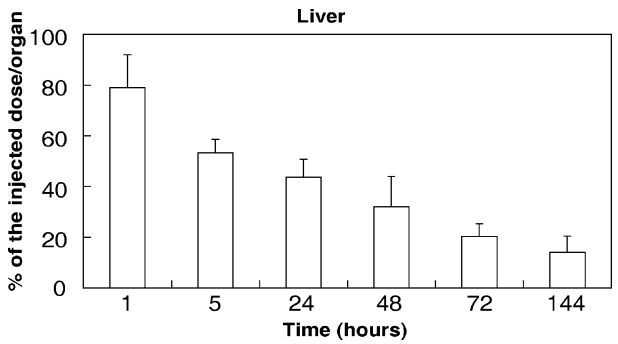


Fig. 6. Distribution profiles of PHEA-2′-*O*-succinylpaclitaxel conjugate in liver. The standard deviations (±SD) were calculated on the basis of 10/animals point.

show that the polymeric conjugate is cleared from the bloodstream more rapidly than the free paclitaxel. After 30 min, about 35% of the PHEA-2′-*O*-succinyl-paclitaxel injected dose was found whereas at the same time over 75% of free paclitaxel was detected.

The disappearance of the polymeric conjugate from the blood was accompanied by its massive disposition in the liver while the amounts detected in heart, lungs, kidneys and spleen were negligible. Fig. 6 shows that the prodrug is taken up quickly by liver since about 80% of the injected dose was found in this organ after 1 h from injection and high conjugate concentration was retained in this organ throughout 144 h.

The PHEA-2′-*O*-succinyl-paclitaxel pharmacokinetic and biodistribution are significantly different to the ones obtained with naked PHEA. A previous study demonstrated, in fact, that PHEA has a half-life in plasma of about 25 min and no more than 35% of the injected dose accumulates in the liver (data not shown). The in vivo behaviour of the naked polymer was attributed either to its high molecular weight and hydrophilic character, which reflect in high hydrodynamic volume that prevents the rapid kidney ultrafiltration. However, the conjugation of a high amount of paclitaxel can modify the PHEA physicochemical properties significantly with consequent dramatic alteration of the carrier pharmacokinetic behaviour. In particular, the covalent linking of about 20% (w/w) of paclitaxel to PHEA, corresponding to about 15 paclitaxel molecules per macromolecule, does not modify the physico-chemical properties of the construct in comparison with that of hydrophylic naked carrier only, but also its conformational arrangement giving a copolymer bearing hydrophobic moieties. Therefore PHEA changes in secondary and tertiary structures are expected to promote the liver up-take by a passive mechanism and retention throughout a long period.

The distribution study evidenced a lean conjugate accumulation in the tumor, as compared to the liver. Probably, the rapid liver up-take, which represents the main disposition route of conjugated paclitaxel, limits the extensive accumulation in the extra hepatic tumour mass, which takes place more slowly.

4. Conclusions

The conjugation to water soluble polymeric carriers seems to be a promising strategy to enhance the physico-chemical and biopharmaceutical properties of paclitaxel. The results reported in the present study show that PHEA can be properly used for this purpose. Actually, PHEA conjugation increases paclitaxel stability and solubility while the drug binding through a succinic ester bond guarantees the drug release. The synthetic strategy adopted in the present research was simple and effective since it furnished a conjugate characterized by high drug loading, solubility, stability and prolonged drug release. Furthermore, the derivative was demonstrated to maintain high pharmacological activity. The pharmacokinetic data demonstrate that paclitaxel conjugation to PHEA modifies dramatically the in vivo fate of paclitaxel, carrying very quickly most of conjugate (and of the drug consequently) to the liver. If on one hand this pharmacokinetic behaviour can render this derivative useful for anticancer treatment of the liver, on the other the further derivatization of the PHEA-2′-succinylpaclitaxel conjugate might give a more favorable drug pharmacokinetic profile. Indeed, the possibility of introduction of physico-chemical modifiers or active targeting moieties into the polymeric backbone of the paclitaxel conjugate paves the way for the exploitation of this derivative which can selectively accumulate in a particular region of the body where they can display their activity with reduction of systemic toxicity.

Acknowledgements

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