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Folate-mediated targeting of albumin conjugates of paclitaxel obtained through a heterogeneous phase system

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ABSTRACT

The study developed cytotoxic macromolecular conjugates that specifically target the folate receptor and deliver the drug into cell cytoplasm. The anticancer agent paclitaxel was conjugated to human serum albumin (HSA) and this drug–albumin conjugate was further equipped with folic acid, linked via an extended poly(ethylene glycol) spacer. Preparation was carried out in a heterogeneous phase system exploiting the binding ability of Cibacron Blue dye to HSA. Unreacted reagents were easily removed and, after purification by gel filtration, the conjugate was fully characterized. Binding and in vitro cytotoxicity studies on human nasopharyngeal epidermal carcinoma KB and colorectal carcinoma HT-29 cells (as negative control) demonstrated increased selectivity and anti-tumoral activity.

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1. Introduction

Paclitaxel (PTX), a major anticancer drug isolated from the bark of *Taxus brevifolia*, has been adopted in the therapy of a wide variety of cancers, including breast, non-small cell lung, epithelial, and ovarian. It is also recommended in the treatment of AIDS-related Kaposi's sarcoma as second-line treatment and in the prophylaxis of coronary stent stenosis.

Owing to its low solubility it is administrated under slow infusion with castor oil/ethanol solution as surfactant, and hypersensitivity reactions occur (Szebeni et al., 1998). The second limitation of taxoids is their systemic toxicity, which causes severe adverse side effects (Michaud et al., 2000). This toxicity has restricted dosage, which often leads to incomplete tumor eradication.

Different approaches have been proposed (including micellar carriers, soluble polymers, paclitaxel soluble prodrugs and polymeric nanocapsules) [*reviews*: (Skwarczynski et al., 2006; Dosio et al., 1997; Pawar et al., 2004; Marupudi et al., 2007; Singla et al., 2002)].

Some significant therapeutic results have been obtained with polyglutammic acid paclitaxel (polyglumex) (Chipman et al., 2006), and more recently with a docosahexaenoic acid–paclitaxel conjugate (Taxoprexin®) (Fracasso et al., 2009) or with nanoparticles of human serum albumin–paclitaxel bound (nab™ technology) (Abraxane®) (Gradishar, 2006).

Other recent applications of albumin (HSA) have demonstrated some advantages as a natural and therefore biocompatible and biodegradable carrier to construct targeted cytotoxic conjugates with apoptosis-inducing drugs (Temming et al., 2006, 2007). In these cases the albumin-based targeted drug delivery system has increased the disease tissue/normal tissue drug concentration ratio.

Among targeting agents directed to membrane-bound tumor-associated receptors, folic acid has been widely utilized as a ligand for the selective targeting and delivery of macromolecular drugs into tumor cells.

The overexpression of folate receptor (FR) on many cancer cells obviously identifies that receptor as a potential target for a variety of ligand- and antibody-directed cancer therapeutics (Gruner and Weitman, 1999). Folate receptors exist in three major forms, namely FR-α, FR-β, and FR-γ. The FR-α form may be further qualified as a tumor-specific target overexpressed by many types of tumor cell, including those of the ovary, brain, kidney, breast, myeloid cells and lung, since it generally becomes accessible to intravenous drugs only after malignant transformation (Lu and Low, 2002). Furthermore, folate receptor density appears to increase with progression of the cancer stage/grade.

In previous reports we presented an approach involving albumin prodrugs of paclitaxel with improved solubility and increased in vitro anti-tumoral activity (Dosio et al., 1997). We also found that the shielding induced by surface modification with poly(ethylene glycol) (PEG) may be important to further reduce clearance and liver and spleen uptake, maintaining satisfactory activity (Dosio et al., 2001).

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The aim of this study was to increase the selectivity of HSA–paclitaxel conjugates, developing macromolecules targeted against folate receptors. To achieve this goal, a thiol-reactive Folate-PEG derivative was first synthesized, in which the extended linker is used to increase circulation time and as a spacer to improve exposition of the targeting agent. An albumin–paclitaxel targeted conjugate was also prepared, purified and characterized. In order to reduce the number of purification steps and in view of automating preparation, the use of heterogeneous phase system exploiting the capability of Cibacron Blue dye to bind to HSA was investigated.

The selectivity and in vitro anti-tumoral activity of the targeted conjugate were compared on a human nasopharyngeal epidermal carcinoma KB cell line, used as positive control, and on HT-29 tumor cells lacking FR-α, as negative control.

2. Materials and methods

2.1. Materials

Paclitaxel was supplied by Indena (Milan, Italy), 3,3′ labeled [^{3}H]-paclitaxel (10 Ci/mmol), L-[4,5-^{3}H]-leucine (58 Ci/mmol), ^{3}H folic acid potassium salts (50 Ci/mmol) and Nonidet P-40 were from Amersham, Italy. Human serum albumin (Fluka Chimica, Milan, Italy) was purified by gel filtration chromatography on a Sephadex G-100 column before conjugation. PEG-bisNH_2 (MW 3400) was purchased from Nektar Therapeutics (Huntsville, AL); Folic acid, dicyclohexylcarbodiimide, dithiothreitol, 5,5′-dithiobis-2-nitrobenzoate, 6-maleimidohexanoic acid *N*-succinimidyl ester and solvents were obtained from Sigma–Aldrich (Milan, Italy). Sephadex PD-10 and DEAE-Sepharose gels were from Pharmacia (Uppsala, Sweden); Affi-gel Blue was from Bio-Rad (Richmond, VA).

2.2. Synthesis of amino-PEG-Folate

Folic acid (FA) (52.97 mg, 0.12 mmol) was suspended in dry dimethysulphoxide (DMSO) with 24.76 mg (0.12 mmol) of dicyclohexylcarbodiimide (DCCD) and 12 μl of dry pyridine. After dissolution 400 mg (0.12 mmol) of PEG_{3400}-bisNH_2 in dry DMSO were added and the mixture was maintained under stirring at 25 °C for 12 h in the dark. After dilution with water the insoluble by-product was removed by filtration. The solution was extensively dialyzed (cut off 3500 Da) against phosphate buffered solution (PBS) 10 mM pH 7.0 and then lyophilized. To remove unreacted PEG-bisNH_2 and PEG-bis folate the crude product was purified on a DEAE-Sepharose column (1.8 mm × 200 mm) eluted with a gradient of 100 mM ammonium acetate buffer pH 10.0 and NaCl 20 mM at a flow rate of 1 ml/min. The fractions comprising a broad peak that eluted after 90–180 min were collected and lyophilized, giving 312 mg of amino-PEG-Folate as yellow powder (yield 68%).

1H NMR (400 MHz, DMSO-d6) δ (ppm): 8.64 (s, C7–H), 7.67 (d, *aromatic protons*), 6.72 (d, *aromatic protons*), 4.39 (d, C9–H2), 4.31 (dd, C19–H), 1.85–2.3 (m, C20–C21), 3.40–3.85 (broad, methylene PEG).

2.3. Synthesis of MH-PEG-Folate

To 200 mg of amino-PEG-Folate (0.05 mmol), dissolved in 2 ml of dry dimethylformamide (DMF), 10 μl of triethylamine and 24.7 mg (0.08 mmol) of 6-maleimidohexanoic acid *N*-succinimidyl ester (MH-NHS), previously dissolved in DMF, were added. The reaction was maintained under stirring for 4 h at room temperature in the dark. Solvent and reagent were removed by extensive dialysis and the compound was then lyophilized, giving 170 mg of MH-PEG-Folate (yield 82%).

1H NMR (400 MHz, DMSO-d6) δ (ppm): 8.64 (s, C7–H), 7.67 (d, *aromatic protons*), 6.95 (s, CH=CH maleimido), 6.6–6.7 (m, *aromatic* and, 3′,5′-H,) 4.60 (9-CH_2N), 4.39 (d, C9–H2), 4.31 (dd, C19–H), 1.85–2.6 (m, C20–C21, CH_2–NH_2), 3.40–3.85 (broad, methylene PEG), 1.3–1.6 (m 6H hexanoic acid).

2.4. Preparation of albumin conjugates with paclitaxel and evaluation of HSA adsorption on Affi-gel Blue and disulphides reduction

The method to prepare the drug–albumin conjugate was essentially as described previously (Dosio et al., 2001) with some modifications. Briefly: gel filtration-purified human serum albumin (10 mg, 0.015 mmol) dissolved in 1 ml of PBS solution was reacted with a 30 M excess of 2′-sulpho-NHS-succinyl-paclitaxel dissolved in 80 μl of dry dimethylsulphoxide, containing 5 μCi of [^{3}H]-labeled paclitaxel molecule. The reaction mixture was maintained for 1 h at 25 °C under vigorous stirring, then, after centrifugation, the mixture was loaded onto 1 ml Affi-gel Blue previously washed with degassed PBS 0.1 M pH 7.6 and maintained in end-over-end stirring for 30 min in a 2 ml plastic conical vial. The unconjugated drug was removed by washings. Successively dithiothreitol (DTT) 30 mM in PBS was added in different HSA molar excesses (from 30 to 100) and the thiol reduction was carried out for 3 h at 20 °C then the reagent was removed by washings. To evaluate the molar thiol content, each preparation was washed with 4 ml of PBS plus NaCl 1.5 M and the eluted fraction was checked by the 5,5′-dithiobis-2-nitrobenzoate (DTNB) reagent (ε = 14,200 at 412 nm). The mean recovery of the HSA is the total amount of protein (E0.1% at 280 nm = 0.6) eluted from the gel after washings. Each experiment was repeated three times.

2.5. HSA–paclitaxel derivatization with MH-PEG-Folate

Freshly prepared gel immobilized disulphide-reduced HSA–paclitaxel, with a mean of 3.8 thiol free content, was reacted with a seven molar excess of MH-PEG-Folate dissolved in PBS. The suspension was maintained in end-over-end stirring for 3 h, then washed with PBS to remove reagent excess. The HSA–(paclitaxel)-PEG-Folate conjugate and by-products were eluted from the gel by washing with 4 ml of PBS supplemented with NaCl 2 M. The salt from the solution was removed by Centricon® Centrifugal Filter 30 kDa (Millipore, Milan, Italy) and finally concentrated to a final volume of 1 ml.

Purification and size characterization of the conjugates were performed on a TSK G-3000SW (1.5 mm × 600 mm) (Beckmann Instr. San Ramon, CA) eluted with buffer 0.01 M sodium phosphate, 0.1 M sodium sulphate pH 7.0 at a flow rate of 0.5 ml/min and 20 °C. All chromatograms were generated on a Merck-Hitachi 655A-12 Liquid Chromatographer equipped with L5000 LC Controller (Merck, Milan, Italy) and the eluting fractions were monitored at 280 nm using an L4000 UV detector. Peak heights and areas were recorded and processed on a CBM-10A Shimadzu interface (Shimadzu, Milan, Italy). Fractions eluted after 22–25 min were collected and concentrated by Centricon®.

Mass spectra were determined using the matrix-assisted laser desorption/ionization time-of-flight MALDI-TOF mass spectrometry (Bruker Ultroflex TOF model).

The degree of paclitaxel derivatization was determined from the ratio between radioactivity and protein concentration; the latter was determined by a modified Lowry method (DC Protein assay, BioRad, Hercules, CA). The product was stored at 5 °C in the dark and the stability was checked as dialyzable radioactivity against PBS solution.

The extent of modification of the MH-PEG-Folate derivatized HSA was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels under non-reducing conditions; Coomassie blue staining was used to visualize proteins

and radiochromatographic analyses were performed using a System 200 Imaging Scanner (Canberra Packard, Milan, Italy).

The UV spectrum of the conjugate showed absorption in the range between 230 and 400 nm with two maximum peaks at 277 nm (ε = 11,100) and 340 nm (ε = 7340).

2.6. Stability experiments

The stability of conjugates (HSA–paclitaxel and Folate derivatized HSA–paclitaxel) prepared with the above method was evaluated in buffered solutions at pH 7.4 or by incubation in sterile fetal calf serum. The test was repeated three times.

The method was essentially as described elsewhere (Dosio et al., 1997). The conjugate solutions (1 mg/ml and containing 70–90 μg of paclitaxel) were maintained at 37 ± 0.1 °C and analyzed at scheduled intervals by HPLC. Released paclitaxel was extracted from the samples by adding t-butyl methyl ether and the organic layer was evaporated to dryness. Aliquots were injected into an Agilent Lichrospher 100 RP-18 column (Agilent Technologies Italia, Milan, Italy) and analyzed using a Merck-Hitachi HPLC system as described above. The mobile phase was acetonitrile–water (60:40, v/v) and the flow rate was 1 ml/min. Paclitaxel and internal standard (freshly prepared N-octyl benzamide) were detected by monitoring absorbance at 227 nm.

2.7. Cell culture and binding studies

Human nasopharyngeal epidermal carcinoma KB cells (positive for FR-α) and colorectal carcinoma HT-29 cells (as negative control) were cultured and maintained in RPMI 1640 medium containing 10% fetal calf serum and 0.1% antibiotics, at 37 °C in a 5% CO_2 humidified atmosphere. In order to obtain cells overexpressing folic acid receptors, the KB cells were also grown in low-folic acid (3 nM instead of 2 μM) medium.

The relative affinity of HSA conjugates was determined by the method described by Westerhof et al. (1995) with some modification. KB cells (0.1×10^6) were seeded in 24-well Falcon plates and allowed to form adherent monolayers overnight in low-folic acid medium. 100 nM of ^{3}H-folic acid (0.5 μCi) in the presence or absence of increasing concentration of HSA conjugates or Folate-PEG derivative. The cells were incubated for 1 h at 37 °C then thoroughly washed in cold PBS, and lysed in 0.85 ml of buffer (PBS containing 1% Triton X-100). After 30 min, cell lysates were collected, transferred to individual vials containing 5 ml of scintillation cocktail, and then counted with a Packard-2500 TR Liquid Scintillation Analyzer. The experiments were repeated five times. The cells exposed to the ^{3}H-folic acid plus 1 mM unlabeled folic acid served as positive control. From all samples, both the radioactivity due to paclitaxel, evaluated before the test, and that measured in the positive controls, were subtracted.

2.8. Cytotoxicity tests

KB and HT-29 cells were cultured for 7 days in folate-free medium before any viability test was carried out. The cells were then seeded 3×10^4/well in microtiter plates and incubated for 16 or 4 h to allow cell adhesion. Various dilutions of paclitaxel and HSA conjugates (expressed as paclitaxel concentration), in the presence or absence of 1 mM free folic acid (as binding site competitor), were added in triplicate and incubated for 4 h. For the purpose of comparison with previous work, PEG-HSA–paclitaxel, containing 6 mol of drug and 2 mol of PEG 2000, was also tested and, as control comparison, albumin and folic acid were tested up to a concentration of 0.1 mM. The supernatants were removed and, after washing, the cells were chased in fresh medium for 48 h. Spent medium was aspirated and replaced with fresh medium containing 1 mCi of L-[4,5-^{3}H]-leucine (58 Ci/mmol). After a further 4 h at 37 °C incubation, cells were harvested using Nonidet P-40 (1‰) with a Skatron Harvester and the incorporated radioactivity was measured using a Packard-2500 TR Liquid Scintillation Analyzer. The results were expressed as percentage L-[4,5-^{3}H]-leucine incorporation, versus controls; background values were subtracted. Data are means of three experiments, in which each individual value is the average of triplicate samples (<7% standard error).

3. Results and discussion

3.1. Preparation and characterization of Folate-PEG -HSA–paclitaxel conjugate

Fig. 1 shows the general strategy of the synthesis. MH-PEG-Folate conjugate was prepared via a dicyclohexylcarbodiimide mediated coupling of FA to diamino PEG and, after purification by ion-exchange chromatography, further reaction with maleimidohexanoic acid *N*-succinimidyl ester.

Although carbodiimide-activated FA can couple with diamino PEG via either alpha- or gamma-carboxyl groups of its glutamate residue, it is known that only the alpha-conjugate is capable of binding to the FR; it is widely reported that 80–90% of FA polymer conjugates are linked through the gamma-carboxyl group (Gabizon et al., 1999; Cavallaro et al., 2006).

The folate was extended with PEG linker in order to allow maximum exposure of the targeting agent to the HSA surface; folate was also chosen for its known ability to shield the modified HSA, prolonging circulation time and thus increasing passive targeting through the enhanced permeability and retention (EPR) effect (Tanaka et al., 2004).

Human serum albumin has been used in many studies as delivery agent, and its thiol groups have frequently been used as drug linking point. Several reports describe linkage with the naturally available HSA Cys 34 thiol group (Leger et al., 2004; Shechter et al., 2005) to obtain a stable covalent bond between maleimido-derivatives of biologically active peptides or drugs (Wang et al., 2008; Unger et al., 2007).

But to achieve a dual purpose (transport of both the drug and the targeting agent) surface derivatization is required (Temming et al., 2006). Furthermore, in order to improve folate's availability in the in vitro binding study, it is necessary to link more than one folate per HSA molecule, taking in account the increased heterogeneity of the conjugates obtained. For this reason, Leamon and Low (1991) initially used 10 mol folate/HSA, but further reduced the amount to 4 mol, and used only 1–3 groups for momordin conjugates (Leamon et al., 1999). In view of these data, our goal was to insert 2–3 FA groups per HSA molecule. We also chose an approach in which only the amino lysine groups were used to link the drug, as in a previous report (Dosio et al., 2001), and for this reason, linkage with the targeting moiety was achieved using reduced disulphide bonds.

3.2. Interaction of HSA with Affi-gel Blue and its use for 'heterogeneous phase' system derivatization

Affi-gel Blue is an affinity gel composed by agarose derivatized with the monochlorotriazine dye Cibacron Blue F3G-A. It has been reported that the reactive dye Cibacron Blue 3GA modified adsorbent can bind specifically to dehydrogenases and kinases, as well as binding non-specifically to a wide range of serum albumins (Denizli and Piskin, 2001)

A linear gradient elution by increasing ionic strength has widely been employed in varying circumstances (Gianazza and Arnaud, 1982) because adsorption of most proteins decreases as their ionic strength increases; this type of interaction was used in previous

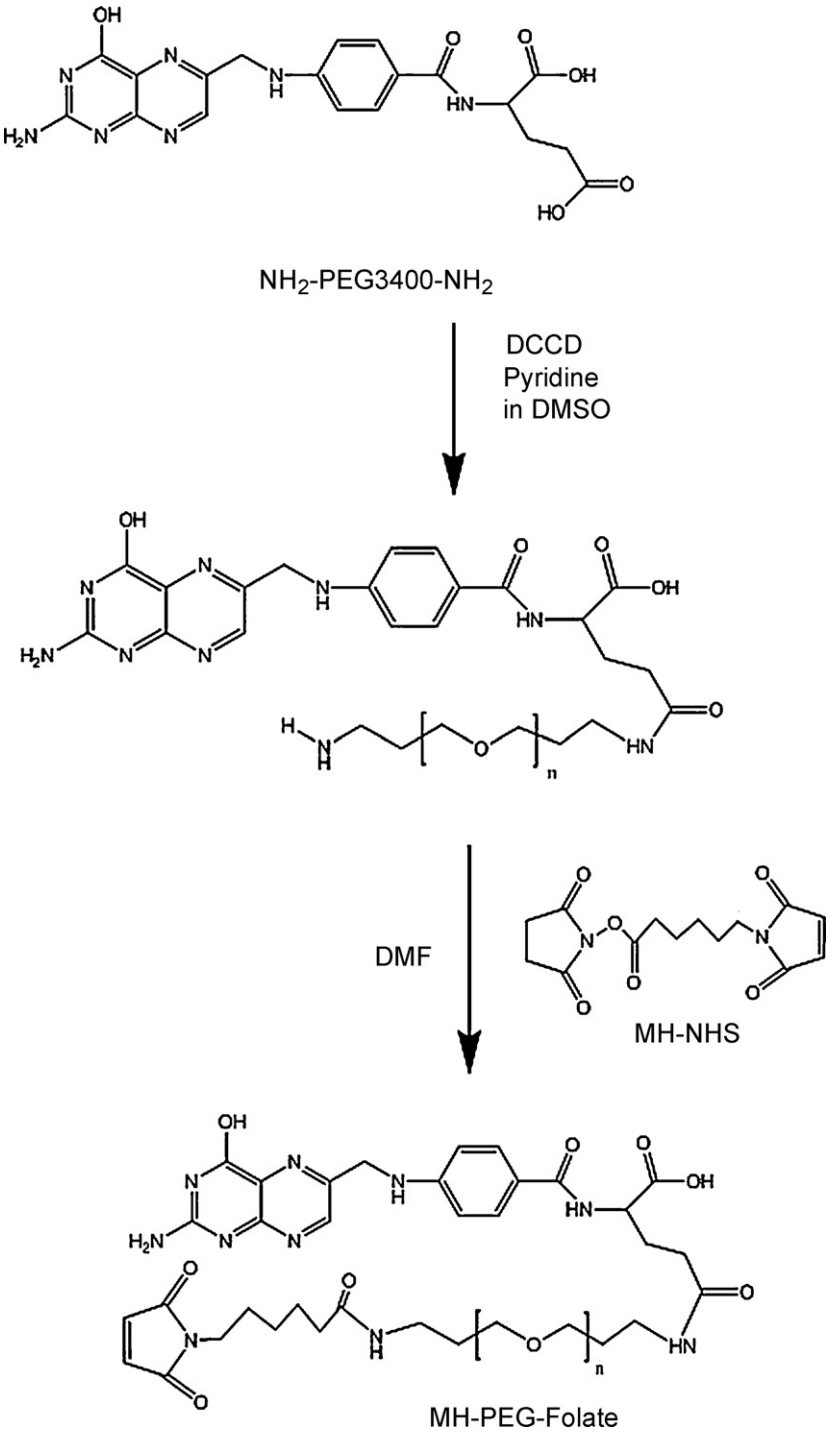

Fig. 1. Synthesis of Folate-PEG-maleimido-derivative.

works to prepare conjugates between adsorbed toxins and monoclonal antibodies (Dosio et al., 1993, 1994).

In this study the heterogeneous phase interaction was used to allow an easy reaction of HSA, previously derivatized with paclitaxel, both with reducible reagent and with the PEG reagent, removing the excess of all unreacted materials. Using serum albumin, a theoretical adsorption of up to 15 mg/ml with phosphate buffer at pH 7.6 has generally been reported, thus the reaction can be conducted in micro-device such as micro-centrifuge tubes, using high protein concentrations. The preparation scheme is shown in Fig. 2.

The covalent linkage with paclitaxel was achieved as reported elsewhere (Dosio et al., 2001), and a 35 M excess of 2′-sulpho-NHS-succinyl-paclitaxel was suitable to allow linkage of an average value of 5.5 ± 0.5 drug/HSA molecule.

The paclitaxel content was determined by comparison of results from DC Protein assay and radioactivity. Using HSA-bound paclitaxel, the binding ability to the dye phase decreased to 10.5 mg/ml at pH 7.6 after 30 min of adsorption.

The reducing agent dithiothreitol was the reactive of choice, and some parameters were tested to increase the reproducibility of the method: reaction time, DTT molar excess and pH value.

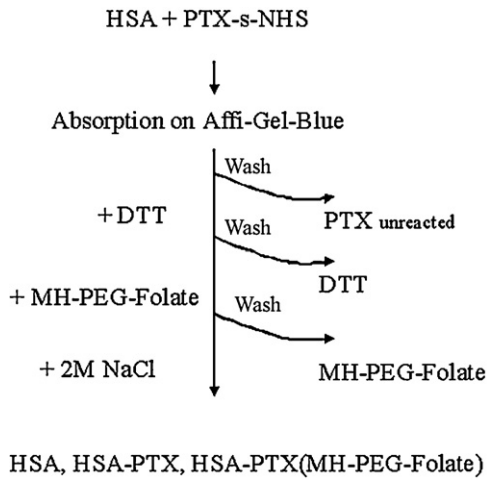

Fig. 2. Scheme of conjugate preparation using absorption on Affi-gel Blue. PTX-s-NHS is 2′-sulpho-NHS-succinyl-paclitaxel; HSA-PTX(MH-PEG-Folate) is the albumin–paclitaxel conjugate derivatized with a thioether linkage to PEG-Folate.

It is important to note that the reducing power of DTT is limited to pH values above ~7, since only the thiolate form is reactive (pK_a of thiol groups is ~8.3). On the other hand, adsorption is at its maximum at acidic pH, maintaining a plateau of around 80% for pH values up to 8.0 and a steep loss for pH values above 8.5. Thiol reduction is proportional to reaction time, and a stable value is obtained after 2.5 h.

With a DTT molar excesses of 30, 50, 70 or 100, the thiol groups exposed were 2.0, 3.8, 4.4 and 4.90 respectively. The mean recovery for reaction at pH 7.6 was around 78.5%. Based on these observations, a 50× excess was used in a typical preparation of targeted macromolecular paclitaxel. The results obtained show that the protein is protected by the Affi-gel matrix: the reduced thiol groups are fewer than 50% of those obtained through a solution reaction (Norez et al., 2008).

Linkage of the targeting moiety was easily achieved by addition of an excess of MH-PEG-FA obtaining a stable thioether linkage. After removal of unbound reactive by washing, the conjugate mixture was eluted from the gel by increasing the ionic strength. An HPLC separation based on gel filtration was necessary to remove most of the unmodified albumin and dimers. MALDI-TOF spectra of HSA–paclitaxel and of PEG conjugate exhibited a major peak centered on 71,902 Da for the former, and a bell-shaped distribution centered on 80,142 Da for FA-PEG-HSA–paclitaxel conjugate; these data indicate a mean of two Folate-PEG groups inserted per mol of HSA (Fig. 3). Different batches confirmed good reproducibility and a mean purity of 80%. SDS-PAGE, in non-denaturing conditions (Fig. 4), showed that the grafted species behaved with an apparent diffusion around the molecular weight value, this effect being due to the flexibility of PEG and the higher degree of hydration (Monfardini and Veronese, 1998). The presence of paclitaxel in the lanes was confirmed by radiochromatographic analyses of dry gel (Fig. 4).

The mean recovery of purified conjugate, after derivatization with MH-PEG-Folate, ranged between 8 and 10% (as albumin moles), starting from a batch of 10 mg of HSA. The instability of purified conjugate, measured as paclitaxel dialyzable radioactivity, did not exceed 10%/month under storage at 5 °C.

3.3. Stability studies

The release of paclitaxel from targeted and non-targeted conjugates was estimated both in aqueous buffered solution and after incubation with serum. As shown in Fig. 5, the stability of the conjugates under physiological conditions (pH 7.4 and 37 °C) was high, and after 72 h at least 95% of the paclitaxel was still linked to the carrier protein. After the same time interval in the presence of serum, release was 11.7% and 15.8%, for HSA–paclitaxel and folate-targeted compound, respectively. However, comparing the data obtained for the conjugates with or without PEG-folic acid, it may be suggested that the presence of the PEG/folate group in the macromolecular structure may increase the in vitro stability of the paclitaxel/HSA linkage. It is important to note that linkage of the promoiety to the 2′-position of paclitaxel is only feasible if the resulting bond is biologically stable and does not lead to premature drug release. Although in literature the 2′ ester linkage was reported to be labile (Safavy et al., 1999; Greenwald et al., 1996) the HSA seems to stabilize the ester linkage allowing a slow release in buffer and in plasma (Dosio et al., 1997).

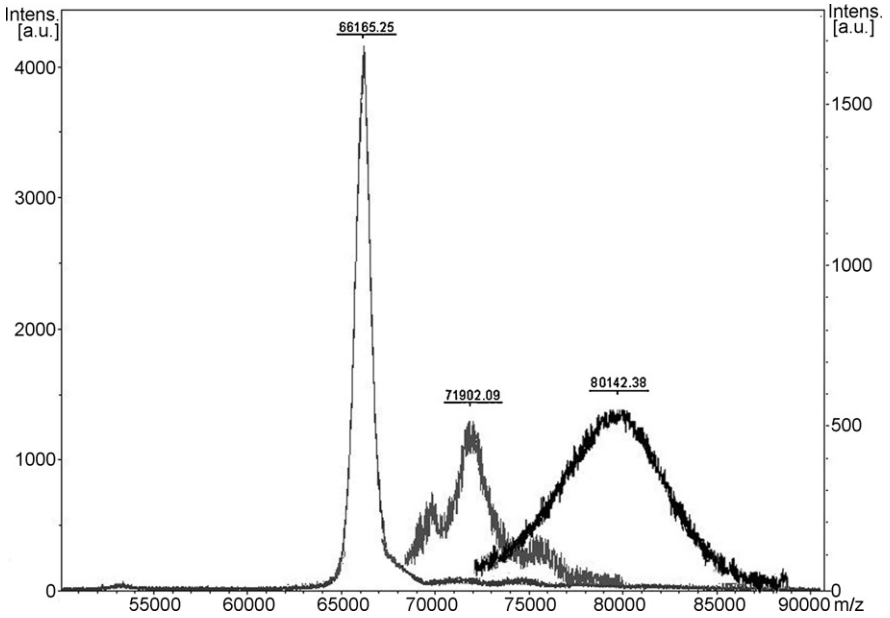

Fig. 3. MALDI-TOF was used to estimate molecular weight of conjugate HSA–paclitaxel (mean value 71,902 Da) and the Folate-PEG-HSA–paclitaxel conjugate (mean value 80,142 Da).

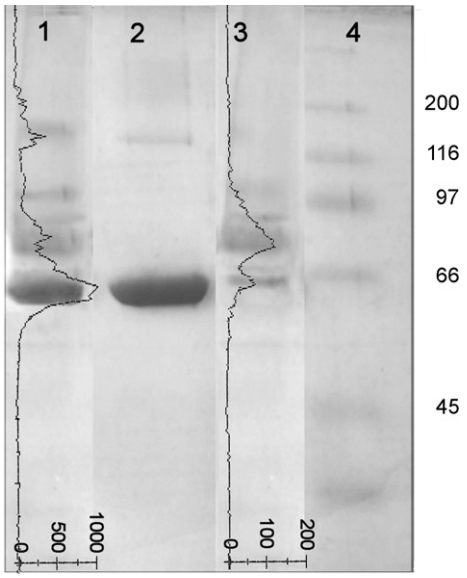

Fig. 4. SDS-PAGE in non-reducing conditions of the mixture of eluted from the Affigel Blue before (1) and after purification on TSK G-3000SW (3), HSA (2), molecular weight (expressed in kDa) markers (4). In lanes (1) and (3), the radioactivity scansion of the gel (scale expressed as counts per minute) has been superimposed. The radioactivity was due to [^{3}H]-labeled paclitaxel used in the synthesis.

3.4. Binding affinity of Folate-PEG-HSA conjugates to KB cells

Binding affinity of drug-targeting conjugates was determined in competition experiments with folic acid, as shown in Fig. 6. The conjugates displaced folic acid, the binding affinity of Folate-PEG-HSA being higher than that of Folate-PEG. Furthermore, a clear relationship was observed between the average number of folic acids/albumin and affinity for the target cells.

3.5. In vitro cytotoxic activity of paclitaxel-HSA conjugates

The cytotoxicity of conjugates was studied using human nasopharyngeal epidermal carcinoma KB cells, a cell line which overexpresses the folate receptor (Saikawa et al., 1995) and colorectal carcinoma HT-29 cells, lacking FR-α, as negative control (Gravier et al., 2008). Cell proliferation was evaluated after 4 h exposure to different concentrations of free drug or of conjugates. This time was selected because allows a complete binding and uptake of the folate and a possible recycling (Paulos et al., 2004). As shown in Fig. 7, paclitaxel's cell killing activity was found to be concentration-dependent, with an IC50 of 3 nM when cells were treated for 4 h and maintained for a further 48 h. Among the HSA conjugates, only the targeted one maintained the cytotoxic activity of free drug (5 nM)

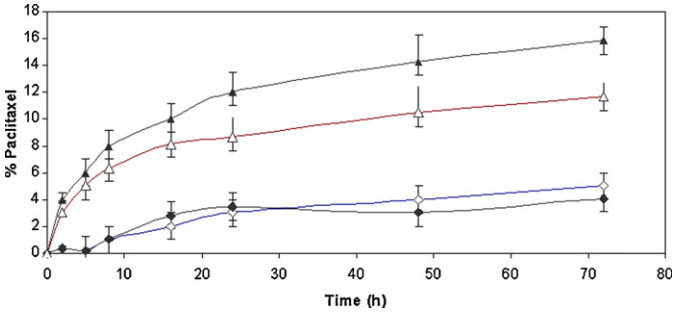

Fig. 5. Release of paclitaxel from HSA–paclitaxel (filled symbols) and from HSA-PTX(MH-PEG-Folate) (open symbols) conjugates at pH 7.4 (diamond) and in serum (triangle).

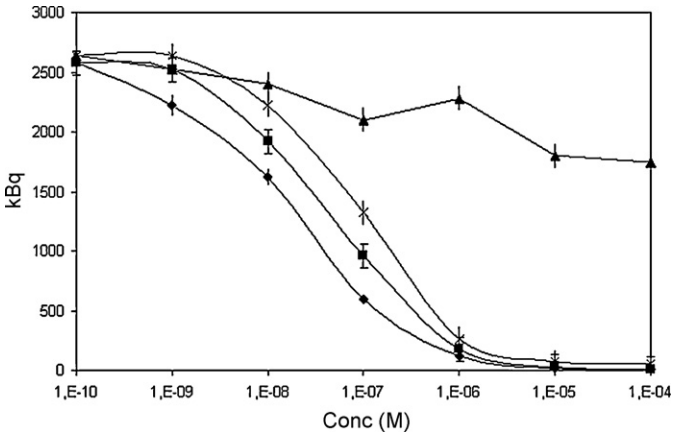

Fig. 6. Relative folate receptor binding affinity of conjugates. KB cells were incubated for 1 h at 37 °C with 100 nM ^{3}H-folic acid (♦) in the presence and absence of increasing competitor concentrations of HSA-PTX(MH-PEG-Folate) (■), PEG-Folate-MH (X) and HSA–paclitaxel (▲) (n = 5).

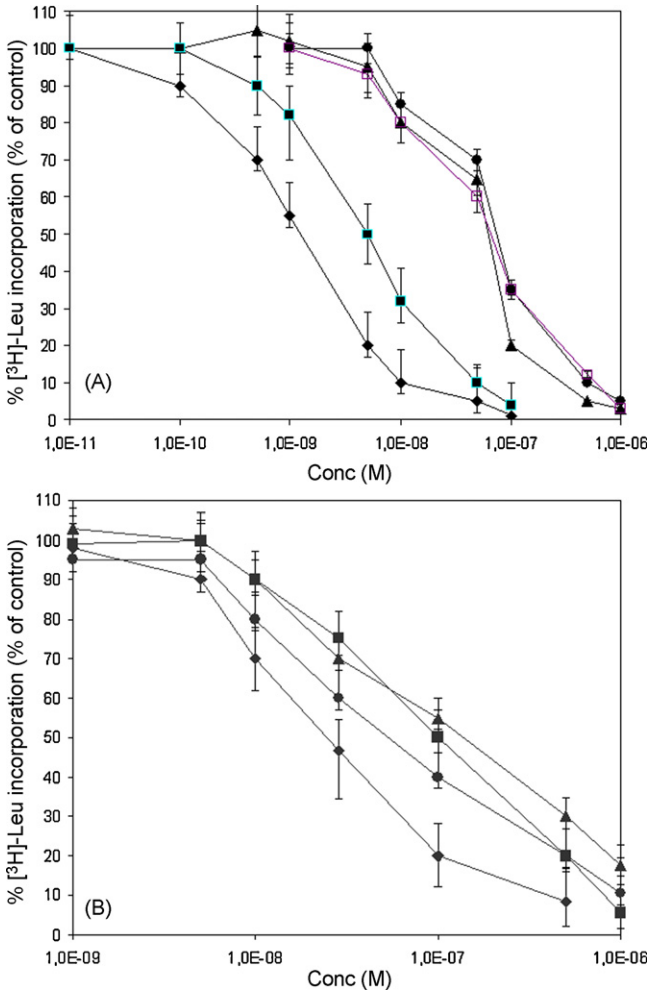

Fig. 7. Cytotoxicity test. KB cells, positive to folate receptor (panel A), and HT-29 cells, negative control (panel B), were pulsed for 4 h with various dilutions of paclitaxel (♦), HSA–paclitaxel (✹), HSA-PTX(MH-PEG-Folate) (■), PEG-HSA–paclitaxel (▲), and Folate-PEG-HSA–paclitaxel in the presence of 1 mM free folic acid (as binding site competitor for KB cell line) (□). The results are expressed as percentage L-[4,5-^{3}H]-leucine incorporation, versus controls; background values were subtracted. Data represent the average ± 1 S.D.

while the untargeted conjugates ranged between 70 and 95 nM. No significant differences between untargeted conjugates appeared and no activity showed free HSA or folic acid (not shown). The excess of free folic acid was found to block the activity of the targeted conjugate, which decreases by from 5 to 75 nM. This means that a short time is sufficient to achieve efficient binding, then, after rapid conjugate uptake, the paclitaxel is released by 2′ ester hydrolysis, allowing a cell intoxication.

In comparison, on HT-29 cells the activity of paclitaxel (27 nM) appeared lower than on KB cells but, more importantly, the highest activity (50 nM) of the conjugates was obtained with HSA–paclitaxel, while pegylated and targeted conjugates showed more reduced activity (100 and 120 nM respectively). HT-29 cells did not show any change in behavior after exposure to HSA or to folic acid (up to 0.1 mM) or on removal of folic acid from the medium.

4. Conclusion

The preparation of targeted conjugates using a method that reduce the number of purification steps, increasing the yield and uniformity of conjugate, is reported. The method, which is based on HSA bound on 'solid phase', may enable an automatic procedure to be developed. The folate targeting moiety is exposed through a PEG spacer that increases biodistribution of the conjugate, enabling binding and increasing specific toxicity. The conjugation was essential to the biological activity of the therapeutic system. The study shows that the targeting agent can enable binding, promote cell uptake and thus increase toxicity on the FR positive KB cell line, and that it appears to be specific. Further studies are warranted to investigate the potential therapeutic advantages of the FR-targeted HSA conjugate and determine possible mechanisms for in vivo tumor targeting.

Acknowledgements

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