Biochimica et Biophysica Acta 1798 (2010) 433–441

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Design of peptide-targeted liposomes containing nucleic acids

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ARTICLE INFO

Article history:
Received 28 July 2009
Received in revised form 8 November 2009
Accepted 1 December 2009
Available online 11 December 2009

Keywords:
Liposomes
Antagonist G
Targeted delivery
Oligodeoxynucleotide
siRNA
Cancer

ABSTRACT

Anticancer systemic gene silencing therapy has been so far limited by the inexistence of adequate carrier systems that ultimately provide an efficient intracellular delivery into target tumor cells. In this respect, one promising strategy involves the covalent attachment of internalizing-targeting ligands at the extremity of PEG chains grafted onto liposomes. Therefore, the present work aims at designing targeted liposomes containing nucleic acids, with small size, high encapsulation efficiency and able to be actively internalized by SCLC cells, using a hexapeptide (antagonist G) as a targeting ligand. For this purpose, the effect of the liposomal preparation method, loading material (ODN versus siRNA) and peptide-coupling procedure (direct coupling versus post-insertion) on each of the above-mentioned parameters was assessed. Post-insertion of DSPE-PEG-antagonist G conjugates into preformed liposomes herein named as stabilized lipid particles, resulted in targeted vesicles with a mean size of about 130 nm, encapsulation efficiency close to 100%, and a loading capacity of approximately 5 nmol siRNA/μmol of total lipid. In addition, the developed targeted vesicles showed increased internalization in SCLC cells, as well as in other tumor cells and HMEC-1 microvascular endothelial cells. The improved cellular association, however, did not correlate with enhanced downregulation of the target protein (Bcl-2) in SCLC cells. These results indicate that additional improvements need to be performed in the future, namely by ameliorating the access of the nucleic acids to the cytoplasm of the tumor cells following receptor-mediated endocytosis.

1. Introduction

Systemic administration of anticancer gene silencing therapy has been so far limited by the inexistence of adequate carrier systems. Ideally, they should efficiently encapsulate the nucleic acids, be stable in blood and present prolonged circulation times, without causing any immune reactions. Moreover, carrier systems must be internalized by cancer cells, where they should destabilize in the endosomal compartment for an efficient release of the active nucleic acids. The best equilibrium between these properties is a true challenge, so far difficult to meet.

Different poly(ethylene glycol) (PEG)-grafted cationic liposomes encapsulating antisense ODN such as coated cationic liposomes (CCL) [1], stabilized antisense lipid particles (SALP) [2], or the related stabilized nucleic acid lipid particles (SNALP) encapsulating siRNA [3–6], have been developed in order to fulfill some of the above-mentioned key requirements. However, in the treatment of cancer there is still the need of more efficient intracellular delivery of nucleic acids, aiming at reaching an adequate pharmacodynamic performance. In this context, one of the most promising strategies involves the covalent attachment of a targeting ligand, at the extremity of PEG chains grafted onto liposomes, which will specifically promote intracellular accumulation of the nucleic acid-containing lipid particle

Abbreviations: ODN, oligodeoxynucleotide; SCLC, small cell lung cancer; siRNA, small interfering RNA; PEG, poly(ethylene glycol); CCL, coated cationic liposomes; SALP, stabilized antisense lipid particles; SNALP, stabilized nucleic acid lipid particles; EGF, epidermal growth factor; PFA, paraformaldehyde; NaN_3, sodium azide; AD, actinomycin D; 7-AAD, 7-aminoactinomycin D; BSA, bovine serum albumin; C12E8, octaethylene glycol monododecyl ether; HSPC, hydrogenated soy phosphatidylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; $CerC_{16}$-PEG, N-palmitoyl-sphingosine-1-succinyl(methoxypolyethylene glycol)$_{2000}$; DSPE-PEG, N-palmitoyl-sphingosine-1-[succinyl(polyethylene glycol)]$_{2000}$; DSPE-PEG-Mal, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)]$_{2000}$; rhodamine-PE, L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl); DOTAP, 1,2-dioleyl-3-trimethylammonium-propane; DODAP, 1,2-dioleyl-3-dimethylammonium-propane; MES, 2-(n-morpholino)ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; CHOL, cholesterol; MBS, MES buffered saline; HBS, HEPES buffered saline; SLP, stabilized lipid particles; SLP^{ODN}, SLP encapsulating ODN; SLP^{siRNA}, SLP encapsulating siRNA

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doi:10.1016/j.bbamem.2009.12.001

into the target tumor cells and further leading to improved gene silencing [7]. Such strategy is also particularly important when dealing with tumor cells that are difficult to transfect, as for example SCLC [8,9], as internalization of the nucleic acids can be itself a limiting step in this process.

The potential of ligand-mediated targeting of liposomes for the treatment of SCLC has been assessed for the first time by Moreira et al [10]. The targeting ligand was a hexapeptide known as antagonist G ([Arg^6,D-$Trp^{7,9}$,$MePhe^8$]substance P[6–11]), first described as a broad-spectrum antagonist, active against several receptors of the G-protein coupled receptor family [11], and more recently described to be a biased agonist of gastrin-releasing peptide and vasopressin V_{1A} receptors [12]. It was used as a targeting agent for sterically stabilized liposomes containing doxorubicin in human SCLC, either upon coupling to preformed liposomes (direct coupling) [10], or by post-insertion of the conjugate DSPE-PEG-antagonist G from a micellar phase into preformed liposomes [13]. One of the challenges posed by the preparation of ligand-targeted liposomes containing nucleic acids, with the direct coupling procedure, comes from the susceptibility to hydrolysis of maleimide groups, currently used in PEG-derivatized lipids for bioconjugation. It might lead to the formation of non-reactive groups, thus compromising the efficiency of the coupling procedure [14]. Moreover, a ligand like antagonist G, short and highly hydrophobic, that easily insert into membranes [15,16], might lead to aggregation of the liposomes during the coupling reaction, thus compromising their systemic administration. Therefore, the post-insertion of ligand-derivatized conjugates onto preformed liposomes is expected to provide a simpler and more flexible method to prepare antagonist G-targeted liposomes containing nucleic acids. However, when this approach was tested before with liposomes containing doxorubicin, it was observed that the transfer of antagonist G conjugates caused the release of a high percentage of drug. This was circumvented by remote loading of doxorubicin after the post-insertion had taken place [13]. In the case of similar problems with the encapsulation of nucleic acids, the previous solution would not be applicable.

The present work aims at designing an antagonist G-targeted non-viral lipid-based particle containing nucleic acids, with small size, high encapsulation efficiency and able to be actively internalized by SCLC cells (as compared to non-targeted particles). For this purpose, the effect of the preparation method (CCL versus SALP), loading material (ODN versus siRNA) and coupling method (direct method versus post-insertion) in each of the previously mentioned issues was assessed. These are important parameters that need to be characterized before the *in vivo* application of antagonist G-targeted liposomes.

2. Materials and methods

2.1. Materials

All salts were either SIGMA® (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or Merck (Darmstadt, Germany). Actinomycin D (AD), 7-aminoactinomycin D (7-AAD), bovine serum albumin (BSA), RPMI 1640 with L-glutamine and without sodium bicarbonate, penicillin–streptomycin solution (10,000 U/ml and 10 mg/ml, respectively), 2-iminothiolane hydrochloride, digitonin, sodium salt of 2-(n-morpholino)ethanesulfonic acid (MES), mouse epidermal growth factor (EGF), hydrocortisone, cholesterol (CHOL), gel filtration media Sephadex® G-50 and DEAE Sepharose® CL-4B, 2-iminothiolane hydrochloride, LY-294,002, Hoechst 33342, and silicon coating reagent (Sigmacote) were SIGMA® (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Fetal bovine serum (FBS) was Gibco® (Invitrogen S.A., Barcelona, Spain). Octaethylene glycol monododecyl ether (C12E8) was Fluka® (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Fully hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), N-palmitoyl-sphingosine-1-succinyl(methoxypolyethylene glycol)$_{2000}$ ($CerC_{16}$-PEG), ammonium salts of N-palmitoyl-sphingosine-1-[succinyl(polyethylene glycol)]$_{2000}$ (DSPE-PEG), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)]$_{2000}$ (DSPE-PEG-Mal), and L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE), and chloride salts of 1,2-dioleyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleyl-3-dimethylammonium-propane (DODAP), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

The hexapeptide antagonist G (*NH_2*-Arg-[D-Trp]-N^{me}Phe-[D-Trp]-Leu-Met-*$CONH_2$*) was synthesized by Prim srl, (Milan, Italy) with a purity >95%. ODN 2009 (desalted 20-mer phosphorothioate [17]) was purchased from University Core DNA services, (University of Calgary, Calgary, AB, Canada). The carboxyfluorescein-conjugated ODN 2009 was purchased from Microsynth (Balgach, Switzerland). The anti-*BCL2* siRNA and the control non-targeting siRNA (siRNA *nt*) were purchased from Dharmacon Inc. (Lafayette, CO) in the form of deprotected, annealed and desalted duplexes. Upon solubilization in ultra pure water or Dharmacon's siRNA buffer (20 mM KCl, 0.2 mM $MgCl_2$, 6 mM HEPES, pH 7.5), the concentration of the stock solutions of both ODN and siRNA was confirmed by absorbance at 260 nm using the corresponding extinction coefficient.

2.2. Cell lines

The human variant SCLC cell line SW2 was kindly provided by Drs. U. Zangemeister-Wittke and R. Stahel (University Hospital of Zurich, Switzerland). The human classic NCI-H69 SCLC cells, and the human non-small cell lung cancer (NSCLC) A549 cells were from the American Type Culture Collection (Manassas, IL). The human microvascular endothelial cell line HMEC-1 was a generous gift from the Centers for Disease Control and Prevention (Atlanta, GA). Cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES and 0.2 % $NaHCO_3$, at 37 °C, in a humidified atmosphere (90%), containing 5% CO_2. HMEC-1 culture medium was also supplemented with 10 ng/ml mEGF and 1 μg/ml hydrocortisone. Cells were maintained in exponential growth phase and periodically tested for mycoplasma contamination with MycoAlert® Mycoplasma Detection kit (Cambrex Bio Science Verviers, Inc., Liege, Belgium).

2.3. Preparation of coated cationic liposomes

The procedure of CCL preparation by reverse phase evaporation was adapted from Stuart et al. [1]. The ODN 2009 at 2 mg/ml in 250 or 500 μl of water was incubated with the cationic lipid DOTAP in an equivalent volume of chloroform, at an ODN/DOTAP (−/+) charge ratio of 1, upon addition of methanol (in a small excess of 0.5 or 1 ml) to form a Bligh-Dyer monophase where hydrophobic ODN-lipid particles are formed. After 30 min of incubation, separation of phases was achieved through addition of another 250 or 500 μl of water and chloroform, followed by centrifugation (7 min at 800×*g*). The organic phase containing the formed hydrophobic ODN-DOTAP particles was then mixed with the neutral lipids solubilized in chloroform (HSPC: CHOL:[DSPE-PEG or DSPE-PEG-Mal] at the molar proportion of 3:2:0.2, relative to DOTAP, with or without 1 mol% rhodamine-PE, achieving a total lipid concentration of around 32 mM. When CCL were submitted to post-insertion of PEG-conjugates, the amount of DSPE-PEG originally incorporated was reduced to half. The resulting organic solution was transformed into a stable w/o emulsion by addition of water (1/3 of the chloroform volume) and sonication for 30 min. The emulsion was reversed upon chloroform evaporation under reduced pressure until a gel phase was formed, time at which the addition of more water (the necessary volume to reach 10 mM of lipid) and further evaporation, completed gel reversion, resulting in the formation of liposomes. During this process, samples were

frequently homogenized by agitation in vortex and the pressure was raised with N_2. The resulting liposomes were then extruded 11 times sequentially through polycarbonate membranes of 400, 200 and 100 nm pore size using a LipoFast mini extruder pre-heated at ~65 °C (Liposofast, Avestin Europe GmbH, Mannheim, Germany) to obtain a uniform size distribution [18]. The liposomes were then run down a Sephadex G-50 column equilibrated either with MES-buffered saline (MBS) (25 mM MES, 129 mM NaCl) pH 6.5, or with HEPES buffered saline (HBS) (25 mM HEPES, 140 mM NaCl), pH 7.4, depending on the coupling method the formulation was planned to be used for (section 2.6).

2.4. Preparation of stabilized antisense lipid particles

In this work, a second method of preparation of targeted lipid-based nanoparticles was used. The procedure to prepare SALP was adapted from Semple et al. [2]. The lipid mixture (either formulation A, DODAP:DSPC:CHOL:DSPE-PEG-Mal, 25:20:45:10, or formulation B, DODAP:DSPC:CHOL:CerC_{16}-PEG, 25:20:45:8) prepared in 100% ethanol at approximately 50 mM total lipid was added to ODN diluted in a 20-mM citrate-buffered solution at pH 4, preheated at 60 °C, under vortex agitation. The final proportion of ethanol was ~40%. The resulting vesicles were then extruded 21 times through polycarbonate membranes of 100 nm pore size at ~65 °C using a LipoFast mini extruder. Liposomes were either directly chromatographed with a DEAE Sepharose® CL-4B column equilibrated with MES, pH 6.5 and submitted to direct coupling of antagonist G (formulation A), or dialyzed using a regenerated cellulose tubular membrane Cellu.Sep T2, of nominal MW cut off of 6000–8000, for 3 h against 25 mM HEPES buffer, pH 7.4, and then submitted to post-insertion (formulation B), as described in Section 2.6.

2.5. Modified procedure for the preparation of stabilized lipid particles

A modified formulation based in the previous described SALP, named stabilized lipid particles (SLP), was used for the encapsulation of either ODN (SLP^{ODN}) or siRNA (SLP^{siRNA}). The liposomes were formed by DOTAP:DSPC:CHOL (16:24:45 mol%), and CerC_{16}-PEG + [DSPE-PEG or DSPE-PEG-Mal] (6 + 4 mol%) or CerC_{16}-PEG only (8 mol%), and with or without rhodamine-PE (usually 1 mol%). The lipid mixture prepared in 2/5 of the final volume in 100% ethanol, at approximately 50 mM of total lipid, was added in a charge ratio of 1:1 to the ODN or siRNA diluted in 25 mM citrate buffer, pH 6, preheated at 60 °C, under vortex agitation. The resulting vesicles, now in ~40% ethanol, were then extruded 21 times through polycarbonate membranes of 100 nm pore size at ~65 °C using a LipoFast mini extruder. Liposomes were either dialyzed for 3 h against MBS pH 6.5, or submitted to size exclusion chromatography in a DEAE Sepharose® CL-4B column, equilibrated with MBS pH 6.5, and then submitted to post-insertion or direct coupling procedures, as described in Section 2.6.

2.6. Covalent coupling of antagonist G to micelles of DSPE-PEG-Mal, post-insertion, and direct coupling

Coupling of antagonist G to PEG-derivatized DSPE has been adapted from previous reports [13]. Peptide thiolation [19] took place upon reaction of 0.35 mM antagonist G with 2-iminothiolane at 1:4 molar ratio in 12.5 mM HEPES, 1 mM EDTA, pH 8, for 1 h at room temperature, in an inert N_2 atmosphere, and in a silicon-coated glass vial. Thiolated antagonist G was then added to 0.35 mM DSPE-PEG-Mal micelles, suspended in MBS, pH 6.5, at a lipid/peptide molar ratio of 1:1. Coupling reaction took place overnight at room temperature in an inert N_2 atmosphere. For post-insertion, micelles of targeted conjugate (or control DSPE-PEG micelles) were incubated with the liposomes for 1 h at 60 °C, at a conjugate/total lipid ratio of 2 mol%.

Alternatively, in the so-called direct coupling procedure, the thiolated peptide was added to DSPE-PEG-Mal-grafted liposomes in MBS and the reaction took place as described in the previous paragraph. When required, excess maleimide was quenched by reaction with an excess of 2-mercaptoethanol for 30 min. The resulting liposomes were purified by size exclusion chromatography in Sepharose CL-4B columns equilibrated with HBS, pH 7.4, in both coupling procedures. Separation of free siRNA was always performed with Sepharose CL-4B.

2.7. Physicochemical characterization of the liposomal formulations

The size dispersion of liposomes was evaluated by photon correlation spectroscopy with a N5 submicron particle size analyzer (Beckman Coulter, Inc. Fullerton, CA), upon dilution in filtered HBS. The encapsulation efficiency (EE) is the percentage of the ratio between the nucleic acid to lipid molar ratio in the final liposome dispersion and that one in the initial mixture (liposome dispersion before extrusion). ODN and RNA were quantified by using the Quant-iT™ RiboGreen® RNA reagent (Invitrogen S.A. Barcelona, Spain) in the presence of C12E8 detergent. The total lipid concentration was inferred from cholesterol concentration determined with Infinity™ cholesterol liquid stable reagent (ThermoTrace Ltd, Victoria, Australia).

2.8. Quantitative evaluation of liposomes cellular association

Tumor cells (0.5×10^6) were incubated with rhodamine-labeled liposomes in cell culture medium for 1 h and further washed three times with ~3 ml of PBS. Washed cells were transferred to 96-well plates in 100 µl PBS, and lysed with 100 µl of lysis buffer (25 mM HEPES, 2 mM EDTA, 1.2 mM C12E8, pH 8). Rhodamine-PE fluorescence was measured in the supernatant in a SpectraMax Gemini EM plate reader fluorimeter (Molecular Devices, Sunnyvale, CA) at 545 nm/570 nm/587 nm excitation/cut-off/emission wavelengths. The amount of cell-associated lipid was drawn from calibration curves comparing lipid concentration and relative fluorescence units. In experiments with adherent cells, (plated the day before), the results were normalized by determining the total protein of each well with the BCA™ Protein Assay Kit (Pierce, ThermoFisher Scientific, Rockford, IL, USA).

2.9. Qualitative evaluation of liposome association to SCLC cells by flow cytometry and confocal fluorescence microscopy

SCLC SW2 cells (1×10^6), suspended in the culture medium mentioned in Section 2.2, were incubated with SLP^{ODN} prepared by post-insertion, labeled with rhodamine-PE (membrane marker) and containing carboxyfluorescein-labeled ODN (25% of total ODN content), at 0.6 mM of total lipid, for 1 h at 37 °C. At the end of the incubation, half of the SW2 cells were washed and resuspended in PBS, and a minimum of 10000 events were immediately acquired in a flow cytometer (BD FACSCalibur™, BD Biosciences, Erembodegem, Belgium). The other half of SW2 cells was centrifuged and resuspended in fresh medium, and incubated at 37 °C for another 5 h. Following this period, SW2 cells were fixed with 4% paraformaldeyde in PBS for 20 min at room temperature, washed with PBS, and stained with Hoechst 33342 (1 µg/ml in PBS, for 5 min). After a final washing step, cells were gently spread over the glass and mounted on Mowiol® mounting medium (Calbiochem®, Bad Soden, Germany). Confocal images were acquired in a Zeiss LSM-510 Meta laser-point scanning confocal microscope (Zeiss, Jena, Germany), with a 63× Plan Apochromat oil immersion objective, using a diode (405 nm), an argon (488 nm) and a DPSS (561 nm) excitation lasers, and LSM-510 Meta software. Emission of Hoechst, carboxyfluorescein and rhodamine were measured with BP 420–480, BP 505–550 and 572–700 nm

filters, respectively. All instrumental parameters pertaining to fluorescence detection and image analyses were held constant to allow sample comparison.

2.10. Evaluation of the mechanism of internalization of antagonist G-targeted liposomes

Aiming at evaluating the cell-entry pathway of antagonist-G targeted liposomes, 1×10^6 SCLC H69 cells, suspended in the culture medium mentioned in section 2.2, were preincubated for 30 min at 37 °C with non-toxic concentrations of drugs that inhibit different cell entry pathways, including: hypertonic medium (0.45 M sucrose), 200 μM genistein or 50 μM LY-294,002; or at 4 °C for 1 h. Liposomes were then added at 0.1 mM of total lipid, and further incubated for 1 h at 37 or 4 °C. The cellular association for each one of the tested conditions was expressed in percentage and normalized for the uptake of targeted liposomes at 37 °C for 1 h, without pretreatment with drug inhibitors. In parallel, similar cellular association experiments were performed with Alexa Fluor® 546 transferrin conjugate (AlexaF-transferrin), (Molecular Probes, Invitrogen S.A., Barcelona, Spain), at 100 μg/ml, aiming at assessing the extent of the drug inhibitors effect in the clathrin-mediated endocytosis pathway.

2.11. Evaluation of Bcl-2 knockdown

SW2 cells ($1–2\times10^6$ cells/ml), suspended in the culture medium mentioned in Section 2.2, were incubated with 2 μM anti-BCL2-siRNA or siRNA *nt* encapsulated in non-targeted or antagonist-G targeted SLP for 6 h, and further centrifuged and resuspended in fresh medium. Bcl-2 levels were evaluated at 72 h post transfection. At the end of the experiment, approximately 0.2×10^6 tumor cells were incubated with 7-AAD (20 μg/ml) for 15 min at 4 °C in 0.1 ml of PBS, then washed once and fixed in 1% PFA for 20 min at 4 °C. Afterwards, cells were washed with 1 ml of buffer containing 1% BSA, 0.1% NaN_3 and 20 μg/ml AD, and incubated for 15 min in 0.2 ml of the previous solution containing 0.5 mg/ml digitonin and 8 μl of phycoerythrin-conjugated mouse anti-human Bcl-2 mAb (IgG1k, clone Bcl-2/100, BD Pharmingen™, BD Biosciences, Erembodegem, Belgium) or the same volume of the correspondent phycoerythrin-conjugated isotype control (IgG1k, clone MOPC-21, BD Pharmingen™, BD Biosciences). After washing once with PBS containing BSA and NaN_3, about 30,000 events cells were acquired in a flow cytometer (BD FACSCalibur™, BD Biosciences, Erembodegem, Belgium) and analyzed as described elsewhere [20].

2.12. Statistical analysis

Plots display data as mean ± SEM or SD, as mentioned in the legends of the figures. Differences were evaluated by one-way or two-way analysis of variance (ANOVA), with post-test analysis, depending on the study design, and significance levels indicated with symbols ($*p<0.05$; $**p<0.01$; and $***p<0.001$).

3. Results and discussion

Different liposomal formulations have been used to deliver nucleic acids to cancer cells. One of the most widely used systems involves the mixture of preformed cationic liposomes with nucleic acids (originating lipoplexes). The excess of positive charge provides an efficient encapsulation of the negatively charged molecules as well as an efficient delivery of the associated nucleic acid, following electrostatic interaction with the negatively charged target cell membrane [21]. Although lipoplexes are efficient transfection agents for *in vitro* applications, they are usually not adequate for systemic administration. Lipoplexes present toxicity and inadequate biodistribution due to their instability, propensity to form large aggregates, and extensive clearance by the mononuclear phagocyte system (reviewed in [22]). *In situ* administrations using lipoplexes have been studied [23–25], but are not adequate to address a systemic disease such as SCLC.

CCL [1] and SALP [2] are examples of formulations that encompass a number of physicochemical features, such as efficient encapsulation of nucleic acids, good physical and biological stability as well as small size (100–200 nm), which make them suitable for systemic administration. For this reason, these formulations were selected to develop antagonist G-targeted sterically stabilized liposomes containing nucleic acids.

3.1. Effect of the liposomal preparation method and coupling procedure on the nucleic acid encapsulation efficiency

In a first stage, the effect of two different coupling methods and of the liposomal preparation procedure on the encapsulation efficiency of both ODN and siRNA was assessed. One method involved the covalent coupling of the thiolated antagonist G to the distal end of Mal-PEG-derivatized lipid grafted onto CCL or SALP (herein designated as *direct coupling* method). In the procedure referred as *post-insertion*, thiolated antagonist G was coupled to DSPE-PEG-Mal micelles, and the resulting DSPE-PEG-peptide conjugates (DSPE-PEG-G) were then transferred to preformed liposomes, as described in Section 2.

The preparation of antagonist G-targeted CCL containing ODN, either through direct coupling or post-insertion, led to encapsulation efficiencies around 60% (Fig. 1A). When targeted liposomes were prepared from SALP, also by both direct coupling and post-insertion methods, encapsulation efficiency of ODN decreased to 40% and 20%, respectively (Fig. 1A). The post-insertion to SALP took place at pH 7.4, at a temperature above the melting temperature (~60 °C), which might have led to partial liposomal destabilization. Such destabilization, at a pH where DODAP is neutral (intrinsic pK_a of ~6.6–7 [26]), might have favored ODN leakage. In fact this was the reason why, in order to make the insertion of DSPE-PEG-G conjugates possible, DODAP was replaced by DOTAP (giving rise to the herein referred *Stabilized Lipid Particles*, SLP), a lipid that is protonated during post-insertion. It was thus possible to significantly improve the encapsulation efficiency of ODN, from about 20% to close to 60% (Fig. 1A). The ODN encapsulation efficiency observed between the antagonist G-targeted CCL, SALP and SLP^{ODN} and each of the corresponding non-targeted formulations was not significantly different (data not shown).

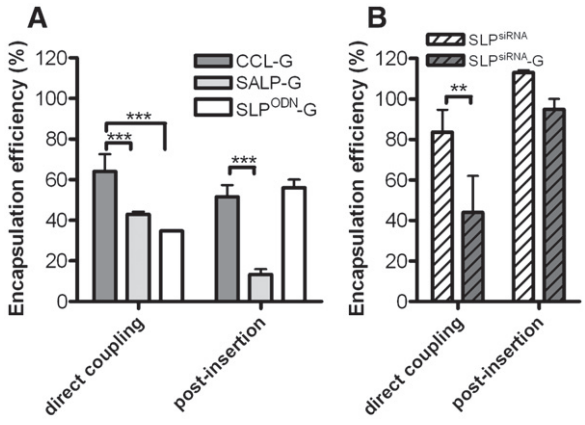

Fig. 1. Encapsulation efficiency of different antagonist G-targeted and non-targeted formulations as a function of the preparation method and coupling procedure. The ODN encapsulation efficiency was compared among antagonist G-targeted CCL, SALP and SLP^{ODN} prepared by either the direct coupling or post-insertion methods (A). The encapsulation efficiency of siRNA was compared between non-targeted (SLP^{siRNA}) and antagonist G-targeted (SLP^{siRNA}-G) SLP, prepared by either the direct coupling or post-insertion methods (B). Bars are the mean ± SD of independent experiments. '*' symbols represent the significance level of the difference between formulations (two-way ANOVA, Bonferroni's post test).

When the encapsulation of siRNA was attempted with the developed SLP formulation (SLP^{siRNA}), the resulting antagonist G-targeted liposomes presented higher encapsulation efficiency when prepared by the post-insertion as compared with the direct coupling method (Fig. 1B). It is important to point out that using the direct coupling procedure, the incubation of the thiolated antagonist G with preformed liposomes led to a 2-fold decrease of the encapsulation efficiency relative to non-targeted liposomes (Fig. 1B). A similar effect was also observed when this coupling procedure was performed with liposomes containing doxorubicin, although at a lower extent (10% of drug leakage; JN Moreira, unpublished observations). These observations are therefore a strong indication that the free targeting peptide, interfered with the final encapsulation yield of siRNA. Overall, regarding encapsulation efficiency, the use of CCL or SLP, followed by post-insertion of antagonist G conjugate, were the best methodologies to prepare antagonist G-targeted liposomes. The overall loading of nucleic acids (5–6 nmol ODN or 4–5 nmol siRNA/μmol total lipid) was comparable with the values obtained by others [1,5].

3.2. Mean size of the different non-targeted and antagonist G-targeted liposomes

The size of any non-viral particle aiming at systemic gene silencing plays a major role in its pharmacodynamic performance, affecting both the circulation time in the blood and the passive accumulation into a tumor (EPR effect) [27].

In line with what has been shown in the previous section, direct coupling of antagonist G to both CCL and SLP was also disadvantageous in terms of the vesicles final size, as compared to the non-targeted counterparts. It led to a significant increase of the mean size ($p = 0.001$, two-way ANOVA; Fig. 2A), polydispersity index (data not shown) as well as average size dispersion, relative to non-targeted formulations, particularly for CCL (185 ± 24 nm and 326 ± 135 nm, for CCL and CCL-G, respectively). Both non-targeted and antagonist G-targeted SLP prepared by direct coupling were significantly smaller than the corresponding CCL ($p = 0.004$, two-way ANOVA). The mean size of antagonist G-targeted SLP was approximately 200 nm (Fig. 2A), a value still suitable for systemic administration.

The preparation of antagonist G-targeted CCL upon incubation of preformed liposomes with 2 mol% DSPE-PEG-G micelles led to vesicles with a mean size that was not significantly different from that of non-targeted liposomes (190 ± 34 nm and 155 ± 3 nm, respectively, Fig. 2B). Similarly, the post-insertion of antagonist G conjugate into SLP did not change the size of the resulting vesicles (131 ± 8 nm and 126 ± 3 nm, respectively, Fig. 2B) and the overall mean size was even slightly lower than the one obtained with CCL ($p = 0.002$, two-way ANOVA; Fig. 2B). The reduced mean size dispersion was an indication of the reproducibility of the liposomal preparation process. The same observation can be extended to SALP (data not shown), although the encapsulation efficiency was only around 20%, as demonstrated in the previous section.

Overall, the results gathered regarding mean size and encapsulation efficiency of antagonist G-targeted liposomes containing nucleic acids, demonstrate that the post-insertion of DSPE-PEG-G into either CCL or SLP results in physico-chemical features that better meet the requirements of a targeted non-viral particle aiming at systemic gene silencing for cancer treatment.

3.3. Cellular association of antagonist G-targeted CCL and SLP liposomes

All the formulations used in the present work are characterized by the presence of an amount of PEG that ranges from 3.2 mol% (CCL) to 10 mol% (SALP and SLP) relative to total lipid. The advantages of using PEG are undoubtedly recognized. It provides the nanoparticle with long circulation times in blood and further accumulation in distant sites of disease such as solid tumors [28]. In the case of formulations like CCL and SLP/SALP, the presence of PEG also allows the final size of the nucleic acid-containing lipid-based nanoparticle to be controllable within a range adequate for systemic administration [1,26]. The downside of the presence of PEG is the significant reduction in the extent of the liposomal cellular association [29], as it was also evidenced for non-targeted SALP (data not shown), CCL, and SLP formulations (Fig. 3). In addition, SCLC cells are intrinsically difficult to transfect [8,9], and antagonist G can, therefore, provide an added value to the nanoparticle. Antagonist G was first described as a broad-spectrum antagonist, active against several receptors of the family of G-protein coupled receptor family [11], and more recently has been shown to be a biased agonist of gastrin-releasing peptide and vasopressin V_{1A} receptors [12]. Indeed, receptors recognized by this peptide, such as vasopressin and gastrin-releasing peptide receptors, are expressed by classic and variant SCLC cells, as well as by tumor cells from other histological origins [30,31]. As a targeting ligand, antagonist G has the additional advantage of being readily available, inexpensive to manufacture and easy to handle.

Based on the results presented in the previous section, the cellular association of antagonist G-targeted CCL or SLP, prepared by the post-insertion coupling method, was assessed in SCLC cells in order to evaluate whether such formulations would be selectively targeted to these cells. The extent of association to SCLC H69 cells for both types of targeted liposomes was, approximately, 4- to 10-fold higher than that

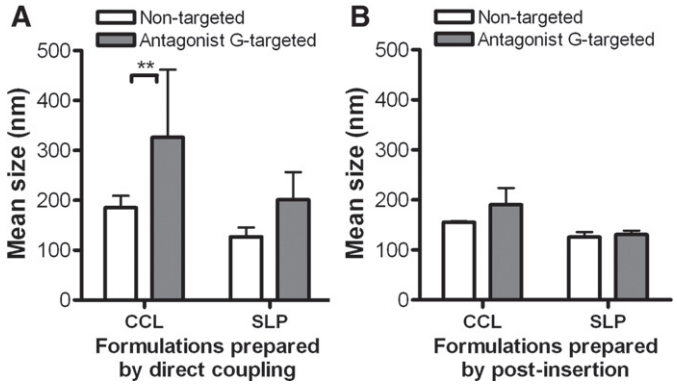

Fig. 2. Mean size of different non-targeted and antagonist G-targeted formulations as a function of the preparation method. Antagonist G-targeted liposomes were prepared from CCL or SLP (encapsulating either ODN or siRNA) using the direct coupling (A) or post-insertion method (B). The size dispersion of liposomes was evaluated by photon correlation spectroscopy. Bars represent the mean ± SD of 3 to 10 independent experiments. '*' symbols represent the significance level of the difference between formulations (two-way ANOVA, Bonferroni's post-test).

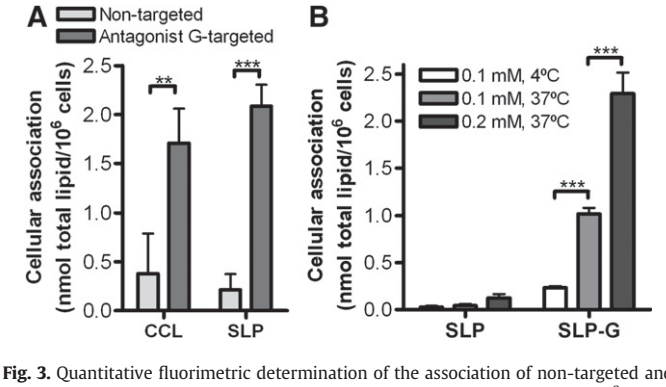

Fig. 3. Quantitative fluorimetric determination of the association of non-targeted and antagonist G-targeted CCL and SLP to SCLC H69 cells. Tumor cells (0.5×10^6) were incubated with: rhodamine-labeled non-targeted, targeted CCL or SLP prepared by post-insertion (2 mol%), at 0.2 mM of total lipid for 1 h at 37 °C (A), or rhodamine-labeled non-targeted or targeted SLP at 0.1 or 0.2 mM of total lipid for 1 h at 4 °C or 37 °C (B). In A, bars are the mean ± SD of independent experiments, each one performed in triplicate. In B, bars are the mean ± SEM of triplicates of a typical experiment '*' symbols represent the significance level (two-way ANOVA, Bonferroni's post-test).

for the non-targeted counterparts (Fig. 3A). Furthermore, the extent of cellular association was dependent both on the lipid concentration and temperature (Fig. 3B). The significant increase observed for the extent of cellular association upon raising the temperature from 4 °C (a non-permissive endocytosis condition) to 37 °C is a strong indication that the particles are actively internalized by tumor cells (Fig. 3B).

The increased cellular association of antagonist G-targeted CCL or SLP was also verified in other SCLC cell lines, such as U1285 (data not shown) and SW2 cells. For all these cells, the homogeneous cellular association shown by flow cytometry indicated that the liposomes targeted all tumor cells in a similar extent (shown for SW2 cells, Fig. 4A). Confocal microscopy analysis of the uptake of the liposomes labeled with rhodamine-PE (membrane marker, red label) and encapsulating carboxyfluorescein-labeled nucleic acid (green) confirmed that targeted liposomes were internalized by tumor cells in a higher extent than non-targeted liposomes (Fig. 4B).

Having shown that the targeted liposomes are actively endocytosed, we were interested in elucidating the main endocytosis pathway implicated. Different mechanisms of endocytosis can be considered. Phagocytosis is a very specialized mechanism, usually present in cells of the immune system [32]. The others are broadly considered pinocytosis mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and the less studied endocytosis mechanisms, which are independent of both clathrin and caveolin. The mechanism of internalization of antagonist G-targeted liposomes was assessed by pre-incubating SCLC H69 cells with drugs described to selectively inhibit different cell entry pathways before targeted liposomes were added. Control experiments were first performed to address the cellular association of fluorescein-labeled dextran (a marker of macropinocytosis), BODIPY-LactoCeramide (a marker of the caveolae-mediated cell entry pathway), and AlexaF-transferrin, a "classical" marker of clathrin-mediated endocytosis, as well as the effect of the selected endocytosis inhibitors on the uptake of these control markers [33–36]. There was a marked difference in the uptake of dextran at 4 and 37 °C, indicating the presence of macropinocytosis mechanisms in SCLC H69 cells, and a moderate inhibitory effect of LY-294,002 or wortmannin (described as selective macropinocytosis inhibitors, data not shown). The extent of cellular association of BODIPY-LactoCeramide was neither statistically different at 37 °C and 4 °C, nor in the presence of inhibitors such as genistein and Filipin (inhibitors of caveolae-mediated endocytosis, data not shown). These results suggest that caveolae-mediated endocytosis is not present in SCLC H69 cells, in accordance with caveolin-1 being not detected in a large proportion of SCLC cell lines [37,38]. On the other hand, cellular association of AlexaF-transferrin was partially inhibited upon pre-incubation of the cells with LY-294,002 and genistein, as well as with hypertonic medium (0.45 M sucrose, described to selectively inhibit clathrin-mediated endocytosis) (Fig. 5A). These results show that there is lack of LY-294,002 and genistein selectivity for macropinocytosis and caveolae-mediated endocytosis in H69 cells.

Similarly to the observed with AlexaF-transferrin, both genistein and, to a less extent, hypertonic medium, inhibited partially the cellular association of antagonist G-targeted liposomes (40 and 20% inhibition, respectively, Fig. 5B). However, as shown in the same Figure, LY-294,002 did not exhibit any significant effect on the cellular association of antagonist G-targeted liposomes, in contrast to its partial, although significant, inhibitory effect on the cellular association of AlexaF-transferrin, thus indicating that macropinocytosis and clathrin-mediated endocytosis do not play an important role in the uptake of these targeted liposomes.

Taken together, the results from this systematic analysis strongly suggest that clathrin- and caveolae-independent endocytosis mechanisms are involved in the internalization of antagonist G-targeted liposomes into SCLC H69 cells. This is in accordance with the reports stating that neuroendocrine cells, like SCLC cells, have clathrin-independent mechanisms of endocytosis, with an important role in the recovery of membrane proteins after secretion (reviewed in [32]). However, a small contribution of clathrin-mediated endocytosis, or even of macropinocytosis, for the uptake of antagonist G-targeted liposomes into SCLC H69 cells cannot be excluded.

Growth factor receptors, such as gastrin releasing peptide and vasopressin receptors, are not exclusively expressed by SCLC cells [39,40]. In several types of tumor and endothelial cells of angiogenic vessels, expression, or even overexpression, of growth factor receptors is an important mechanism supporting cell proliferation [41,42]. Therefore, antagonist G-targeted CCL or SLP could be hypothesized to target cells from different histological origins. Indeed, antagonist G significantly increased the extent of association of CCL or SLP to cell lines from other types of cancer (relative to non-targeted liposomes), such as Namalwa (Burkitt's lymphoma), RL (non-Hodgkin's lymphoma), MCF-7 (breast cancer) and MDA-MB-435S cells (data not shown). Interestingly, this observation was not extended to NSCLC A549 cells (Fig. 6A), which shows some selectivity in the cellular association of targeted formulations. In fact, autocrine signaling with neuropeptides involving gastrin releasing peptide receptor, or the expression of V_{1A} receptors, is not present in NSCLC cells [43,44]. Impressively, however, non-targeted liposomes associated much more extensively to A549 cells than to SCLC H69 cells (approximately 7-fold), association that was inhibited upon incubation of cells at 4 °C (Fig. 5B), and may be, therefore, attributed to active cell internalization. This suggests that antagonist G-mediated targeting might be important in cells that exhibit low capacity for the uptake of non-targeted sterically stabilized liposomes. Also of interest, was the observation of the significantly increased association of

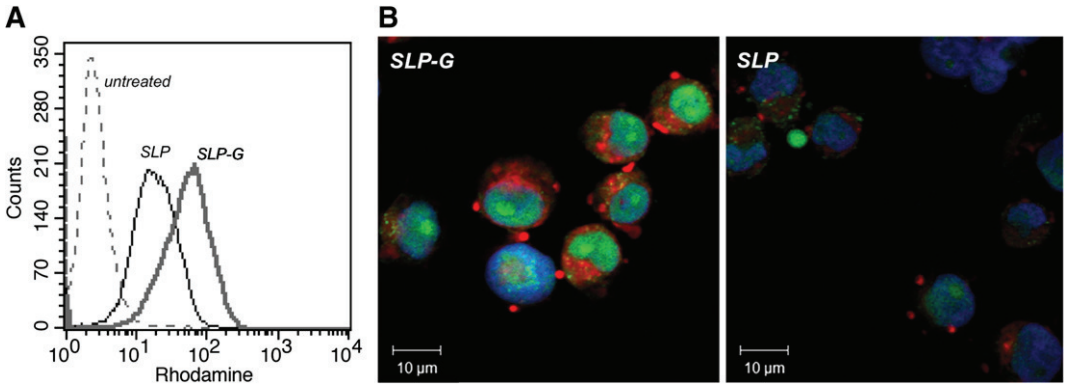

Fig. 4. Qualitative evaluation of the association of non-targeted and antagonist G-targeted SLP^{ODN} to SCLC SW2 cells. Tumor cells were incubated with the liposomes at 0.6 mM total lipid, for 1 h at 37 °C, followed by assessment of cellular association by flow cytometry (A), or incubated for an additional 5 h in fresh medium at 37 °C before observation by confocal scan fluorescence microscopy (B). The nucleus was stained with Hoechst (blue), lipid was labeled with rhodamine-PE (red) and ODN were labeled with carboxyfluorescein (green).

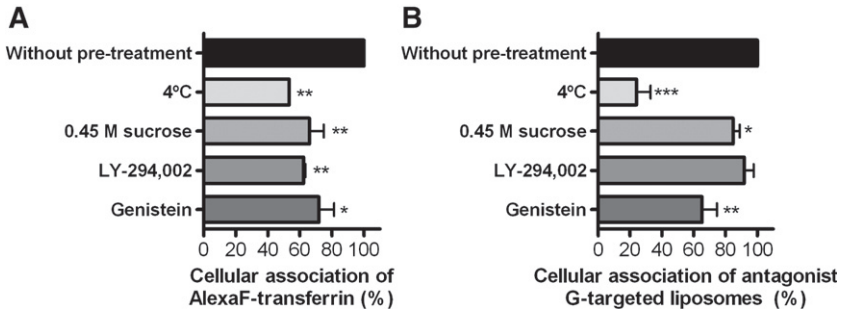

Fig. 5. Evaluation of the mechanisms of cellular internalization of antagonist G-targeted liposomes in SCLC H69 cells. The effect of several endocytosis inhibitors on the association of AlexaF-transferrin (A) and of antagonist G-targeted liposomes (B) to tumor cells was evaluated by fluorimetry. Tumor cells (1×10^6) were incubated with inhibitors of endocytosis for 30 min, and then incubated with either AlexaF-transferrin at 100 μg/ml or antagonist G-targeted liposomes at 0.1 mM of total lipid, for 1 h, as described in Section 2. The results of cellular association evaluated by fluorimetry were expressed in percentage relative to those obtained for control cells (without pre-treatment), at 37 °C. Bars are the mean ± SD of at least 3 independent experiments. '*' symbol represents the significance level of the difference of the mean of inhibitory treatments from 100% (one-sample *t* test).

antagonist G-targeted liposomes to HMEC-1 cells, a model of proliferating microvascular endothelial cells (Fig. 6B). Overall, these results suggest that antagonist G-mediated internalization of liposomes might be valuable in cancer types with different histological origins and in endothelial cells of angiogenic vessels.

3.4. Assessment of Bcl-2 protein levels in SCLC cells upon transfection with antagonist G-targeted liposomes containing anti-BCL2 siRNA

We have previously selected an anti-*BCL2* siRNA that significantly downregulated Bcl-2 in SCLC SW2 cells in a sequence-specific manner, both at the protein and mRNA levels at a concentration of 50 nM (unpublished observations). Such sequence was therefore selected to test the transfection ability of the developed antagonist G-targeted liposomes (SLP) in SCLC cells. Surprisingly, the improved cellular association of antagonist G-targeted SLP containing siRNA did not have any impact on the Bcl-2 protein levels of SCLC SW2 cells relative to untreated cells (data not shown), as neither did the non-targeted liposomes nor the antagonist G-targeted liposomes containing a control *non-targeting* (*nt*) sequence. The outcome was the same when the concentration of siRNA was increased to 4 μM or when double treatments were attempted (data not shown). These results clearly indicate that there are a number of biological obstacles, besides liposomal internalization, that compromise gene silencing. The absence of Bcl-2 downregulation might be due to an inadequate release of the nucleic acids from the endocytic pathway, due to the presence of 10 mol% of PEG incorporated in the SLP formulation [45] and/or to the absence of membrane destabilizing/fusogenic lipids [3]. PEG plays the double role of preventing aggregation during liposomal preparation and of modulating biodistribution and pharmacokinetics of the liposomes [46].

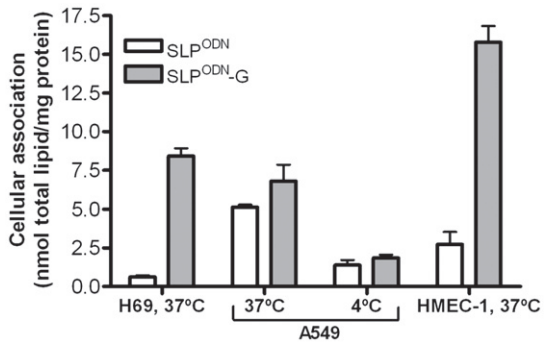

Fig. 6. Association of non-targeted and antagonist G-targeted SLP^{ODN} with SCLC H69 and NSCLC A549 cells (A), or HMEC-1 endothelial cells (B). Cells were incubated with rhodamine-labeled SLP^{ODN}, either targeted or non-targeted, at 0.2 mM of total lipid, for 1 h at 4 or 37 °C, as indicated. Bars are the mean ± SEM, $n=3$.

Tekmira developed SNALP formulations containing siRNA, which are a good example of a competent-transfecting system targeting the liver [5,6]. These particles are prepared at an initial −/+ charge ratio of 1:2 or lower, and incorporate a high proportion of cationic lipid (30 to 40 mol%). The excess of positive charges likely stabilizes the liposomes in the step where ethanol is present, in such extent that this procedure demands the incorporation of only 2 mol% of a PEG-derivatized lipid [4,6], which might, in part, justify their transfection capabilities. At physiological pH, however, even if the assumed final neutrality of the particles, due to the low pK_a of the cationic lipid, makes them adequate for systemic administration, the reduced amount of PEG, grafted with a C14 lipid anchor, dramatically limits their circulation half-life in the blood (72 min in cynomolgus monkeys and 38 min in mice, [5]). These formulations, while efficiently transfecting the liver, are not so efficient in transfecting subcutaneous tumors [6]. Targeting distant disease sites (beyond the liver), like small cell lung cancer, with liposomes, probably does not cope with such low levels of PEG.

Moreover, the main endocytosis pathways used by antagonist G-targeted SPL (caveolin- and clathrin-independent endocytosis) might lead to degradation of the encapsulated nucleic acid. In this respect not much is known, just that the caveolae-mediated endocytosis pathway, absent in this type of cell lines [37,38], has been described to be more efficient in terms of cell transfection than the clathrin-mediated one [36,47]. The cell entry pathway used by targeted liposomes incorporating receptor ligands has never been an issue when delivering small molecular weight drugs, like doxorubicin [48,49]. However, the scenario seems to be different when this same system is used to deliver nucleic acids. Only a few examples of successful targeting of liposomes containing nucleic acids designed for systemic antitumor therapy have been reported. Even in such reported cases, the success was limited. GD2-targeted CCL containing anti-c-myb or anti-c-myc ODNs were active only when used in multiple treatments every two days (for a minimum of 3 treatments), at 10 or 20 μM ODN [50,51]. Furthermore, EpCAM-targeted CCL encapsulating a bispecific anti- (*BCL2* and *BCL-xL*) gapmer ODN (with enhanced potency compared to phosphorothioate), required double treatments of 5 μM ODN, for 4 h each, to achieve 50% to 60% target silencing (with 25% cytotoxicity) [52]. However, these can be considered extreme concentrations, difficult to be reached at the target tumors *in vivo*.

In the present paper, we were able to generate peptide-targeted liposomes containing nucleic acids, with high encapsulation efficiency and loading capacity, which significantly increased intracellular accumulation of the encapsulated nucleic acid as compared to non-targeted liposomes. However, the enhanced cellular association is merely one of the components towards an efficient transfection of tumor cells. The past effort to find a way to improve cell transfection has led to the discovery of agents (e.g. fusogenic lipids, peptides or

drugs) that facilitate liposomal-content release from the endocytotic pathway [24,53,54]. It is interesting to notice that some targeting ligands themselves, such as transferrin, also actively promote the endosomal release of the nucleic acids [55]. In the present work, the absence of Bcl-2 downregulation indicates that additional improvements need to be performed on the liposomal formulation, namely aiming at ameliorating the access of the nucleic acids to the cytoplasm of the tumor cells following receptor-mediated endocytosis.

Acknowledgements

The authors would like to acknowledge Sagid Hussain, PhD student in the laboratory of Molecular Oncology of the University Hospital of Zurich, Switzerland for his advice on CCL preparation, Prof. Theresa M Allen from the University of Alberta, Edmonton Canada, for her advice on SALP preparation , and Luisa Cortes from CNC, for her assistance with the confocal fluorescence microscopy.

A. Santos and L.C. Gomes da Silva were students of the international PhD program on Biomedicine and Experimental Biology, from the Center for Neuroscience and Cell Biology, and recipient of fellowships from the Portuguese Foundation for Science and Technology (FCT) (ref.: SFRH/BD/11817/2003 and SFRH/BD/33184/2007, respectively). The work was supported by a Portuguese grant from FCT, POCTI and FEDER (ref.: POCTI/FCB/48487/2002), and Portugal-Spain capacitation program in nanoscience and nanotechnology (ref.: NANO/NMed-AT/0042/2007).

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