

This is the peer reviewed version of the following article:

pH-Promoted Release of a Novel Anti-Tumour Peptide by “Stealth” Liposomes: Effect of Nanocarriers on the Drug Activity in Cis-Platinum Resistant Cancer Cells / Sacchetti, Francesca; Marverti, Gaetano; D’Arca, Domenico; Severi, Leda; Maretti, Eleonora; Iannuccelli, Valentina; Pacifico, Salvatore; Ponterini, Glauco; Costi, Maria Paola; Leo, Eliana. - In: PHARMACEUTICAL RESEARCH. - ISSN 0724-8741. - 35:11(2018), pp. 206-207. [10.1007/s11095-018-2489-z]

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

26/04/2026 03:31

(Article begins on next page)

pH-Promoted Release Of A Novel Anti-Tumour Peptide By “Stealth” Liposomes: Effect Of Nanocarriers On The Drug Activity In Cis-Platinum Resistant Cancer Cells

Running Title: Effect Of Liposomes On The Intracellular Drug Activity

*Francesca Sacchetti^a, Gaetano Marverti^b, Domenico D’Arca^b, Leda Severi^a, Eleonora Maretti^a,
Valentina Iannuccelli^a, Salvatore Pacifico^c, Glauco Ponterini^a, Maria P. Costi^{a1} and Eliana Leo^{a*1}.*

^aDept. Life Sciences, Via Campi 103, University of Modena and Reggio Emilia, 41125, Modena, Italy. ^bDept. Biomedical, Metabolic and Neurosciences, Via Campi 287, University of Modena and Reggio Emilia. ^cDept. of Pharmaceutical Sciences, University of Ferrara, via Fossato di Mortara 17-19, 44100 Ferrara, Italy

Abstract

Purpose: To evaluate the potential effects of PEGylated pH-sensitive liposomes on the intracellular activity of a new peptide recently characterized as a novel inhibitor of the human thymidylate synthase (hTS) over-expressed in many drug-resistant human cancer cell lines. **Methods:** Peptide-loaded pH-sensitive PEGylated (PpHL) and non-PEGylated liposomes (nPpHL) were carefully characterized and delivered to cis-platinum resistant ovarian cancer C13* cells; the influence of the PpHL on the drug intracellular activity was investigated by the Western Blot analysis of proteins involved in the pathway affected by hTS inhibition. **Results:** Although PpHL and nPpHL showed different sizes, surface hydrophilicities and serum stabilities, both carriers entrapped the drug efficiently and stably demonstrating a pH dependent release; moreover, the different behavior against J774 macrophage cells confirmed the ability of PEGylation in protecting liposomes from the reticuloendothelial system. Comparable effects were instead observed against C13* cells and biochemical data by immunoblot analysis indicated that PEGylated pH-sensitive liposomes do not modify the proteomic profile of the cells, fully preserving the activity of the biomolecule. **Conclusion:** PpHL can be considered as efficient delivery systems for the new promising anti-cancer peptide.

¹ These Authors contributed equally to this work

*Corresponding Author: Eliana Leo, PhD; Office phone: + 39 59 205 8558;
e-mail: eliana.leo@unimore.it

Abbreviations

hTS: human thymidylate synthase

PpHL: PEGylated peptide-loaded pH-sensitive liposomes

nPpHL: non-PEGylated peptide-loaded pH-sensitive liposomes

EPR: enhanced permeation and retention

RES: reticuloendothelial system

PEG poly-ethylene-glycol

PBS phosphate buffer solution

[D-Gln⁴]LR: new synthesized octapeptide (amino acid sequence: LSCQLYQR) with inverted chirality at position 4 (corresponding to the amino acid glutamine) (LSCqLYQR)

TRAP1, Tumour Necrosis Factor Receptor Associated Protein 1.

HSP 90-alpha: heat shock protein (HSP90AA1)

DHFR dihydrofolate reductase

Introduction

Liposomes are highly biocompatible and biodegradable drug carriers that can protect entrapped compounds from chemical or enzymatic degradation and enhance the drug permeation across membranes [1]. However, adsorption of serum proteins (opsonins) onto the liposomal surface plays a critical role in the liposome clearance from blood [2]. The opsonized liposomes are recognized by the mononuclear phagocytic system, known as the reticuloendothelial system (RES), and are thus removed from circulation [3].

To overcome this drawback, a change in the surface properties of the particles may be useful. Because a hydrophilic coating is necessary for a successful intravenous administration, binding poly-ethylene-glycol (PEG) chains to the particle surface is the most used strategy to preserve vesicles from rapid clearance by the RES [4]. In fact, PEG creates a hydration envelope on the liposomal surface where

its hydrophilic chains attract water molecules; this prevents both the interaction of liposomes with serum components ("stealth" effect) and, due to steric hindrance, the fusion and aggregation with other liposomes, with an efficacy related to the density of the polymer on the particle surface [5,6]. The prolonged circulation enables a "passive" accumulation of liposomes in the tumor tissue, thus promoting enhanced permeation and retention (EPR) based on spontaneous penetration of circulating particulate drug carriers into the *interstitium* of solid tumors [7]. However, although characterized by long blood circulation, the PEGylated liposomes must feature efficient tumor penetration, cellular uptake and endosomal escape in order to passively target tumor tissues with good efficiency and specificity. A high degree of PEGylation screens the surface charge of liposomes and hence facilitates tumor penetration but it hinders their uptake by targeted tumor cells [8]. Indeed, the drawback of PEGylation is a decrease in cellular uptake and endosomal escape that results in a decrease in the efficiency of the delivery systems. These issues are currently referred to as the "PEG dilemma" [9]. An additional issue related with the delivery of an anti-cancer drug by PEGylated liposomes is the possible interference of the PEGylated liposome components with the intracellular mechanism of action of the drug: to our knowledge, this possibility remains unexplored.

To address such issues, we have encapsulated the octapeptide [D-Gln⁴]LR, LSCqLYQR, into pH-sensitive PEGylated and non-PEGylated liposomes (further on quoted as PpHL and nPpHL, respectively) and have delivered it to cisplatin-resistant cancer cells. (LSCqLYQR) is an analogue of the LR (LSCQLYQR) peptide used in previous work [10], with the same aminoacidic sequence but with inverted chirality at glutamine 4. The anti-proliferative activity of these peptides and their inhibition of human thymidylate synthase (hTS), a validated anti-cancer target, by an innovative mechanism of action have been recently investigated [11,12]. In order to deliver the peptide to the cytosol, we have designed pH-sensitive liposomes and have characterized their surface hydrophilicity, stability in serum, interaction with macrophages, drug encapsulation efficiency and drug release. Moreover, the cytotoxicities of the peptide-loaded PpHL/nPpHL have been assessed on a cisplatin-resistant cell line. Finally, to test the impact of the employed PEGylated liposomes on the

drug's intracellular mechanism of action, we performed a western blot analysis of four proteins, namely, thymidylate synthase itself, the TNF receptor associated protein 1 (TRAP1), the heat shock protein HSP 90-alpha (HSP90AA1) and dihydrofolate reductase (DHFR). These proteins are involved in the pathways affected by these peptidic hTS inhibitors, and their differential expression modulation pattern was closely associated with their intracellular mechanism of action [13].

2. Materials and method

2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), [N-(Carbonyl-methoxypolyethylene glycol-2000)]-1,2-distearoyl-sn-glycero-3-phospho-ethanolamine sodium salt (DSPE_PEG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE) were purchased from Lipoid (Ludwigshafen, Germany), while cholesteryl hemisuccinate (CHEMS) from Sigma Aldrich (USA). The fluorescent 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) (ammonium salt) used for the macrophage internalization assay was obtained from Avanti Lipids (Alabaster, AL, USA). The active [D-Gln⁴]LR peptide along with the internal standard (IS) LR-Ala7, used for quantitative analysis, were synthesized as previously described in [12]. HEPES was obtained from Alfa Aesar, (Karlsruhe, Germany), while NaCl and Phosphate Buffer Saline (PBS) containing KH₂PO₄/Na₂HPO₄ buffers at different pH value (7.4, 6.5, 5.5, 4.0) were purchased from Carlo Erba Reagenti s.p.a., (Milan, Italy). Cell culture reagents were purchased from EuroClone (Milan, Italy), except otherwise indicated. All other reagents were of analytical grade.

2.2 Liposome formulation

Liposomes were prepared using the reverse phase evaporation (REV) technique followed by homogenization as described previously [10]. Briefly, phospholipid solutions in chloroform at fixed concentration, DOPE:CHEMS:DSPE (22.8: 15.2: 2mM) for nPpHL liposomes and

DOPE:CHEMS:DSPE_PEG (22.8: 15.2: 2 mM) for PpHL liposomes, were placed into a glass flask and the solvent was removed under vacuum. The phospholipid thin film was re-dissolved in diethyl ether and mixed with HEPES Buffer Saline (HBS) medium (20 mM HEPES, 135 mMNaCl) containing or not 2 mg of the [D-Gln⁴]LR peptide (theoretical loading) to a final diethyl ether:water phase ratio of 3:1, obtaining 5 mL of final suspensions. After diethyl ether evaporation, the suspension was homogenized by Ultraturrax (Ika-euroturax T 25 basic, IkaLabortechnik, Staufen, Germany) for 3 minutes at 24,000 rpm. The liposome formulation was dialyzed for 2 h (Dialysis Tubing - Visking MWCO-12-14000 Daltons, Medicell International Ltd, London) versus HBS buffer and stored at 4°C in vials. For macrophage internalization studies, the composition of liposomes included the fluorescent 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) at 0.2 molar % of total phospholipids, obtaining Rhod_PpHL and Rhod_nPpHL with PEGylated and non-PEGylated liposomes, respectively [14,15].

2.3 Size and morphology analysis

The liposome size and surface charge were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS analyzer system (Zetasizer version 6.12; Malvern Instruments, Worcs, U.K.) equipped with a 4 mW He–Ne laser (633 nm) and DTS software (Version 5.0). Measurements were performed in triplicate and each measurement was averaged over at least 12 runs. The morphology of liposomes was observed by AFM (Atomic Force Microscope, Park Instruments, Sunnyvale, CA, USA). The experiments were performed in water at room temperature at atmospheric pressure operating in the non-contact mode, depositing liposomes onto a small mica disk. The final pictures were obtained processing the topographical AFM images with the ProScan Data Acquisition software and Gwyddion software.

2.4 Surface hydrophobicity measured by the Rose Bengal test

Liposomes surface hydrophobicity was determined by the Rose Bengal test as described in refs. [16,17] with some modifications. Briefly, to a sequence of PpHL and nPpHL suspensions with

increasing concentrations (from 0.1 to 0.7 mg/mL at steps of 0.2 mg/mL), an aliquot of dye solution was added obtaining a concentration of 6 µg/mL and a final volume of 1.5 mL. After 3h of incubation in dark conditions at room temperature, the resulting solutions were centrifuged at 20,000 g at 3°C for 45 minutes (Rotina 380R, Hettich, Germany) and the supernatants were spectrophotometrically analyzed at 548 nm (Lambda 3B Perkin-Elmer, Waltham, USA). The recorded absorbance was corrected by subtracting the absorbance of the supernatants of the liposome suspensions, at the same concentration but without dye, centrifuged in the same conditions. A partitioning quotient (PQ), defined as the ratio of the Rose Bengal amount bound on the surface to that in the dispersion medium, was calculated and plotted as a function of the liposome concentration. The slopes denoted the degree of surface hydrophobicity. Each measurement was performed in triplicate and two different preparations for each sample were used.

2.5 Stability in serum

In order to evaluate the stability of the liposomes in serum, their size was monitored in 100% FBS at 37°C. Briefly, 500 µL of the nPpHL and PpHL liposomal formulations were added to 1 mL of FBS solution. At incubation times of 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h, aliquots of the suspensions were diluted with MilliQ water (1/10 v/v) and size, PDI and Z-Potential were measured by the DLS analysis (Malvern Z-Sizer, Zetasizer version 6.12, Malvern Instruments, Worcs, U.K). Each measurement was performed in triplicate and two different preparations for each sample were used.

2.6 Macrophage cell culture

Cells of the murine macrophage J774 line, from IZSLER (Brescia, Italy) (number of passages 25), were cultured as a monolayer in Dulbecco's modified Eagle essential medium (DMEM) High Glucose complemented with 2mM GlutaMAX Supplement, 100 UI/mL penicillin, 100 µg/mL streptomycin, 1% Non-Essential Amino Acid (NEAA) and 10% Fetal Bovine Serum (FBS) at 37 °C in humidified 5% CO₂ atmosphere. Cells were sub-cultured when the confluence was ≥80%.

2.7 Cytofluorimetric analysis of internalization by macrophages

J774 cells were seeded at 400,000 cells/well in 6-well plates and incubated in complete medium at 37 °C in 5% CO₂ for 24 h. Cells were incubated for 0.25, 0.5, 2, 4, 8 and 24 h with nPpHL and PpHL labeled or not with rhodamine diluted in complete DMEM to a final concentration of 0.125 mg/mL. After incubation, cells were washed three times with PBS, collected by gently scraping, centrifuged at 188 g for 5 minutes, and the resulting pellet was re-suspend with 1mL of PBS for flow cytometric analysis. Flow-cytometry evaluation of intracellular uptake was performed by a COULTER® EPICS® XL™ (Beckman Coulter Inc., 250 S Kraemer Blvd Brea, CA, 92821 United States) flow cytometer equipped with a 488 nm argon laser. Analyses were done after recording at least 10,000 events for each sample. Results were expressed as percentages of rhodamine-positive cells compared to non – rhodamine stained cells used as basal control. Data were analyzed using EXPO 32 ADC Analysis (Advanced Cytometry Systems). For confocal microscopy analysis, J774 cells were seeded on BD Falcon™ Culture Slides (Fisher-Scientific) at a density of 50,000 cells/well. After 24 h, the cells were incubated for 0.25, 2, 8 and 24 h with nPpHL and PpHL labeled or not with rhodamine at 0.125 mg/mL. Then the cells were washed three times with PBS and fixed in paraformaldehyde (3%, w/v) for 30 minutes at room temperature, re-washed with PBS and subjected to cell nucleus staining by Hoechst 33342 stain (blue) (2 µg/mL) (Frankfurt, Germany) for 10 minutes at room temperature. After a further PBS washing, cells were observed by confocal laser scanning microscopy (DMIRE2, Leica Microsystems GmbH, Wetzlar, Germany).

2.8 [D-Gln⁴]LR loading and encapsulation efficiency

To evaluate the encapsulation efficiency (EE%) and the drug loading (DL), liposomes were disrupted and the drug quantified by liquid chromatography coupled with triple-quadrupole mass spectrometry (LC-MS/MS). Because of the structural similarity, the quantitative determination of the [D-Gln⁴]LR peptide was performed with the method set up previously for the LR (LSCQLYQR) peptide [10].

Drug loading (DL), expressed as the amount of drug (μg) encapsulated in 1 mg of liposomes, was calculated by the following equation (Eq. 1)

$$\text{DL (w/w)} = \text{W loaded [D-Gln}^4\text{]LR peptide} / \text{W total lipids} \quad (\text{Eq. 1})$$

where “W loaded [D-Gln⁴]LR peptide” is the weight of the peptide encapsulated while “W total weight of lipids” is the total amount of lipids used in the formulation.

The encapsulation efficiency (EE%), which is a measure of the percentage of the peptide entrapped within the liposome as a function of the total quantity operated, was calculated by the following equation (Eq. 2).

$$\text{EE (\%)} = \text{Amount of encapsulated [D-Gln}^4\text{]LR peptide} / \text{total amount of [D-Gln}^4\text{]LR peptide} \times 100$$

(Eq. 2)

where the “amount of encapsulated [D-Gln⁴]LR peptide” is the amount of [D-Gln⁴]LR peptide loaded into liposomes and the “total amount of [D-Gln⁴]LR peptide” is the initial amount of [D-Gln⁴]LR peptide used in the formulation.

Briefly, 300 μL of aqueous liposome suspension were spiked with 300 μL of acetonitrile and 700 μL of trichloromethane (organic phase), and ultrapure water (aqueous phase) to yield a final organic:water phase ratio of 1:3. Then, two cycles of vortex-mixing (60 s) and centrifugation (12,000 g for 15 minutes at 20°C) (Rotina 380R, Hettich, Germany) were carried out to perform a liquid/liquid extraction of the peptide from the lipid matrix. The aqueous solution was analyzed according to the method previously reported [10].

2.9 [D-Gln⁴]LR release from liposomes

The release rate of [D-Gln⁴]LR peptide from nPpHL and PpHL was analyzed in PBS medium (pH 7.4). Briefly 800 µL of liposome suspension placed in a semipermeable membrane (Dialysis Tubing - Visking MWCO-12-14000 Daltons, Medicell International Ltd, London) was immersed in 30 mL of PBS. The system was maintained at 37°C under suitable magnetic stirring to maintain dialysis bag immersed (900 r.p.m., Heating magnetic stirrer, Velp Scientifica) and 80 µL of PBS medium were withdrawn at fixed time intervals (0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 24 h) and kept at -20°C until further LC-MS/MS analysis. Two aliquots were analyzed for each time point and the study was performed in triplicate.

2.10 [D-Gln⁴]LR leakage from liposomes at acidic pH

The peptide leakage from PEGylated or non-PEGylated [D-Gln⁴]LR-loaded liposomes was measured in different acidic buffers, in order to correlate the release of the peptide with the pH sensitivity of the liposomes. Liposomes were incubated with KH₂PO₄/Na₂HPO₄ buffer at pH 7.4, 6.5, 5.5, 4.0 (1:5 dilution of liposome suspension with buffer) at 37°C under stirring for 15 minutes. After incubation, the samples were centrifuged for 90 minutes at 24,000 g at 4°C, the supernatants were collected and liquid/liquid extraction was performed prior to LC-MS/MS quantification. Two aliquots were analyzed for each pH value and the study was performed in triplicate.

2.11 Cell culture and MTT cytotoxicity assay

The activity of [D-Gln⁴]LR-loaded liposomes was evaluated on C13* cell line. C13* is the cisplatin-resistant cell line clone of the 2008 cells, derived from a human ovary carcinoma [18]. This cell line was developed by monthly exposure to cisplatin, followed by chronic exposure to stepwise increased cisplatin concentrations [19]. The cell line (number of passages 35) was cultured as a monolayer in RPMI-1640 containing stable glutamine complemented with 10% heat-inactivated fetal bovine serum, penicillin 100 UI/mL and streptomycin 100 µg/mL at 37°C in a humidified 5% CO₂

atmosphere. The cisplatin-resistant human ovarian carcinoma C13* cells express elevated hTS levels [20] which is relevant for studying the hTS inhibitor [D-Gln⁴]LR peptide

The cytotoxicity assay was performed by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) assay (MTT assay). Briefly, cells were seeded at a density of 30,000 cells/well in 24-well plate in complete RPMI-1640 medium for at least 24 h. Immediately prior to cell treatment, the culture medium was aspirated from each well and cells were treated for 15 h (overnight) with 500 µL of complete medium, containing [D-Gln⁴]LR-loaded or -unloaded PEGylated or non-PEGylated liposomes at concentrations of 0.05, 0.075, 0.125 and 0.250 mg/mL. Then, cells were washed with PBS, added with 500 µL of complete RPMI-1640 medium and incubated for additional 48 h. After this additional time, the MTT test was performed to assess cell viability with a multiplate reader (TecanGenios Pro with Magellan 6 software) at 535 nm wavelength. The results are expressed as percentage of cell growth with respect to the control (untreated cells) and performed in triplicate.

2.12 Protein extraction and western blot analysis

C13* cells were seeded at 300,000 cells/well in a 6-well plate in complete RPMI medium for 24h to allow complete cellular adhesion. Then cells were incubated with liposomes, the SAINT-PhD peptide-delivery system (Synvolux Therapeutics, NL) and 5-fluorouracyl (5-FU) (Sigma Aldrich, Milan), used as a reference drug, each according to its treatment protocol. SAINT-PhD is a surfactant molecule bearing a cationic pyridinium head and a lipid chain. The cationic surfaces of the vesicles formed by this surfactant have a high affinity for the negatively charged cell surface. The peptide is then released from the lipid chains of the molecule into the cytoplasm. This delivery system did not alter cell growth when used at low concentrations. In the case of liposomes, both [D-Gln⁴]LR-loaded and -unloaded liposomes (the latter used as a control) were incubated at 0.25 mg/mL concentration for 15 h. The medium was then withdrawn and replaced with fresh one, maintaining the cell culture for 48 h before cell lysis for protein extraction. In the case of the SAINT-PhD delivery system, the treatment was performed according to the standard transfection protocol (see supplementary

material). For each treatment, peptide/SAINT-PhD complexes were prepared by diluting the appropriate amount of peptide to reach the concentration of 5 μM in 120 μL of HBS, and then, 80 μL of SAINT-PhD was added; the mixture was incubated for 5 minutes at room temperature and then added with medium up to 500 μL . The culture medium was aspirated from the cells and the SAINT-PhD/peptide complex was added to the wells, and incubated for 4 h. After this time, complete RPMI was added for maintaining the cell culture for 48 h before performing the experiments. A solution of the delivery system at the same concentration was used as the control treatment. A 5 mM stock solution was obtained by solubilizing the drug in saline solution (0.9% NaCl) and then stored at -20°C before use. The drug was diluted to a 5 μM final concentration in complete RPMI medium and was incubated for 48 h with the cells. Cells treated with saline solution were used as the control. For protein extraction, cells were washed twice in ice-cold PBS, lysed in RIPA, a radioimmuno precipitation assay, buffer (composed of 20 mM TRIS-HCl, pH 7.5; 150 mM NaCl; 1 mM Na_2EDTA ; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 1 mM Na_3VO_4 ; 1 mM PMSF; Complete Mini Protease inhibitor cocktail (Roche) and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma) and then centrifuged at 14,000 g at 4°C for 30 minutes to remove debris. The protein concentration in each lysate was determined using the Bradford protein assay reagent (Sigma-Aldrich). 80 μg of the cell extracts was subsequently loaded on a polyacrylamide gel after denaturation according to the method of Laemmli [21]. The membranes were blocked in non-fat dry milk (2% and 5%) in TBS-T buffer (Tris Buffer Saline containing 0.1% Tween-20) at room temperature for 1 h. Primary antibodies were incubated overnight in non-fat dry milk (2% and 5%) in TBS buffer containing 0.1% Tween-20. The following antibodies were used: anti-DHFR (clone A-4, Santa Cruz Biotechnology, 1:250 dilution), anti-TS (clone TS106, Abnova, 1:250 dilution), anti-HSP90AA1 (clone 4F10, Abnova, 1:5000 dilution), anti-TRAP1 (clone TR-1A, Santa Cruz Biotech, 1:500 dilution), and anti- β -actin (Santa Cruz Biotechnology, Inc., 1:1500 dilution). After washing, membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Biosciences, 1:5000 dilution) for 1 h at room temperature in TBS-T with 5% dry milk. Antibody staining was

performed with a chemiluminescent detection system (ECL Plus, Amersham), and the signal was detected with X-ray film (Amersham). Densitometric quantification of the obtained western blot bands was performed using an Epson Photo Perfection 4180 scanner (Epson) and ImageJ densitometry software (1.48 version, NIH, USA). For each sample, we acquired densitometric values of three replicates; the latter are expressed in arbitrary units.

2.13 Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA). The data are represented as means \pm SD. Differences were considered statistically significant at p-values less than 0.05 (*p<0.05; **p<0.01; ***p<0.005).

3. Results

3.1 Size and morphology analysis

Sizes, polydispersity indexes (PDI) and Z-potentials of the unloaded PpHL and nPpHL were measured in water by DLS and reported in Table 1.

The PDI value of non-PEGylated liposomes was higher than 0.3 indicating a not good enough size uniformity probably due to the method used for the final size homogenization of liposomes (Ultraturrax). However, the PDI value as well as the size of the vesicles decreases from 280 to 190 nm with the use of DSPE_PEG and this occurrence may be related to a decrease in the interfacial tension with respect to non-PEGylated heads, as discussed more in detail below. Moreover, also a decrease in the surface Z potential from - 52.1 to - 14.54 mV was observed for the PpHL with respect to the non-PEGylated homologue formulation.

AFM topographical images of PpHL (Fig. 1a) show overall size of the particles in good agreement with the DLS values (Table 1).

The above diameters and simple geometric considerations, combined with the approximation that the hydrophobic chain(s) of a lipid in the liposome has a length of 2 nm and occupy an average volume of 1 nm³ [22], allow us to estimate average numbers of lipid molecules per liposome of 5 x 10⁵ and 1.1 x 10⁶ for PEGylated and non-PEGylated liposomes, respectively.

3.2 Liposome surface characterization

The use of DSPE_PEG rather than DSPE in the liposome formulation is expected to produce, among other changes in the surface properties, an increase in hydrophilicity that is responsible for the escape of these particles from the RES. In order to evaluate the surface hydrophilicity of our liposomes, we employed an adapted version of the Rose Bengal test [16]. According to the method, a partitioning quotient (PQ), defined as the ratio of the amount of Rose Bengal bound on the surface to the amount in the dispersion medium, was measured and plotted as a function of the liposome concentration (Fig.S1). The decrease in the slope obtained with PpHL relative to nPpHL (1.9 vs. 2.6) confirms the expected higher surface hydrophilicity of the former and indicates that the PEG chains tie at the external surface of the liposomes, producing a hydrophilic coating of the particle surface that is expected to promote elusion of adsorption of plasma protein (opsonines) in the bloodstream [23]. To test this, we monitored the dimensional stabilities of PpHL and nPpHL suspended in complete fetal bovine serum (FBS) *in vitro*. Size (Z-Average) and zeta potential of serum suspensions of liposomes were evaluated at different times, up to 72 h from suspension (Fig.1b). In the first few minutes, nPpHL underwent doubling of the average size, from 200 to 400 nm, a value that remained constant within error until the end of the experiment. In parallel, the zeta potential became less negative in the first minutes, denoting either a partial neutralization of the particle surface or dielectric screening of the surface charge. On the contrary, PpHL maintained almost the same size and zeta potential during the first 8 hours, and subsequently showed only slight modifications, less than 10% of the initial value, clearly indicating weak interactions with the serum proteins.

3.3 Cytofluorimetric and confocal analysis of internalization by macrophages

For the macrophage uptake experiments, we employed the J774 cell line, a validated *in vitro* model for predicting the kinetics of blood clearance of colloidal drug delivery systems [15,24]. Before the macrophage internalization test, the two types of liposomes were assayed for their cytotoxicity against J774 cells by the MTT test and the results revealed no alteration of cell viability (*data not shown*). Unlabeled nPpHL and PpHL liposomes, both used as negative controls, exhibited a negligible fluorescence emission intensity. The results of flow-cytometry experiments performed using rhodamine-labeled liposomes (Rhod_PpHL and Rhod_nPpHL), expressed as percentages of positive cell counts, are shown versus the incubation time in Fig. 2. Internalization in J774 cells occurred with largely differing efficiencies and in different time scales for the two types of liposomes. nPpHL were already significantly internalized after only 15 minutes, in accordance with reported data [25], and showed the highest internalization level after 8h when 45% of the cells showed rhodamine fluorescence. On the contrary, PpHL were poorly internalized, with only 5% of positive cells detected after 24h of incubation. A confocal-microscopy analysis confirmed this result (Fig. 2). Since unlabeled liposomes revealed a negligible fluorescence in the experimental conditions employed (*images not reported*), red spots unequivocally identify labeled liposomes. The non-PEGylated liposomes approach the cell membrane already after 15 minutes and are internalized shortly afterwards. On the other hand, with PEGylated liposomes the fluorescence intensity within cells was much smaller. The maximum fluorescence was observed at 24 h of incubation while at 8 h of incubation a slight adsorption onto the cell membranes was visible for few cells. Overall, these findings prove that the amount of PEGylated phospholipid used in the liposome composition was suitable for avoiding a fast macrophage uptake.

3.4 Influence of PEGylation on [DGLn⁴]LR loading and release

The micrograms of peptide internalized per milligram of liposome (drug loading, DL) and the percentage of peptide encapsulated (encapsulation efficiency, EE) obtained in loading experiments are reported in Table 2. Use of DSPE_PEG, instead of DSPE, in liposomes did not affect drug loading as this turned out to be $11.73 \pm 2.98 \mu\text{g}/\text{mg}$ for PpHL and $12.06 \pm 0.65 \mu\text{g}/\text{mg}$ for nPpHL,

corresponding to EE values of 36-38%. Drug loading caused only a minor relative increase, 4%, in the vesicle average diameter, corresponding to +12 and +8 nm for nPpHL and PpHL, respectively. In addition, drug loading did not alter the zeta potentials. From these DL values and the average numbers of lipid molecules per liposome deduced in paragraph 3.1, we can estimate the average number of peptide molecules internalized in each liposome. We calculate an average molecular mass of the lipid mixture employed (57% DOPE, 38% CHEMS and 5% DSPE-PEG) of 749 a.m.u., *i.e.*, 1.25×10^{-21} g, and obtain average liposome masses of 6.2×10^{-16} and 1.4×10^{-15} g for PpHL and nPpHL, respectively. From the DL values of 12 μ g of peptide per mg of liposome, we easily estimate that 4×10^3 and 1×10^4 peptide molecules are encapsulated into each PEGylated and non-PEGylated liposome, respectively. Based on the previous numbers, we estimate that the equilibrated liposomes host about 1 peptide molecule per 100 lipid molecules, or per 50 tail-to-tail lipid chain pairs in the double layer, almost irrespective of PEGylation. However, being the peptide extremely target-specific, this relatively low EE should not represent a problem regarding its ability to exert a therapeutic action.

To verify the stability of the encapsulated peptide, [D-Gln⁴]LR_PpHL and [D-Gln⁴]LR_nPpHL samples were incubated at 37°C at pH 7.4 in phosphate buffer for 24h. As reported in Fig. 3a, the release profiles of the two samples are similar. Both ones show a small initial release of peptide, 14% and 11% for the non-PEGylated and PEGylated samples, respectively, attributable to drug molecules weakly adsorbed at the outer surface of the liposomes. These data suggest that the remaining portion of the drug was stably incorporated into the liposome vesicles, in keeping with a hypothesis of insertion of the prevalently hydrophobic N-terminus region of the peptide into the hydrophobic-tail double layer, and could therefore reach the target site.

Another serious issue of liposome PEGylation is the possible stabilization of the lipid envelope resulting in a decrease in its pH sensitivity and a resultant poor endosomal escape. In order to test this possibility, we assessed the ability of the liposomes to leak the drug when incubated at increasingly acidic pH values, corresponding to those of the endosome maturation, namely, pH 7.5, 6.5, 5.5 and

4. At the same time, we monitored the size of the liposomes to evaluate the modification of the lipid envelope. The results reported in Fig. 3b show that incubation of [D-Gln⁴]LR_PpHL at acidic pH values leads to a rapid and marked increase in the average diameter, from 186 (pH 7.4) to 466 nm (pH 5.5), a finding likely correlated with a destabilization of the liposome architecture. Macroscopically, a well visible increase in the turbidity of the systems indicated a collapse of the liposomes. Simultaneously, a considerable loss of drug was observed at pH 5.5 that further increased at pH 4, confirming the strong destabilization of the liposomal membrane. Because non-PEGylated liposomes had shown a very similar behavior (see [10]), we conclude that use of DSPE_PEG in the formulation does not substantially modify pH sensitivity and expect PEGylated liposomes to escape lysosome degradation and release the encapsulated peptide at suitably low pH values within cells.

3.5 Cytotoxicity of liposome-delivered [D-Gln⁴]LR on C13* ovarian cancer cells

The cytotoxicity assays were performed on C13* cells, an ovarian cancer cell line resistant to cisplatin against which it has activated several defense mechanisms including over-production of hTS. First, we checked the cytotoxicity of the naked peptide. Fig. 4A shows the survival data of C13* cells obtained at increasing concentrations of the naked peptide incubated with the cells under the same conditions used in the experiments with the liposomes. The data showed no differences in cell growth compared to control, confirming the inability of the peptide itself to enter the cells [11].

In order to assess the cytotoxic effect of the peptide delivered by pH-sensitive liposomes, we compared the cytotoxicities of unloaded and loaded liposomes (Figs. 4B and 4C). Both types of liposomal systems, unloaded and loaded with the peptide, were incubated with the cells for 15 hours. Unloaded liposomes, whether non-PEGylated or PEGylated, were not cytotoxic as, in both cases, cell viabilities higher than 80% were found. However, careful inspection of the data shows that unloaded PEGylated liposomes were slightly less toxic than non-PEGylated ones, a finding already reported [26] and probably due to the hydration film on the PEGylated vesicle surface. So, according to this finding, the observable cytotoxicity for loaded liposomes is to be attributed to the activity of the peptide.

The peptide-loaded non-PEGylated liposomes (Fig. 4B) display their cytotoxic activity already at the lowest concentration (0.05 mg/mL). The activity of the drug at such low dosage is likely attributable to an efficient endosomal escape which allows the peptide to reach its target without undergoing degradation. On the other hand, the data obtained with the drug-loaded PEGylated samples (Figure 4C) show a clear cytotoxic activity (i.e. a significant difference between the unloaded and loaded samples) only starting from the 0.075 mg/mL concentration. Although non-significant difference between the two types of peptide-loaded liposomes was found, the cytotoxicity of the peptide delivered by PpHL results only slightly smaller than that measured with nPpHL. This is probably due to a slight reduction of the PEGylated liposome endosomal escape ability or to a different behavior in intracellular uptake, in keeping with the finding reported in the literature as the “PEG dilemma” [27].

However, our data clearly show that the PEGylated liposomes correctly transport the drug to its target and attend a cytotoxicity comparable with the cytotoxicity achieved by using a different, artificial peptide carrier, SAINT-PhD, a membrane barrier crossing agent, studied previously [11–13].

3.6 Protein Extraction and Western Blot Analysis

We finally addressed the issue of the possible interference of the PEGylated liposomes on the mechanism of action of the peptide. To do this, we used western blot analysis to investigate the modulation of four proteins in C13* cells following treatment with the [D-Gln⁴]LR peptide delivered by the PpHL. The four proteins, thymidylate synthase (hTS), TNF receptor associated protein 1 (TRAP1), heat shock protein HSP 90-alpha (HSP90AA1) and dihydrofolate reductase (DHFR), had been selected from a previously investigated protein set to characterize the cellular mechanism of action of these peptides [13]. In parallel, cells treated with the peptide internalized by SAINT-PhD, which allows peptide internalization without interfering with its intracellular mechanism of action, and with 5-FU, a well known inhibitor of hTS that, unlike the peptide, binds at the enzyme catalytic site, were used as reference systems. The elaborated immunoblot results are depicted in Fig. 5.

While 5-FU caused an increase of about 60% in the hTS protein level, this remained unmodified with respect to the control ($p > 0.05$) following [D-Gln⁴]LR delivery by the PEGylated liposomes while slightly decrease by SAINT-PhD ($p < 0.05$). This finding represents evidence that the intracellular mechanism of action of [D-Gln⁴]LR, whether delivered by liposomes or by SAINT-PhD, remains different from that of 5-FU and does not include hTS over-expression [13,28,29].

Moreover, delivery of the peptide by non-PEGylated liposomes modulates the levels of the four proteins with a trend similar to that observed when it is delivered by the SAINT-PhD technology. The levels of hTS, HSP90 and DHFR were almost unmodified compared to the control while the level of TRAP1 underwent a slight increase. In the same way, delivery by the SAINT-PhD system yielded expression levels quite similar to those of the controls and of the liposome delivered peptide, apart from a general decrease. This finding is consistent with previous observations [11,12] and suggests that the encapsulation of [D-Gln⁴]LR in PEGylated pH sensitive liposomes did not affect its intracellular mechanism of action.

Discussion and conclusions

Platinum-resistant recurrences of ovarian cancer are a major target of thymidylate synthase inhibitors. Recently [12], the [D-Gln⁴]LR peptide (LSCqLYQR) demonstrated a significant enhanced biological effect towards the hTS target with respect to its analogue, the LR (LSCQLYQR) peptide, investigated in previous work [10,17]. For this reason, it has been selected for the present study. The activity of this peptide had been previously assessed *in vitro* on cisplatin-resistant cells by a delivery method that involved use of SAINT-PhD, an organic cation transporter of proteins mainly used in *in vitro* experiments [12]. However, the idea of using such biocompatible carriers as PEGylated pH-sensitive liposomes to deliver these peptides seems very tempting because, among other benefits, they carry drugs into the target site following parenteral administration thanks to enhanced permeability and retention (EPR), an effect described in the literature and exploited in the clinical practice [30]. While

PEGylation is essential to ensure a passive targeting, the pH sensitivity of the liposomes facilitates the lysosomal escape of the transported peptide [10]. On the other hand, PEGylation of pH-sensitive liposomes might reduce their "lysosome escape" ability and cellular uptake (the "PEG dilemma" [9,27]). As for the possibility that PEG interfere with the mechanism of action of a delivered drug, to our knowledge this remains unexplored.

For these reasons, in this paper we have prepared and characterized PEGylated pH sensitive liposomes loaded with the [D-Gln⁴]LR peptide and have evaluated their action on ovarian cancer cells resistant to cisplatin (C13* cell line) on which the mechanism of action of the drug delivered by SAINT-PhD had already been characterized [11–13]. In a previous work, a pH sensitive DOPE:CHEMS:DSPE liposome formulation was optimized [10]. Here, we employ the same standardized method (REV technique) to obtain 5% PEGylated liposomes using DSPE_PEG [31].

PEGylated liposomes were smaller than the non-PEGylated ones (Table 1). The decrease in the average diameter of the vesicles obtained with the PEGylated DSPE surfactant is consistent with the expected increase in curvature of the liposome associated with the increased average area of the hydrophilic heads [32]. This is likely due to both a decrease in the interfacial tension with respect to non-PEGylated heads [33] and the hydration of the nearby PEG chains, at least partly in the brush regime [22] that increases the lateral steric repulsion. On the other hand, the marked decrease in the Z potential measured for PpHL relative to nPpHL is attributable to dielectric screening of the surface negative charge of DOPE by the PEG shield and represents evidence of its extensive hydration. This phenomenon and the conformational flexibility of the PEG chains [34] are likely responsible for the decrease in the extent of particle-protein interactions in biological fluids. Such interactions lead to formation in serum of a "protein corona" around non-PEGylated liposomes, hence to an increase in their size [35,36], and are responsible for their rapid phagocytosis in the bloodstream [23]. Concerning liposome size, this is also affected by an osmotic shrinkage due to the ionic strength difference between the interior and the exterior of the liposome. In our case, during the time interval in which interaction of liposomes, both nPpHL and PpHL, with serum protein was observed (Fig.

1b), we noticed a marked increase in both the size and the surface charge for nPpHL, but not for PpHL. Probably, the formulated pH sensitive liposomes have a rather rigid structure that is little affected by ionic strength gradients. Thus, enlargement due to formation of a protein corona prevails for the non-PEGylated liposomes [37]. On the other hand, the dimensional stability of PpHL in serum is attributable to the poor deposition of opsonins on the surface, mainly due to the lower electrostatic interaction with the positively charged serum proteins. This is expected to make these nanocarriers "invisible" to macrophages. Indeed, in the experiments performed to show the interactions of liposomes with the murine J774 cell line, previously validated as an *in vitro* model to predict the kinetics of blood clearance of colloidal drug delivery systems [25], the nPpHL entered the macrophage cells in much greater amounts than the PpHL. From a mechanistic point of view, the process of liposome uptake mediated by endocytosis is a sequence of two steps, i.e., binding of liposomes to the cell surface and effective internalization. The images obtained allow these two phases to be distinguished well for the non PEGylated liposomes. They rapidly accumulate around the macrophage membrane and are then internalized (Fig. 2). On the other hand, for the PEGylated liposomes, the uptake was very slow and the selected observation times did not allow to appreciate the intermediate step of crowding at the cell surface.

As for the peptide loading and release ability, PEGylation of the liposomes proved essentially irrelevant. Based on the presence of three hydrophobic amino acids, L, C and L within the first five positions counting from the N terminus, we speculate that the [D-Gln⁴]LR peptide might be hosted with the N-terminus within the hydrophobic region of the liposome double layer and the hydrophilic C-terminus region protruding into the inner aqueous region. PEGylation does not seem to significantly affect the thermodynamics of this molecular arrangement, that is likely controlled by hydrophobic interactions involving the mentioned N-terminus residues of the peptide and the hydrophobic double layer of the liposome.

Similarly, the two kinds of liposomes loaded with the peptide exhibited only small differences in cytotoxicity towards C13* cisplatin-resistant cells. The empty nPpHL were slightly more toxic than

the PpHL, probably due to the lower biocompatibility of the carrier itself [26]. Regarding [D-Gln⁴]LR-loaded liposomes, the non-PEGylated ones proved only slightly more cytotoxic than the PEGylated ones, especially at the lowest concentration. This behavior is in agreement with the observation of a destabilization of the liposomal membrane of the PEGylated liposomes and a release of the drug at decreasing pH values quite parallel to the behavior shown by nPpHL.

In conclusion, the PEGylated liposomes have proved capable of promoting the efficacy of the peptide on ovarian cancer cells in an extent slight lower than that of the non-PEGylated liposomes. Thus, we can conclude that, like the non-PEGylated ones, the PEGylated liposomes modify their lipid membrane once internalized in ovarian cancer cells and release the peptide avoiding its degradation in the lysosome. Finally, analysis of the expression of four mechanistically relevant proteins has made clear that the intracellular mechanism of action of the peptide, compared with the protein carrier SAINT-PhD, is not affected by the PEGylated liposomes. We thus believe these liposomes to be very encouraging molecular devices for escaping clearance by macrophages and safely delivering this drug to ovarian cancer cells. Further *in vivo* studies on rat xenograft models bearing multiresistant ovarian cancer would be extremely useful to understand in depth the potentialities of this therapeutic system, also in comparison with other drugs that inhibit hTS with a different mechanism of action, in order to evaluate any synergistic action.

Acknowledgments & disclosures

This work was financially supported by the Italian Association for Cancer Research (AIRC-2010, IG 10474 and AIRC-2015, IG 16977 to M.P.C.). The authors are grateful to Dr. D. Pinetti and to Dr. F. Genovese (Centro Interdipartimentale Grandi Strumenti, University of Modena and Reggio Emilia), and also to Dr. M. A. Croce (Cell lab - "Paolo Buffa") for their valuable technical support.

The authors declare that they have no conflict of interest.

References

1. Lian T, Ho RJ. Trends and developments in liposome drug delivery systems. *J Pharm Sci.* 2001;90:667–80.
2. Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int J Pharm.* 2006;307:93–102.
3. Scherphof GL, Dijkstra J, Spanjer HH, Derksen JTP, Roerdink FH. Uptake and Intracellular Processing of Targeted and Nontargeted Liposomes by Rat Kupffer Cells In Vivo and In Vitro. *Ann N Y Acad Sci.* 1985;446:368–84.
4. Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv Drug Deliv Rev.* 2016;99:28–51.
5. Abe K, Higashi K, Watabe K, Kobayashi A, Limwikrant W, Yamamoto K, et al. Effects of the PEG molecular weight of a PEG-lipid and cholesterol on PEG chain flexibility on liposome surfaces. *Colloids Surf Physicochem Eng Asp.* 2015;474:63–70.
6. Hak S, Helgesen E, Hektoen HH, Huuse EM, Jarzyna PA, Mulder WJM, et al. The effect of nanoparticle polyethylene glycol surface density on ligand-directed tumor targeting studied in vivo by dual modality imaging. *ACS Nano.* 2012;6:5648–58.
7. Gabizon AA. Liposome circulation time and tumor targeting: implications for cancer chemotherapy. *Long-Circ Drug Deliv Syst.* 1995;16:285–94.
8. Xia Y, Tian J, Chen X. Effect of surface properties on liposomal siRNA delivery. *Biomaterials.* 2016;79:56–68.
9. Hatakeyama H, Akita H, Harashima H. The polyethyleneglycol dilemma: advantage and disadvantage of PEGylation of liposomes for systemic genes and nucleic acids delivery to tumors. *Biol Pharm Bull.* 2013;36:892–9.
10. Sacchetti F, D'Arca D, Genovese F, Pacifico S, Maretta E, Hanuskova M, et al. Conveying a newly designed hydrophilic anti-human thymidylate synthase peptide to cisplatin resistant cancer cells: are pH-sensitive liposomes more effective than conventional ones? *Drug Dev Ind Pharm.* 2017;43:465–73.
11. Cardinale D, Guaitoli G, Tondi D, Luciani R, Henrich S, Salo-Ahen OMH, et al. Protein-protein interface-binding peptides inhibit the cancer therapy target human thymidylate synthase. *Proc Natl Acad Sci U S A.* 2011;108:E542–9.
12. Pelà M, Saxena P, Luciani R, Santucci M, Ferrari S, Marverti G, et al. Optimization of Peptides That Target Human Thymidylate Synthase to Inhibit Ovarian Cancer Cell Growth. *J Med Chem.* 2014;57:1355–67.
13. Genovese F, Gualandi A, Taddia L, Marverti G, Pirondi S, Marraccini C, et al. Mass Spectrometric/Bioinformatic Identification of a Protein Subset That Characterizes the Cellular Activity of Anticancer Peptides. *J Proteome Res.* 2014;13:5250–61.

14. Cheng Z, Al Zaki A, Hui JZ, Tsourkas A. Simultaneous quantification of tumor uptake for targeted and nontargeted liposomes and their encapsulated contents by ICPMS. *Anal Chem.* 2012;84:7578–82.
15. Lee KD, Nir S, Papahadjopoulos D. Quantitative analysis of liposome-cell interactions in vitro: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. *Biochemistry (Mosc).* 1993;32:889–99.
16. Doktorovova S, Shegokar R, Martins-Lopes P, Silva AM, Lopes CM, Müller RH, et al. Modified Rose Bengal assay for surface hydrophobicity evaluation of cationic solid lipid nanoparticles (cSLN). *Eur J Pharm Sci.* 2012;45:606–12.
17. Sacchetti F, Marraccini C, D'Arca D, Pelà M, Pinetti D, Maretti E, et al. Enhanced anti-hyperproliferative activity of human thymidylate synthase inhibitor peptide by solid lipid nanoparticle delivery. *Colloids Surf B Biointerfaces.* 2015;136:346–54.
18. DiSaia PJ, Sinkovics JG, Rutledge FN, Smith JP. Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. *Am J Obstet Gynecol.* 1972;114:979–89.
19. Andrews PA, Murphy MP, Howell SB. Differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion. *Cancer Res.* 1985;45:6250–3.
20. Marverti G, Ligabue A, Paglietti G, Corona P, Piras S, Vitale G, et al. Collateral sensitivity to novel thymidylate synthase inhibitors correlates with folate cycle enzymes impairment in cisplatin-resistant human ovarian cancer cells. *Eur J Pharmacol.* 2009;615:17–26.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680–5.
22. Garbuzenko O, Barenholz Y, Prieve A. Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chem Phys Lipids.* 2005;135:117–29.
23. Allen C, Dos Santos N, Gallagher R, Chiu GNC, Shu Y, Li WM, et al. Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol). *Biosci Rep.* 2002;22:225–50.
24. Miller CR, Bondurant B, McLean SD, McGovern KA, O'Brien DF. Liposome-cell interactions in vitro: effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes. *Biochemistry (Mosc).* 1998;37:12875–83.
25. Martina M-S, Nicolas V, Wilhelm C, Ménager C, Barratt G, Lesieur S. The in vitro kinetics of the interactions between PEG-ylated magnetic-fluid-loaded liposomes and macrophages. *Biomaterials.* 2007;28:4143–53.
26. Yang C, Liu HZ, Fu ZX, Lu WD. Oxaliplatin long-circulating liposomes improved therapeutic index of colorectal carcinoma. *BMC Biotechnol.* 2011;11:21.
27. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int J Nanomedicine.* 2006;1:297–315.

28. Ponterini G, Martello A, Pavesi G, Lauriola A, Luciani R, Santucci M, et al. Intracellular quantitative detection of human thymidylate synthase engagement with an unconventional inhibitor using tetracysteine-diarsenical-probe technology. 2016;6:27198.
29. Taddia L, D'Arca D, Ferrari S, Marraccini C, Severi L, Ponterini G, et al. Inside the biochemical pathways of thymidylate synthase perturbed by anticancer drugs: Novel strategies to overcome cancer chemoresistance. *Drug Resist Updat*. 2015;23:20–54.
30. Maeda H. Toward a full understanding of the EPR effect in primary and metastatic tumors as well as issues related to its heterogeneity. *Adv Drug Deliv Rev*. 2015;91:3–6.
31. de Carvalho Maroni L, de Oliveira Silveira AC, Leite EA, Melo MM, de Carvalho Ribeiro AF, Cassali GD, et al. Antitumor effectiveness and toxicity of cisplatin-loaded long-circulating and pH-sensitive liposomes against Ehrlich ascitic tumor. *Exp Biol Med Maywood NJ*. 2012;237:973–84.
32. Israelachvili JN. *Intermolecular and Surface Forces*; ch. 17. 2nd Edition. London: Academic Press; 1991.
33. Wang R, Xiao R, Zeng Z, Xu L, Wang J. Application of poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE) block copolymers and their derivatives as nanomaterials in drug delivery. *Int J Nanomedicine*. 2012;7:4185–98.
34. Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov AA, Trubetskoy VS, Herron JN, et al. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim Biophys Acta*. 1994;1195:11–20.
35. Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. *Proc Natl Acad Sci U S A*. 2008;105:14265–70.
36. Monopoli MP, Aberg C, Salvati A, Dawson KA. Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotechnol*. 2012;7:779–86.
37. Kharazian B, Hadipour NL, Ejtehadi MR. Understanding the nanoparticle-protein corona complexes using computational and experimental methods. *Int J Biochem Cell Biol*. 2016;75:162–74.