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RESEARCH PAPER

Polyaspartamide-Doxorubicin Conjugate as Potential Prodrug for Anticancer Therapy

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ABSTRACT

Purpose To synthesize a new polymeric prodrug based on α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-d,l-aspartamide copolymer bearing amine groups in the side chain (PHEA-EDA), covalently linked to the anticancer drug doxorubicin and to test its potential application in anticancer therapy.

Methods The drug was previously derivatized with a biocompatible and hydrophilic linker, leading to a doxorubicin derivative highly reactive with amino groups of PHEA-EDA. The PHEA-EDA-DOXO prodrug was characterized in terms of chemical stability. The pharmacokinetics, biodistribution and cytotoxicity of the product was investigated *in vitro* and *in vivo* on human breast cancer MCF-7 and T47D cell lines and NOD-SCID mice bearing a MCF-7 human breast carcinoma xenograft. Data collected were compared to those obtained using free doxorubicin.

Results The final polymeric product is water soluble and easily hydrolysable *in vivo*, due to the presence of ester and amide bonds along the spacer between the drug and the polymeric backbone. *In vitro* tests showed a retarded cytotoxic effect on tumor cells, whereas a significant improvement of the *in vivo* antitumor activity of PHEA-EDA-DOXO and a survival advantage of the treated NOD-SCID mice was evidenced, compared to that of free doxorubicin.

Conclusions The features of the PHEA-EDA-DOXO provide a potential protection of the drug from the plasmatic enzymatic degradation and clearance, an improvement of the blood pharmacokinetic parameters and a suitable body biodistribution. The data collected support the promising rationale of the proposed macromolecular prodrug PHEA-EDA-DOXO for further potential development and application in the treatment of solid cancer diseases.

KEY WORDS antitumor activity · biodistribution · doxorubicin · PHEA-EDA · polymeric prodrug

INTRODUCTION

Doxorubicin is an anthracycline widely used for the treatment of cancer. Unfortunately, severe side effects, such as cardiotoxicity, anaemia and leucopenia, may occur during anticancer therapies. An important drawback associated with the use of this drug is also the multiple drug resistance. For these reasons, several doxorubicin colloidal carriers have been developed in order to overcome some of these limitations,

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exploiting "passive" or "active" targeting mechanisms, *i.e.* the enhanced permeability and retention (EPR) effect or the conjugation to ligands or antibodies for a selective delivery to tumour tissues, respectively (1). Among the various colloidal carriers proposed as delivery devices of doxorubicin, the liposomal system was the most successful in terms of clinical use by showing a longer half-life, a more specific distribution and lower side effects than the free doxorubicin. The doxorubicin-based liposomal nanomedicines, Myocet™, Doxil® and Caelyx,® were approved by FDA and EMA for bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, multiple myeloma and Kaposi's sarcoma therapies (2).

Doxorubicin immunoliposomes, conjugates with several antibodies for epidermal growth factor receptors (3) or transferrin receptor (4), or folate (5), were also studied. Moreover, many other drug delivery systems based on solid lipid nanoparticles, microemulsions, polymeric or protein nanoparticles and dendrimers, were also developed (1).

The clinical use of Myocet™, Doxil® and Caelyx® revealed the presence of some side-effects, even if less severe than the free doxorubicin (6). For this reason, many efforts are still necessary to investigate suitable doxorubicin delivery systems to be applied in the clinical practice. In this scenario, a promising strategy is the so-called macromolecular approach, which is based on the conjugation of drugs to suitable polymer system.

The following requirements have to be pursued for the achievement of a successful macromolecular drug and/or prodrug: i) the chemical bond between the polymer and the drug (eventually connected by an appropriate spacer) should be stable enough to carry the drug in physiological conditions and ii) to be hydrolysable *in vivo*; iii) the residual polymeric backbone has to be biocompatible or biodegradable (7,8). In this sense, some doxorubicin-polymer conjugates were developed, using polysaccharides and synthetic polymers, as HPMA (9), PEG (10) and PEI (11).

The protein-like polymer, α,β-poly(N-2-hydroxyethyl)-d,l-aspartamide (PHEA) functionalized with amine pendant groups by reaction with ethylenediamine (EDA), obtaining the α,β-poly(N-2-hydroxyethyl)(2-aminoethylcarbamate)-d,l-aspartamide (PHEA-EDA) copolymer, was extensively exploited in the last years as drug and gene polymeric carrier (12). The introduction of amino groups on the PHEA backbone, a well known water-soluble, non-toxic, non-antigenic and non-immunogenic polymer, allows nucleic acid complexation to be obtained (13) and several bioconjugations to be performed in order to obtain polymeric prodrugs. Other polymeric multifunctional products were obtained by reaction of PHEA-EDA with polysorbate, polylactic acid, polycaprolactone, polyethylenglycol or squalenyl derivatives, leading to micellar systems able to solubilize and carry several hydrophobic drugs as ribavirin tripalmitate (14), rivastigmine (15) or flurbiprofen (16).

Exploiting the properties of PHEA-EDA as a drug carrier, a novel PHEA-EDA conjugate with doxorubicin (PHEA-EDA-DOXO) was thus prepared. Doxorubicin was derivatized with the ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) linker, thus achieving a derivative which was highly reactive with amino groups of PHEA-EDA and easily hydrolysable *in vivo*, due to the presence of two ester bonds.

The new macromolecular prodrug was characterized in terms of stability and tested *in vitro* and *in vivo* on two human breast cancer cell lines, MCF-7 and T47D. The pharmacokinetics, the biodistribution and the anticancer activity of the macromolecular prodrug was investigated and compared to those of the free doxorubicin.

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride was purchased from 21CEC PX PHARM Ltd, Eastbourne, East Sussex BN22 8PW, UK; ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester), celite AW Standard Super-Cel NF, triethylamine (Et_3N), 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT) dye test (TLC purity P97.5%), dimethyl sulfoxide (DMSO), estrogen and phosphate buffer (PBS) solution were Sigma-Aldrich (Milan, Italy) products. Eagle's minimum essential medium (MEM) culture, fetal calf serum (FCS 10), penicillin (100 UI/ml)-streptomycin (100 lg/ml) solution (1% *v*/*v*), and Trypsin/EDTA (1) solution were obtained from GIBCO (Invitrogen Corporation, Giuliano Milanese (Mi), Italy). Double distilled pyrogen-free water was used throughout experimental investigations. All other materials and solvents used in this study are of analytical grade (Carlo Erba, Milan, Italy). HPLC solvents, acetonitrile, trifluoroacetic acid, dichloromethane (CH_2Cl_2) were purchased from Sigma-Aldrich and used without any further purification.

α,β-Poly(N-2-hydroxyethyl)-d,l-aspartamide (PHEA) was prepared and purified according to the previously reported procedure (12). Spectroscopic data (FT-IR and ^{1}H-NMR) were in agreement with attributed structure: ^{1}H NMR (300 MHz, D_2O, 25°C, δ): 2.82 (m, 2H, -CH-CH_2-CO-NH-), 3.36 (t, 2H, -NH-CH_2-CH_2-OH), 3.66 (t, 2H,-CH_2-CH_2-OH), 4.72 (m, 1H, -NH-CH-CO-CH_2-). PHEA average molecular weight was 32.8 kDa ($M_w/M_n = 1.66$) based on PEO/PEG standards, measured by organic size exclusion chromatography analysis (SEC). Ethylenediamine was purchased from Aldrich (Italy).

PHEA-EDA copolymer was synthesized according to a procedure previously published (12). The pure product was obtained with a yield of 97% (*w*/*w*) based on starting PHEA.

Spectroscopic data (FT-IR, ^{1}H-NMR and SEC) were in agreement with the attributed structure. The degree of derivatization in EDA (calculated according to method reported), was 28±1.5 mol%. M_w of PHEA-EDA was 33.3 kDa and polydispersity was 1.66.

Synthesis and Characterization of Doxorubicin-Ethylene Glycol-Bis(Succinic Acid) -N-Hydroxysuccinimide Ester (DOXO-NHS)

Doxorubicin hydrochloride (90 mg, 0.155 mmol) was suspended in a mixture of CH_2Cl_2 (36 ml) and DMSO (9 ml); then Et_3N (43 µl, 0.310 mmol) was added and the solution was stirred until complete solubilization. Separately, ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (141.5 mg, 0.310 mmol) was dissolved in 45 ml of CH_2Cl_2, and the solution was added dropwise to the doxorubicin solution while stirring at room temperature. After 30 min, the CH_2Cl_2 was removed under reduced pressure and residual DMSO was extracted with diethylether (50 ml×3). The oily residue was dissolved in CH_2Cl_2 (15 ml) and 2 g of celite were added to the solution with gentle stirring. After 30 min the solvent was removed under reduced pressure, and the solid residue was placed inside a 2 cm i.d. glass column. Excess linker was removed by elution with 400 ml of hexane/CH_2Cl_2 50/50 whereas the desired product was obtained by elution with 400 ml of dichloromethane. The red colored fraction was collected and the solvent was evaporated under reduced pressure to give the title product as a red oil.

The structure and the purity of the doxorubicin-ethylene glycol-bis(succinic acid)-N-hydroxysuccinimide ester (DOXO-NHS, Fig. 1S, Supplementary Material) were assessed by means of HR ESI-MS and ^{1}H, ^{13}C NMR experiments (details of the ESI-MS and NMR methods are reported in Supplementary Material).

Synthesis and Characterization of PHEA-EDA-DOXO

PHEA-EDA (100 mg, 163 µmol of EDA) was dissolved in 4 ml of anhydrous DMSO, then Et_3N (68 µl, 490 µmol) was added and the solution was stirred until complete polymer solubilization. Then, DOXO-NHS (30 mg, 34 µmol) dissolved in DMSO (1 ml) was added and the reaction was left for 24 h at room temperature under magnetic stirring. The product was recovered by precipitation in ethanol, redissolved in water, dialysed against distilled water (Visking tubing, cut-off: 12,000–14,000) and recovered by freeze-drying. The derivatization degree of the polymer was evaluated by UV–Vis analysis using a Perkin-Elmer instrument, "Lambda 3A" model (details in Supplementary Material).

In Vitro Stability of PHEA-EDA-DOXO Prodrug

Various physiological mimicking fluids, *i.e.* pH 5.5 and 7.4 phosphate-buffered saline (PBS), bovine serum and human plasma, were used for studying the *in vitro* stability of PHEA-EDA-DOXO prodrug and suitable protocols were carried out for each one of these conditions.

For *in vitro* stability studies in PBS, samples (2 mg of PHEA-EDA-DOXO and 0.2 mg of doxorubicin•HCl) were suspended (1 ml) in pH 5.5 or 7.4, 0.1 M PBS and transferred into a Spectra/Por dialysis membrane (mol. wt. cut-off 12,000–14,000 Da), previously soaked in the experimental medium for 4 h. Dialysis membrane-tube were immersed (50 ml) in pH 5.5 or 7.4 PBS and incubated at 37°C under continuous stirring (100 rpm) in a Benchtop 808C Incubator Orbital Shakermodel 420.

PHEA-EDA-DOXO (2 mg) and doxorubicin•HCl (0.2 mg, as control) were dispersed in 1 ml of human plasma or bovine serum at 37°C under stirring conditions. At appropriate time intervals, 2 ml of 10% trifluoroacetic acid (*v*/*v*) were added in order to precipitate proteins. After immediate mixing and centrifugation for 5 min at 10.000 rpm and 4°C, supernatants were filtered through a 0.2 µm pore-size regenerated-cellulose membrane filter and analyzed by HPLC. At scheduled times, external medium was withdrawn and submitted to HPLC determination using a calibration curve built up by doxorubicin standard solutions (ranging from 0.001 to 0.1 mg/ml). Details of the HPLC method used for the analysis were reported ahead and in Supplementary Material. All experiments were carried out in triplicate.

Cell Cultures

MCF-7 and T47D cells were maintained in culture as previously described (17). Briefly, cells were incubated in culture dishes (100×20 mm) (Guaire® TS Autoflow Codue Water-Jacketed incubator) at 37°C (5% CO_2) using MEM medium with glutamine, penicillin (100 UI/ml), streptomycin (100 µg/ml), amphotericin B (250 µg/ml) and FBS (10%, *v*/*v*). Fresh medium was substituted every 48 h. When 80% confluence was reached, cells were treated with trypsin (2 ml) to detach them from the bottom of dishes and then were harvested using a centrifuge tube containing 4 ml of the culture medium. The dishes were then washed with 2 ml of PBS to remove the remaining cells and then the PBS was transferred into the centrifuge tube. The tube was centrifuged at 1,000 rpm at room temperature for 10 min with a Heraeus Sepatech Megafuge 1.0. The pellet was re-suspended in an appropriate culture medium volume and seeded in culture dishes before *in vitro* investigations (18).

Two-Photon Excitation and Confocal Microscopy

For confocal laser scanning microscopy (CLSM) studies MCF-7 cells were seeded into 22 mm round glass coverslips, with a density of $6 \cdot 10^4$ cells/well, placed into 8-well plate. After 48 h, the cells were incubated with 10 µl per well of cell culture medium containing PHEA-EDA-DOXO and doxorubicin•HCl (as a positive control) at a final drug concentration per well of 40 µM. As a function of the incubation time, the growth medium was removed and the cells were washed twice with DPBS. The cell membranes were labeled by incubating with laurdan dye dissolved in DMSO to a final concentration 1 µM in DMEM +2.5% FBS, for 15 min at 37°C and 5% CO_2. After incubation, the cells were washed twice with DPBS, fixed with 100 µl of the DPBS/glycerol mixture (1:1 *v*/*v*) for 30 min, and then washed once with DPBS. Coverslips were placed onto glass microscope slides and living cells were sequentially imaged in two channels using a Leica RCS SP5 confocal laser scanning microscope with a 63× oil objective NA=1.4 (Leica Microsystems, Germany). 1024×1024 images stack were acquired as a function of time after PHEA-EDA-DOXO and doxorubicin•HCl addition (40 µM), at a scanning frequency of 400 Hz. Two-photon excitation (Spectra-Physics /Mai/-/Tai /Ti:Sa ultra-fast laser) to observe cell membrane stained with laurdan dye were used. Excitation was set at 780 nm. Collection range was 400–500 nm. PHEA-EDA-DOXO and doxorubicin•HCl fluorescence were imaged by confocal microscopy. Excitation was set at 514 nm (Argon laser) and the emission spectral range was set to 570–690 nm; the pin hole was 95 µm.

In Vitro Evaluation of the Cytotoxic Activity

Two independent methods were used to evaluate the cytotoxic activity of PHEA-EDA-DOXO against the MCF-7 and T47D cell lines of human breast cancer in comparison with the un-conjugated drug, *i.e.* the cell viability assay by the MTT test and the cell mortality evaluation by the trypan blue dye exclusion assay.

In the MTT-test (cell viability), the cultured cells were seeded in a 96-well plate in 100 µl of medium (5×10^3 cells/0.1 ml) at 37°C 24 h prior to the cytotoxicity test. The culture medium was then removed, replaced with the different doxorubicin concentrations and incubated for 24, 48 or 72 h. The medium was added to the untreated cells used as controls. After each incubation period, 10 µl of tetrazolium salt solubilized in PBS solution (5 mg/ml) was added to every well and the plates were incubated again for 3 h. The medium was removed and the precipitated formazan salts were dissolved with 100 µl of a mixture of DMSO/ethanol (1:1, *v*/*v*), by shaking the plates for 20 min at 230 rpm (IKA® KS 130 Control, IKA® WERKE GMBH & Co, Staufen, Germany). The solubilized formazan was quantified with a microplate spectrophotometer (Multiskan MS 6.0, Labsystems) at a wavelength of 540 nm with reference at a wavelength of 690 nm. The percentage of cell viability was calculated according to the following equation:

$$\textit{Cell viability}\,(\%) = (Abs_T/Abs_C) \times 100 \quad (1)$$

where Abs_T is the absorbance of the treated cells and Abs_C is the absorbance of the control (untreated cells). The formazan concentration is directly proportional to cell viability, which was reported as the mean of six different experiments ± standard deviation (18).

In the trypan blue dye exclusion assay (cell mortality), cells were transferred into 10 ml plastic culture tubes and centrifuged at 1,200 rpm for 10 min at room temperature using a Megafuge 1.0 centrifuge (Heraeus Sepatech, Osterode/Harz, Germany). Cell pellets were resuspended in culture medium to achieve a final cell concentration of 30×10^4 cell/ml and seeded into six-well plastic culture dishes. After 24 h, cells were treated with various drug concentrations as a function of the incubation time. In all experiments untreated cells were used as controls, and PHEA-EDA were used as the blank. Cells were then detached by Trypsin/EDTA (1×) solution (2 ml), mixed and transferred into 10-ml plastic culture centrifuge tubes. Cellular suspensions were centrifuged (10 min at 1,200 rpm and 22°C), supernatants were discarded and cell pellets were suspended in 100 µl of trypan blue buffer solution. The number of dead cells was determined by a hemacytometer chamber using an optical microscope (Labophot-2, Nikon, Japan). The growth inhibitory activity, expressed as percentage (GIA%), was measured according to the following equation:

$$\mathrm{GIA\%} = \left(1 - \frac{(\mathrm{Tt} - \mathrm{Dt})/\mathrm{Tt}}{(\mathrm{Tc} - \mathrm{Dc})/\mathrm{Tc}}\right) \times 100 \quad (2)$$

where *Tt* is the total number of treated cells, *Dt* is the number of dead treated cells blue colored by the trypan blue dye, *Tc* is the total number of control cells, and *Dc* is the number of dead control cells blue colored by the trypan blue dye. All experiments were carried out in triplicate.

Human Breast Cancer Xenograft Model

Animal experiments were carried out in agreement with the principles and procedures outlined by the local Ethical Committee, as well as the Italian law and the accepted international standards for biomedical research. Care and handling of the animals agreed the European Economic Community Council Directive 86/209, recognized and adopted by Italian Government (D.M. No. 95/2003-D).

MCF-7 cells (5×10^6), an human breast cancer cell line, were diluted in 100 µl of PBS and subcutaneously injected into the flank of 5–6 weeks old female immunodeficient NOD–SCID mice (Harlan Italy s.r.l., San Pietro al Natisone (UD), Italy). The MCF-7 tumor is an estrogen-dependent human breast cancer. Therefore, mice were feeded with 1 mg estrogen per liter of water in order to facilitate the tumor growth. The estrogen administration was stopped prior the beginning of antitumoral treatments (19). After 2–3 weeks, when the tumor volume reached 50–60 mm^3, three groups of mice ($n=10$ each) were treated i.v. (~100 µL) with doxorubicin (5 mg/kg), PHEA-EDA-DOXO conjugate at the same doxorubicin concentration and a solution of the polymer PHEA-EDA every 3 days. Control mice ($n=10$) received 100 µl of saline. The concentrations of doxorubicin and PHEA-EDA-DOXO used in this investigation were below the MTD, which was found at a higher dose, *i.e.* 6 mg/kg (20,21). The sizes of tumor masses were measured with a caliper and tumor volumes were calculated according to the following equation:

$$V = 0.5 \times ab^2 \quad (3)$$

where a and b are the long and short diameter of the tumor, respectively. The body weight, feeding behavior and motor activity of mice were used as indicators of general health. NOD-SCID mice were sacrificed when their tumors reached a diameter of 2 cm, in order to avoid any unnecessary animal sufferance. The survival of NOD-SCID mice submitted to various treatments was calculated from the first day of treatment up to the day of killing.

Biodistribution and Phamacokinetic Animal Models

In order to examine the biodistribution and the pharmacokinetic profile of free doxorubicin and PHEA-EDA-DOXO, a dose of 5 mg/kg of free doxorubicin•HCl or the corresponding drug amount as macromolecular conjugate was injected i.v. through the lateral tail vein in mice bearing sub-cutaneous MCF-7 xenograft.

To obtain the biodistribution profile, at given time intervals (4 and 12 h) post-injection, mice (5 per experimental group) were anesthetized by methoxyflurane. Animals were then sacrificed by blood collection *via* trans-cardiac puncture and blood was placed in microtainer tubes with EDTA (3.75 mg per 2.5 ml blood), and centrifuged at 1,500×*g* for 10 min to obtain plasma. Various organs and tumor masses were also collected, weighed and submitted to analysis.

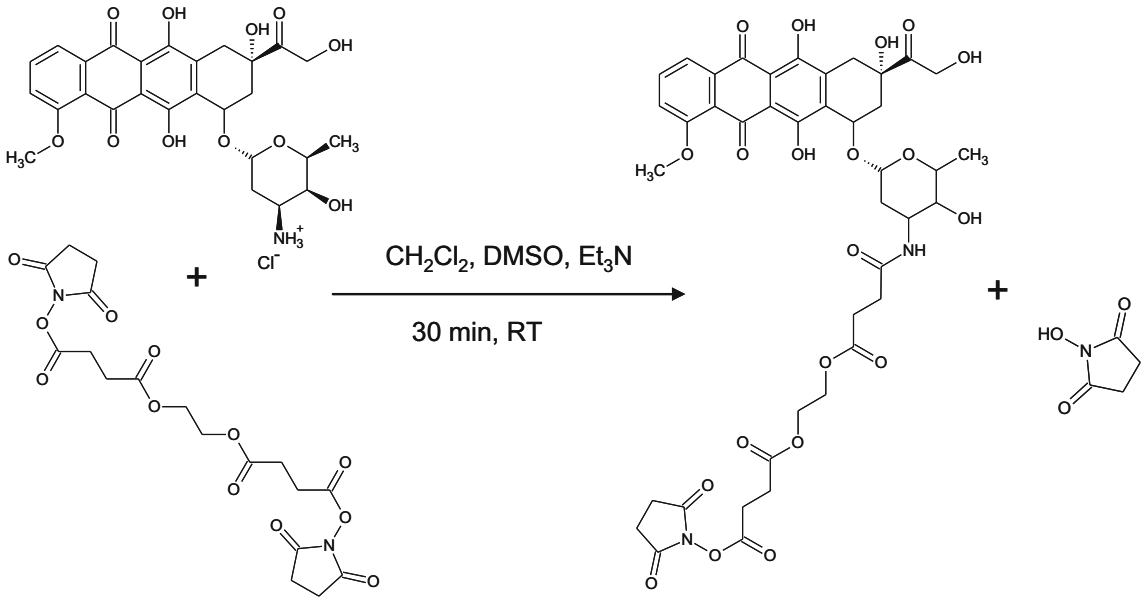
To obtain the pharmacokinetic profile, blood samples (100 µl) were collected from the tail vein into special centrifuge tubes at different times and added with EDTA and Heparin (BD Vacutainer® PLUS Tubes, Becton Dickinson and Company, USA). Blood samples were centrifuged to separate plasma and stored at −80°C until HPLC analysis. Pharmacokinetic parameters were performed using a non-compartmental models. Pharmacokinetic profiles were obtained by determining the amount of doxorubicin as a function of time (5,10,21). More details on the recovery rate of the drug in both blood and tissue samples are reported in Supplementary Material.

Blood and Organ Tissue Analysis

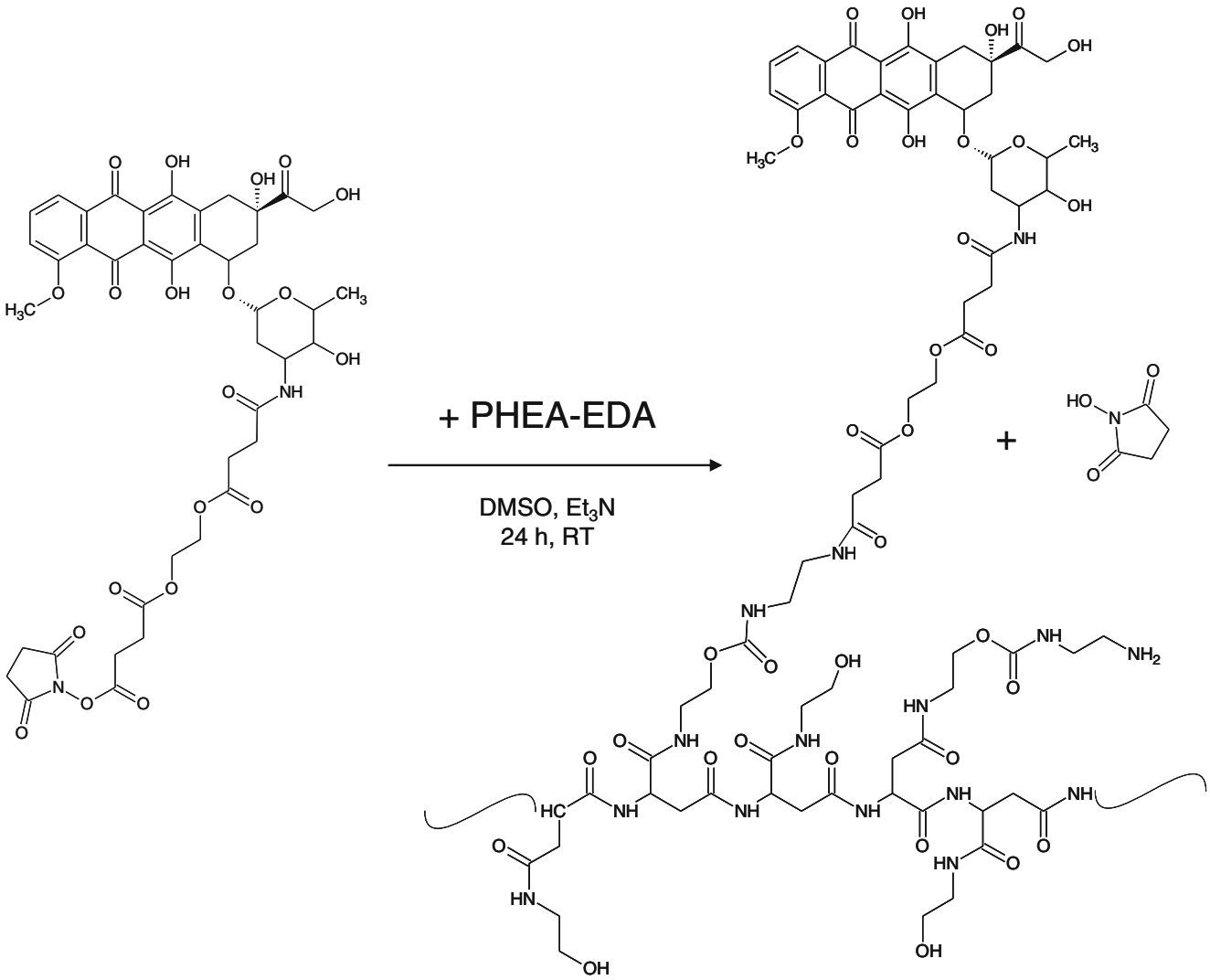
Organs were removed, cleared of blood, weighed and then homogenized. Blood and organ tissue samples were treated for analytical evaluation as elsewhere reported (22). Briefly, the plasma (100 µl) and 200 µl tissue homogenate were solubilized with 500 µl Solvable for 2 h at 60°C and then treated overnight with 200 µl H_2O_2 at room temperature to decolorize samples before the analysis. The samples were diluted with distilled water up to a final volume of 800 µl and then mixed with 100 µl each of 10% (*w*/*v*) sodium dodecyl sulfate and 10 mM H_2SO_4 to reduce the formation of foam and to precipitate organic materials and to allow the hydrolysis of doxorubicin from the polymeric backbone, respectively. Triple extraction was performed after adding chloroform:isopropyl alcohol (3:1 *v*/*v*). The solutions were frozen overnight, thawed, and centrifuged at 16,000×*g* for 10 min. The organic phases were removed and the resulting solutions were evaporated to dryness, and re-dissolved in a mobile phase solution (methanol:isopropyl alcohol:Sorensen's buffer 1:2:7 *v*/*v*/*v*). These samples were analyzed using a Jasco Europe HPLC system equipped with a Jasco FP 920 scanning fluorescence detector (Jasco Europe, Milan, Italy). The chromatographic separation was carried out by using a Phenomenex Jupiter C_{18} column (250×4.6 mm, 5 µm particle size, Phenomenex Inc., Italy). The flow rate of the mobile phase was 1 ml/min. The HPLC analysis was carried out at room temperature and at an excitation wavelength of 480 nm and an emission wavelength of 560 nm. The doxorubicin content in plasma or tissues was determined using a calibration curve built up by adding a known amount of free doxorubicin solution to plasma or tissue samples harvested from control (untreated) mice (22). No interference was observed in the doxorubicin analysis due to any plasma or organ component. Each experimental point was the mean of five different experiments ± standard deviation.

Statistical Analysis

The significance of the experimental results were evaluated by the statistical analysis of the various data using the one-way ANOVA and a posteriori Bonferroni *t*-test was carried out to check the ANOVA test. A p value <0.05 was considered statistically significant. All the values are reported as the average ± the standard deviation.



Scheme 1 Synthesis of DOXO-NHS from doxorubicin and ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester).



Scheme 2 Synthesis of PHEA-EDA-DOXO from PHEA-EDA and DOXO-NHS.

RESULTS AND DISCUS?SION

Synthesis

The synthesis of DOXO-NHS was carried out in CH_2Cl_2 in the presence of 10% *v/v* of DMSO and Et_3N, in order to promote the dissolution of the drug in the hydrochloride form. A twofold molar excess of the homobifunctional linker was used, and the drug solution was added dropwise to the linker solution in order to reduce the formation of a disubstituted DOXO-ethylene glycol-bis(succinic acid)-DOXO product. The reaction (Scheme 1) was fast and quantitative and no unreacted doxorubicin and only traces of the disubstituted product were found after 30 min, as shown by ESI-MS of the crude reaction mixture. After vacuum removing CH_2Cl_2, the residual DMSO was removed by extractions with diethylether, leading, after solvent elimination, to an oily dark red product.

The crude reaction mixture, containing small amounts of the bisubstituted byproduct and excess of unreacted linker was processed using a short pad of celite that retained the mono DOXO-NHS and allowed the sequential elution of the unreacted linker and of the desired product using eluents of increasing polarity. The disubstituted byproduct was not eluted from the column.

ESI-MS analysis in the positive ion mode of the pooled fractions containing DOXO-NHS showed intense signals attributed to the $[M{\cdot}H]^+$ and $[M{\cdot}Na]^+$ species at m/z=885.3 and 907.3 (Fig. 2S and 3S, Supplementary Material). The structure of the DOXO-NHS product was assessed by NMR. In particular, bi-dimensional NMR sequences (COSY, HSQC, HMBC) were used to achieve the complete spectrum assignment of the derivative (Fig. 4S and 5S and Table 2S, Supplementary Material).

The coupling of ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) to doxorubicin, obtained here for the first time, leads to a functionalized drug that can be easily linked to substrates carrying amino groups, with a fast reaction, preserving the integrity of the drug during the synthesis and leading to an *in vivo* hydrolysable prodrug.

The coupling reaction of DOXO-NHS to PHEA-EDA polymer was carried out for 24 h at room temperature in anhydrous DMSO in the presence of Et_3N (Scheme 2). After purification by dialysis against distilled water, the product was recovered by freeze-drying; the polymeric prodrug resulted soluble in water. The degree of derivatization of PHEA-EDA was 9% *w/w* with respect to polymer weight or 7% mol/mol with respect to EDA moieties as assessed by UV–Vis spectroscopy at λ=490 nm (Fig. 6S and 7S, Supplementary Material).

Stability Studies

Stability and drug release studies were carried out at 37°C by incubating PHEA-EDA-DOXO and doxorubicin•HCl in both pH 5.5 and 7.4 phosphate buffer, as well as in bovine serum and human plasma. These media were selected to mimic intra-tumor and extracellular compartments and the physiologic conditions, respectively. Calibration curve of doxorubicin in plasma and chromatographic curves of PHEA-EDA-DOXO and doxorubicin•HCl are reported in Fig. 8S and 9S, Supplementary Material. The free drug degradation profiles at different pH value and in biological fluids are shown in Fig. 1a and b. The results showed that doxorubicin•HCl undergoes partial degradation after 24 h of incubation. The similar degradation profiles obtained in PBS buffers and in relevant biological fluids (*i.e.* serum and plasma) evidenced that doxorubicin•HCl degradation occurs by chemical pathways rather than by enzymatic mechanisms. The profiles of drug released from the PHEA-EDA-DOXO conjugate showed a slightly higher release of doxorubicin in PBS buffer solutions at different pH values with respect to biological fluids. Due to the high stability of the conjugate, the prodrug system can be exploited to provide a prolonged drug

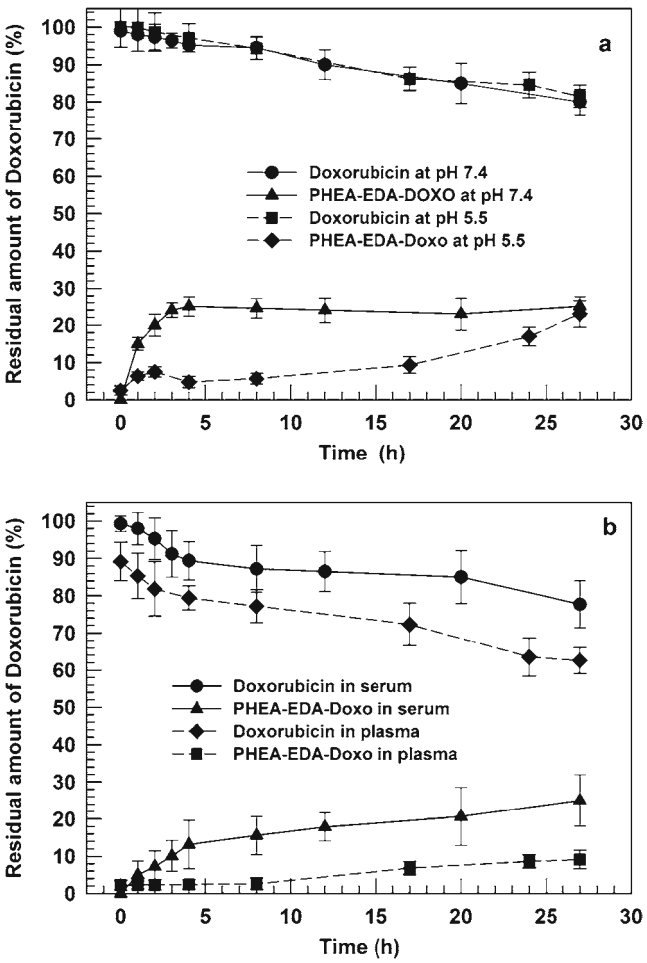


Fig. 1 Stability profile of the PHEA-EDA-DOXO and the un-conjugated drug in pH 5.5 and pH 7.4 PBS buffer solutions (**a**) and in biological fluids (serum and plasma) (**b**) at 37°C. Experiments were carried out at a drug concentration of 1 mg/ml.

release after accumulation in the target site. No degradation or structural changes are apparently promoted or caused to drug from PHEA-EDA backbone.

In Vitro Cytotoxic Activity

Considering that different mechanism of resistance/sensibility can occur as a function of tumour cell phenotype, two different cancer cell lines of human breast cancer were used to evaluate the cytotoxic activity by assaying the time- and dose-dependent effects of doxorubicin-conjugate in comparison with the free drug, thus representing suitable models for different sensibility to doxorubicin and its macromolecular prodrug. PHEA-EDA has not shown a cytotoxic effect *per se*. In contrast, both free drug and PHEA-EDA-DOXO showed a significant time and concentration dependent cytotoxic activity (Fig. 2). In particular, the free doxorubicin showed a cell cytotoxicity of ~50% in the MCF-7 cell line after 24 h incubation at a 10 μM drug concentration, whereas the macromolecular prodrug showed significant effects at the same concentration after 72 h. Probably, these data are due to the slow release of the drug from the conjugate, which means a lower availability at the cellular level during the short time (24 and 48 h). For this reason the conjugate can be exploited for a prolonged drug release after accumulation in the site of action (23). Another factor that contribute to these findings is the different internalization process between the free doxorubicin and the macromolecular prodrug. Doxorubicin•HCl is a small hydrophilic molecule which can be rapidly internalized in cancer cells *via* a pinocytosis process (24); while the macromolecule PHEA-EDA-DOXO is internalized *via* an endocytosis process (25) and hence the free drug internalization is faster than that of the PHEA-EDA-DOXO conjugate.

Analogously, experiments carried out using a different cell model, specifically T47D cells (Fig. 10S, Supplementary Material), showed a similar behavior of the prodrug with respect to the free drug in time-dependent and dose-depended cell viability. The results suggest that the macromolecular features, *i.e.* internalization rate and drug release, prevail on the specific cell sensibility in the final *in vitro* antitumor activity.

The cytotoxic effects of PHEA-EDA-DOXO and free doxorubicin observed by the MTT-test were in very good agreement with the experiments based on cell mortality by the trypan blue dye exclusion assay (Fig. 2). After 24 h of incubation, the free drug showed a significant growth

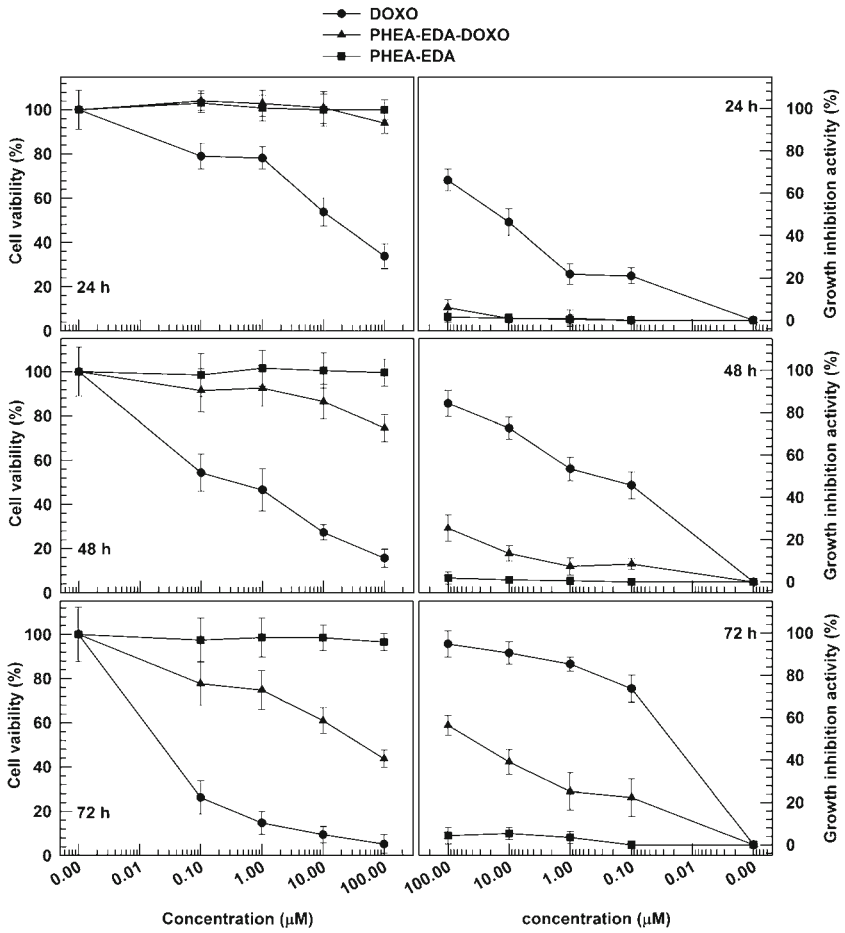


Fig. 2 Time-dependent and dose-depended *in vitro* antiproliferative activity of doxorubicin as free drug or macromolecular prodrug against MCF-7 breast cancer cells. Data are expressed as percentage of cellular viability as determined by MTT-test. Results are the mean of six different experiments ± standard deviation.

inhibitory activity at the 10 μM concentration with a MCF-7 cell deaths of 50%. Nevertheless, the conjugation of doxorubicin with PHEA-EDA dramatically reduces the initial cytotoxicity of the drug even at the highest concentration (100 μM), showing only ~6% of cell death after 24 h. Only after 72 h of treatment, the conjugate showed a significant cytotoxic action on MCF-7 cells, *i.e.* a cell mortality of ~60% was elicited at the highest concentration (100 μM). Therefore, also the trypan blue dye exclusion assay supported the cytotoxic action of PHEA-EDA-DOXO mediated by the slow release of the doxorubicin.

Intracellular Uptake

Confocal microscopy (CLSM) is a suitable tool to investigate the cellular uptake behavior and intracellular distribution of a drug delivery device (26) and hence the interaction of PHEA-EDA-DOXO and the free doxorubicin•HCl with MCF-7 cancer cells was investigated. Our findings showed (Fig. 3) that the free doxorubicin•HCl was largely accumulated in the cell nuclei of MCF7 cells, while the red fluorescence of PHEA-EDA-DOXO was mainly located in the cytoplasm. These findings evidenced that the PHEA-EDA-DOXO conjugate was internalized in MCF-7 cells and agreed the *in vitro* cytoxicity investigation supporting the hypothesis of the action mechanism of the doxorubicin macromolecular prodrug, mainly based on the preliminary macromolecular internalization *via* endocytosis and hence the cytoplasmic cleavage of the doxorubicin from the macromolecule backbone. Considering that the doxorubicin antiproliferative activity is due to its DNA intercalating feature, only the free drug that reaches the nuclei of the tumoral cells can ensure the DNA intercalation, thus justifying the retarded antiproliferative activity observed in the case of doxorubicin macromolecular prodrug.

Biodistribution and Pharmacokinetic Profile

The requirement of a certain tumor targeting efficiency is really relevant for a drug carrier to be successfully proposed in the therapeutic scenario of the treatment of solid tumor diseases (27,28). The targeting ability of a delivery device has to be taken into consideration not only in terms of potentially increased *in vivo* antitumor efficacy but also of a potential reduction of the clinical drawbacks of the delivered drug, which often represent the limiting factor for a suitable clinical use of a drug. In fact, the distribution of an antitumoral drug in healthy tissues and organs (high volume of drug distribution) beside the tumor is the main reason of the rise of toxicity. Therefore, the organ distribution of PHEA-EDA-DOXO in

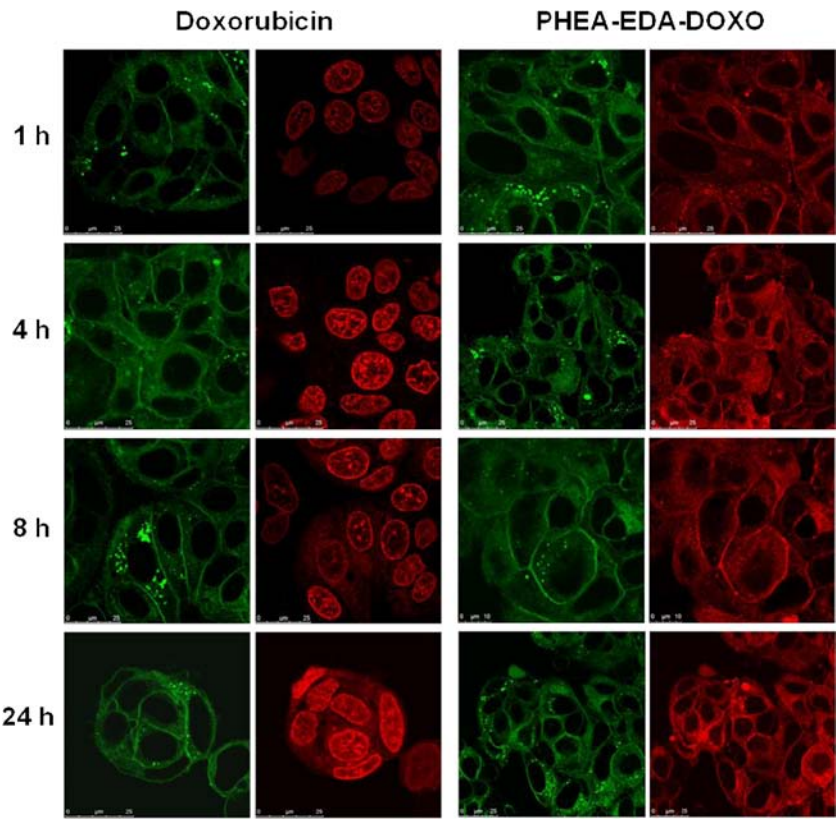


Fig. 3 Confocal microscopy analysis of MCF-7 cells incubated with doxorubicin (*left column*) or PHEA-EDA-DOXO (*right column*) at 1, 4, 8 and 24 h. Cell membranes were stained with Laurdan (*green*).

comparison with the free drug as well as the pharmacokinetic profiles were investigated in animals bearing MCF-7 carcinoma xenograft (Fig. 4a and b).

As shown in Fig. 4b, the blood levels of free doxorubicin and PHEA-EDA-DOXO evidenced that the PHEA-EDA polymeric backbone promoted a longer circulation of the drug. This feature (typical of some therapeutic polymers, *e.g.* polyethylenglycole) could amplify the EPR effect thus providing a passive targeting to the solid tumor. In fact, the PHEA-EDA-DOXO significantly ($p<0.001$) increased the drug concentration in the tumor masses in comparison with the free drug, *i.e.* from 8.16 μg/g organ and 2.93 μg/g organ (for 4 and 12 h following the i.v. administration, respectively) to 17.32 μg/g organ and 12.2 μg/g organ (for 4 and 12 h following the i.v. administration, respectively). As a consequence, the presence of the drug was increased ($p<0.01$) in the RES organs (liver and spleen) by the PHEA-EDA polymer, due to its specific uptake by these tissues determined by the greater molecular weight of the macromolecular prodrug than the free doxorubicin (23). A very interesting finding of the bio-distribution features of the PHEA-EDA-DOXO was the significant ($p<0.001$) decrease of the drug level in the heart in comparison with the free doxorubicin. Therefore, the conjugation of doxorubicin to the macromolecular backbone of PHEA-EDA may elicit a drastic reduction of the free drug cardiotoxicity, similarly to that observed in the case Doxil® where the liposomal carrier ensured the heart avoidance (29).

A more suitable refinement of the drug levels in blood as a function of time following the i.v. administration of free doxorubicin and PHEA-EDA-DOXO was achieved by evaluating the pharmacokinetic profiles and parameters (Fig. 5 and Table I). The pharmacokinetic profiles of both doxorubicin and PHEA-EDA-DOXO were characterized by a biphasic shape with a first part (0–1.5 h for doxorubicin and 0–3 h for PHEA-EDA-DOXO) of rapid drug clearance from the blood stream followed by a more gradual blood disappearance (2–5 h for doxorubicin and 1.5–25 h for PHEA-EDA-DOXO). The doxorubicin half-life ($t_{1/2}$ value) after i.v. administration was 21.3 h. The PHEA-EDA-DOXO showed a 2.2 times longer $t_{1/2}$ value (46.9 h) than doxorubicin. Significant difference between doxorubicin and PHEA-EDA-DOXO were observed not only in the pharmacokinetic profiles but also in the pharmacokinetic parameters. This difference was further evidenced by the AUC values (Table I), *i.e.* PHEA-EDA-DOXO elicited an improvement of the AUC value (93 h·μg/ml) of ~4-fold in comparison with the unconjugated drug (6.8 h·μg/ml). As shown in Table I, the PHEA-EDA-DOXO was also able to ameliorate the pharmacokinetic parameters of doxorubicin by reducing the volume of distribution (*Vd*), thus leading to a drug avoidance from tissues where the drug action is not required and hence probably decreasing the drug drawbacks and in particular its

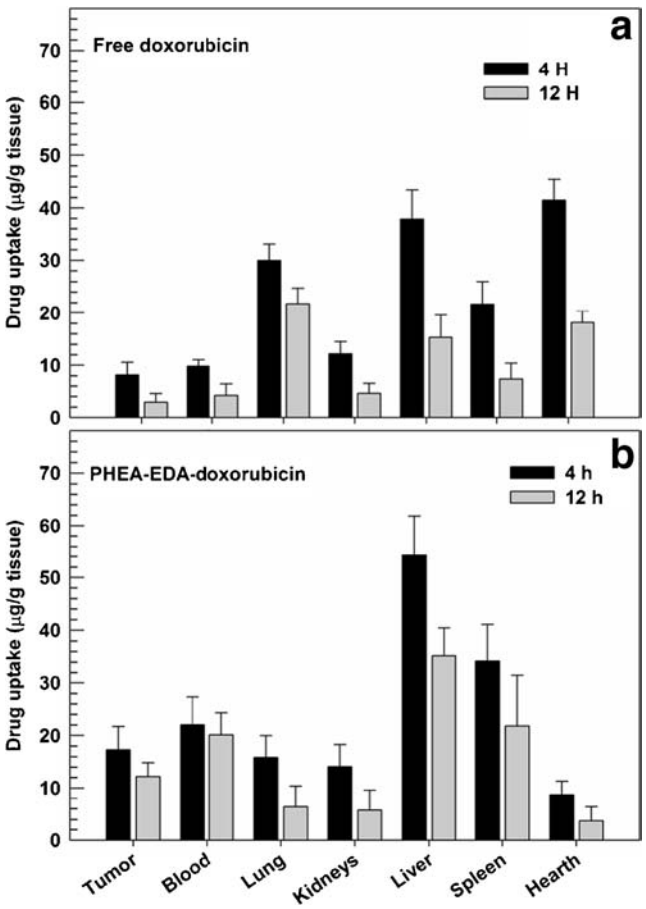


Fig. 4 *In vivo* distribution of doxorubicin (**a**) and PHEA-EDA-DOXO (**b**) in various organs following i.v. administration into xenograft-bearing NOD SCID mice as a function of time. Data are the mean ± standard deviation of five experiments.

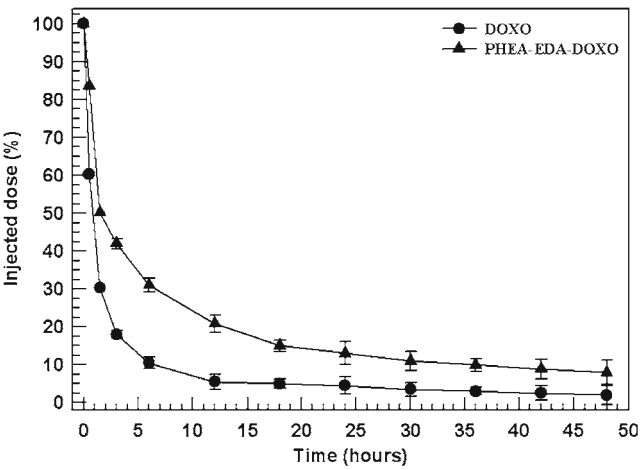


Fig. 5 Pharmacokinetic profile of un-conjugated doxorubicin and PHEA-EDA-DOXO in MCF-7 carcinoma xenograft-bearing NOD-SCID mice following i.v. administration. Data are the mean of five different experiments ± standard deviation. The PHEA-EDA-DOXO showed a pharmacokinetic profile significantly different from that of doxorubicin, ANOVA $p<0.01$.

Table I Pharmacokinetic Parameters of Free Doxorubicin and PHEA-EDA-DOXO

Sample	Pharmacokinetic parameters[a]			
	$T_{1/2}$ (h)	C_{max} (μg/ml)	*Vd* (ml)[b]	*AUC* (μg/ml × h)[c]
Free doxorubicin	21.3 ± 4.0	5.3 ± 1.2	2.6 ± 0.2	6.8 ± 0.5
PHEA-EDA-DOXO	46.9 ± 7.4	10.6 ± 3.0	1.4 ± 0.5	93.3 ± 20.0

[a] Data of pharmacokinetic parameters have an ANOVA significance $P < 0.001$ with respect to the free doxorubicin. No statistical significance was only observed for the C_{max} values

[b] The apparent volume (*Vd*) was calculated according to the equation $Vd = ADD_{t0}/PDC_{t0}$, where *ADD* is the administered drug dose (5 mg/kg) at time $t = 0$ and PDC is the plasma drug concentration (μg ml^{-1}) at time $t = 0$, as extrapolated value on semi-log y-axis of the pharmacokinetic profile

[c] The areas under the plasma concentration-time curve (AUC) (starting from the first to the last sampling time) was calculated using the trapezoidal rule with extrapolation to infinity using the ratio *Cn*/*Ke*, where *Cn* was the last measurable concentration and *Ke* is the constant of drug elimination

cardio-toxicity, one of the severe limiting factor in the clinical use of doxorubicin.

Antitumor Activity in Xenograft Animals

Following the outcome of the *in vitro* and *in vivo* experiments, an *in vivo* investigation on the anticancer efficacy using a human breast carcinoma xenograft model was carried out to shed light on the real therapeutic potentiality of PHEA-EDA-DOXO for the treatment of solid tumor diseases. As shown in Fig. 4b, PHEA-EDA-DOXO was able to provide the EPR effect thus leading to a greater accumulation of the macromolecular prodrug at the level of the tumor masses and hence PHEA-EDA-DOXO should act as an intra-tumor reservoir from which the drug is gradually cleaved following both chemical and enzymatic hydrolysis. Therefore, a delayed *in vivo* antitumor action following an initial accumulation of the PHEA-EDA-DOXO at the tumor site has to be expected.

The *in vivo* experiments, based on the treatment (every 3 days) of NOD-SCID mice bearing a MCF-7 human breast carcinoma xenograft having a tumor volume of 50–60 mm^3, showed (Fig. 6a) that there was no significant difference after 18 days between the treatment with free doxorubicin and PHEA-EDA-DOXO, *i.e.* both ensured a similar *in vivo* antitumor activity by reducing the growth of the tumor volume with respect to the control (untreated animals). After the 18th day, a significant ($p < 0.05$) improvement of the *in vivo* antitumor activity was observed in the case of the PHEA-EDA-DOXO treatment in comparison with both doxorubicin treatment and the control, *i.e.* a remarkable slowing down of the growth of tumor volume was observed.

The improvement of the anti-tumor activity of PHEA-EDA-DOXO with respect to the un-conjugated doxorubicin was also supported by the significant ($p < 0.01$) survival advantage of NOD-SCID mice bearing MCF-7 human breast carcinoma xenografts (Fig. 6b). In fact, the survival of NOD-SCID mice treated with PHEA-EDA-DOXO was longer that the group treated with doxorubicin (70 and 60 days, respectively).

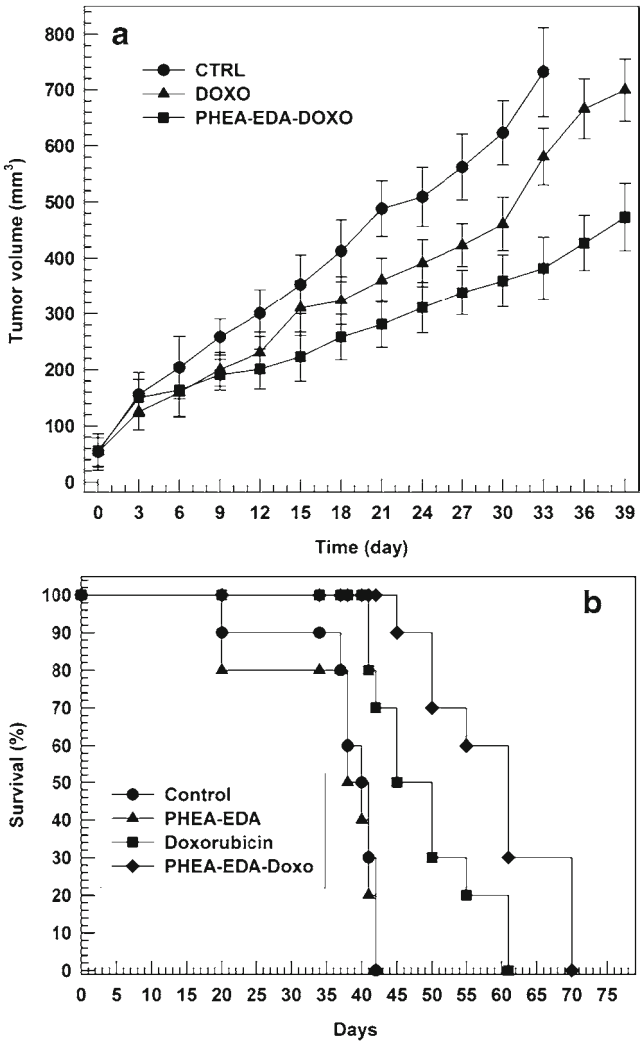


Fig. 6 Tumor growth inhibition of human breast cancer MCF-7 carcinoma xerograft in NOD-SCID mice (**a**) and survival of NOD-SCID mice (**b**). Mice were injected i.v. with 5 mg/kg of doxorubicin or an equivalent dose of PHEA-EDA-DOXO. Untreated animals were the control. Each value is the mean ± the standard deviation of five different experiments.

These findings supported very well our plausible hypothesis of an intra-tumor reservoir-like action of PHEA-EDA-DOXO being able to gradually release the drug within the solid tumor and they were also in very good agreement with other experimental observations (29) on antitumor drug-conjugates used as macromolecular prodrugs. In these cases, no significant improvement of the *in vitro* antitumor activity or even a worsening was observed, but in animal models there was an increase of the drug antitumor efficacy due to an improvement of the drug body-distribution (30).

CONCLUSIONS

A polymeric prodrug based on α,β-poly(N-2-hydroxyethyl)-d,l-aspartamide functionalized with amine pendant groups (PHEA-EDA) coupled with doxorubicin was obtained. Doxorubicin was easily derivatized with ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) leading to a product very reactive towards the amino groups of the PHEA-EDA. After polymer functionalization, a polymeric prodrug having the 9 *w/w* of doxorubicin was obtained; the product resulted stable at physiological pH and it was shown to accumulate into cell cytoplasm for short times of incubations, thus exploiting also a retarded cytotoxic effect on tumor cells *in vitro*. Nevertheless, our investigation evidenced a significant improvement of the *in vivo* antitumor activity of PHEA-EDA-DOXO with respect to the un-conjugated drug, thus leading to a clear survival advantage of the treated NOD-SCID mice. These promising findings can be attributed to the biopharmaceutical feature of the polymeric backbone conjugated with the doxorubicin, thus providing (i) a potential protection of the drug from the plasmatic enzymatic degradation and clearance, (ii) an improvement of the blood pharmacokinetic parameters both in terms of elongation of the blood residence time and a reduction of the volume of distribution and (iii) a suitable body biodistribution with a passive tumor targeting mostly driven by the enhanced-permeation and retention effect.

All together, our findings firmly support the promising rationale of the approach based on macromolecular prodrugs of doxorubicin, *i.e.* PHEA-EDA-DOXO, for further potential development and application as useful tool in the treatment of solid cancer diseases, thus prompting even a potential clinical use.

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