Solid lipid nanoparticles carrying lipophilic derivatives of doxorubicin: preparation, characterization, and in vitro cytotoxicity studies

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Abbreviations:

BA, behenic acid; C_{12}-DOXO ester, lauroyl ester of doxorubicin; C_{12}-DOXO amide, lauroyl amide of doxorubicin; DMF, N,N dimethylformamide; DMSO, dimethyl sulfoxide; DOXO, doxorubicin; %EE, entrapment efficiency; EPR, enhanced permeability and retention; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide; Na-BA, sodium behenate; NPs, nanoparticles; PVA 9000, 80% hydrolyzed polyvinyl alcohol 9000-10000; SLNs, solid lipid nanoparticles.

Chemical compounds studied in this article:

Doxorubicin (PubChem CID: 31703)

Behenic acid (PubChem CID: 8215)

ABSTRACT

Doxorubicin (DOXO) lauroyl ester and amide were proposed as lipophilic derivatives and entrapped in SLNs. DOXO derivatives-loaded SLNs were spherical shaped, had 200-300 nm mean diameters and showed 80-94% w/w drug entrapment efficiencies.

The effect of DOXO derivatives-loaded SLNs and free DOXO on cell growth was examined by MTT and colony-forming assays on four different tumor cell lines: a pancreatic, CFPAC-1, a lung, A549, and two ovarian, A2780 and A2780res (DOXO-resistant).

The results obtained with MTT and colony-forming assay show that although DOXO displayed an inhibition of cell proliferation greater or similar to DOXO lauroyl amide loaded SLNs on all cell types, the effect induced by DOXO lauroyl ester loaded SLNs was higher and concentration-dependent, and it was the only one maintained at 10^{-5} mM concentration.

Only DOXO lauroyl ester loaded SLNs were able to induce a 40% inhibitory effect on A2780 res cell line up to 10^{-4}mM concentration.

INTRODUCTION

Doxorubicin (DOXO) is one of the most effective chemotherapeutics used against a wide spectrum of solid tumors in clinical cancer therapy for over 30 years. However, even when located in the tumor *interstitium*, similarly to other anticancer drugs, its efficacy against several solid tumor types can be limited, owing to the ability of cancer cells to develop drug resistance mechanisms (MDR) and to evade chemotherapy. Transmembrane proteins act as drug efflux pumps, actively reducing intracellular drug to levels lower than the effective cytotoxic concentration. Due to this efflux and to the resulting sub-therapeutic concentrations of active drug in cancer cells, progressively higher doses of the anti-cancer drug are required. The administration of increased DOXO doses enhances the risk of toxicity to normal cells as well as systemic toxicity to most major organs, especially life-threatening cardiotoxicity (Swain et al., 2003) and frequently occurring bone marrow suppression which force the treatment to become dose limiting. Consequently, the clinical application of DOXO is often limited by its severe toxicity and it is therefore preeminent to find effective approaches to overcome MDR in order to reduce adverse drug reactions.

Over the years, many studies were performed to develop drug delivery systems able to target the tumor site and overcome MDR (Barraud et al., 2005; Duggan et al., 2011; Petschauer et al., 2015).

One of the effective approaches is to use nanoparticle (NP)-mediated drug delivery to increase drug accumulation in drug resistant cancer cells.

A possible application of NPs is the entrapment of poorly soluble drugs (Merisko-Liversidge and Liversidge, 2008) and the delivery of multi-agent enhancing therapeutic effects (Devalapally et al., 2007; Ganta and Amiji, 2009). However, the most attractive feature of multifunctional NPs for treating MDR cancers is the obtainment of targeting using target molecules such as antibodies, peptides or aptamers, that can help locate, bind or traffic NPs into the target tumor (Iyer et al., 2013). Moreover, it is well known that nanosystems, such as properly designed NPs, can also passively reach tumors by EPR effect, which can be considered as passive targeting (Moghimi et al., 2001).

Among different NPs types, SLNs have attracted increasing attention as potential drug delivery carriers consequently of their physical stability, their capacity to protect labile drugs from degradation, the easiness of preparation, and the lack of toxicity (Laquintana et al., 2009; Doktorovova et al. 2014; Thukral et al. 2014).

SLNs are disperse systems having size ranging from 1 to 1000 nm and represent an alternative to polymeric particulate carriers. They are composed of physiological or biocompatible lipids or lipid molecules with a history of safe use in therapy and they are generally suitable for intravenous administration, avoiding the toxicity problems caused by polymeric NPs.

SLNs technology represents a promising approach to lipophilic drug delivery, although in recent years, several papers report on SLNs potentially used as carriers of different water soluble drug, i.e. zidovudine, insulin, cisplatin, ciprofloxacin hydrochloride, to improve their therapeutic effects (Gasco et al., 1996; Singh et al., 2010; Trotta et al., 2010; Trotta et al., 2011; Shah et al., 2012).

Moreover, recently, several authors described the ability of SLN formulations to increase both stability and efficacy of water-soluble substances other than drugs (i.e. vitamin C, caffeic acid) allowing their improved use (Dikmen et al., 2015; Güney et al., 2014).

In literature, many authors studied SLNs as systems able to deliver anticancer drugs to the tumor site (Wong et al., 2007). Miao et al. (2013) found that a SLN-based drug delivery system could increase the transport of paclitaxel or DOXO into cancer cells and enhance the cytotoxicity against both sensitive and their multi-drug resistant variant cells, compared with free drug solutions. It can be supposed that SLN lipid matrix can protect the entrapped drug from the P-gp efflux mechanism of the cell and overcome MDR, revealing a potential application of this drug resistance reversal mechanism in drug resistant human cancer cells.

Some authors (Ma et al., 2009) found that SLNs loaded with DOXO were able to overcome Pgp-mediated MDR both *in vitro* in P388/ADR leukemia cells and *in vivo* in the murine leukemia mouse model. The results suggested that SLNs might offer potential to deliver anticancer drugs for the treatment of Pgp-mediated MDR in leukemia.

Recently, a new solvent-free technique, defined as "coacervation" was developed to prepare fatty acids-based SLNs (Battaglia et al., 2010). In a fatty acid alkaline salt micellar solution, in the presence of an appropriate polymeric stabilizer, the pH is lowered by acidification and the fatty acid precipitates as SLNs owing to proton exchange between the acid solution and the sodium salt.

DOXO was studied as anticancer drug to be entrapped in SLNs, but, owing to its hydrophilic nature that hampers its entrapment in SLNs, several strategies were performed to increase its lipophilicity and to favor its entrapment in the SLN core.

In previous studies, lipophilic counter ions were tested to perform lipophilic ion pairing of DOXO (Battaglia et al., 2014); among several screened counter ions, only sodium dioctylsulfosuccinate (AOT) allowed to obtain an ion pair entrapped in SLNs with high efficiency. Although cytotoxicity studies on glioma cell lines and *in vitro* BBB permeation studies revealed a higher performance of DOXO-AOT entrapped in SLNs than free DOXO, the possibility of ion pair dissociation after dilution (occurring after iv administration) is an actual risk that should not be underestimated.

The aim of this work is to prepare, characterize and evaluate the potential of SLNs to release DOXO lipophilic derivatives to tumor cells *in vitro* and to demonstrate that the entrapment of DOXO in SLNs does not negatively influence the cytotoxicity of the native drug.

For these reasons, in this experimental work we decided to synthesize lipophilic prodrugs of DOXO and entrap them in SLNs; lipophilic DOXO prodrugs and analogues have been described in the literature (Chhikara et al., 2011; de Graaf et al., 2004; Ibsen et al., 2010; Wang et al., 2006). Therefore, two different DOXO derivatives, lauroyl ester and lauroyl amide were prepared and proposed as model of DOXO lipophilic derivatives to be entrapped in fatty acid SLNs.

For the above-mentioned purpose, SLNs were prepared and extensively characterized *in vitro* with regard to their physicochemical properties, their capacity to load lipophilic derivatives and their cytotoxicity.

METHODS

Chemicals

Na-BA was purchased from Nu-Chek Prep, Inc. (Elysian, U.S.A.). PVA 9000, acetonitrile, DMF, dioxane, DMSO, lauric acid, THF, trifluoroacetic acid and dichloromethane from Sigma (Dorset, UK); hydrochloric acid, sodium hydroxide and monobasic sodium phosphate from Merck (Darmstadt, Germany); methanol, thionyl chloride, diisopropyl ether, diethyl ether and ethanol from Carlo Erba (Val De Reuil, France); deionized water was obtained by a MilliQ system (Millipore, Bedford, MO).

Synthesis of DOXO derivatives

C_{12}-DOXO ester: KF (0.16 g, 2.70 mmol) was added to the solution of 14-bromo/chloro daunorubicin hydrobromide (0.30 g, 0.45 mmol) and lauric acid (0.27 g, 1.35 mmol) in dry DMF, in one portion and the reaction mixture was stirred at room temperature for 72h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (eluent: gradient from 98/2 to 9/1 dichloromethane /methanol) to give a red solid. The obtained compound was dissolved in freshly distilled dry THF, and two equivalents of HCl 1.7 M solution in dry dioxane was added. The resulting mixture was stirred for 2 h at room temperature, than diluted with diisopropyl ether. Precipitated hydrochloride was filtered, washed extensively with diethyl ether and dried in a vacuum chamber to give a title compound as a red powder (yield 58% w/w). HPLC (Waters Acquity UPLC, Waters Corp., Milford NA, column Xterra MS C18 2.1×150, 3.5 µm; flow rate = 0.3 ml/min; A = 0.1% v/v formic acid in water, B = 0.1% v/v formic acid in acetonitrile, gradient (B%, time (min)): 10,1; 90,9; 10,5): Rt = 10.7 min, 98% (Water Acquity PDA detector, λ 480 nm and 254 nm).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 0.85 (t, 3H, CH_2CH_3); 1.18 (m, 3H, $^{5'}CHCH_3$); 1.24 (m, 16H, $8CH_2$); 1.57 (m, 2H, $OCOCH_2CH_2$); 1.70 (d, 1H), 1.89 (m, 1H) ($^{2'}CH_2$); 2.00 (d, 1H), 2.30 (d, 1H)

($^{8}CH_2$); 2.39 (t, 2H, OCOCH_2); 2.75 (d, 1H), 2.97 (d, 1H) ($^{10}CH_2$); 3.63 (m, 1H, $^{4'}CH$); 3.94 (s, 3H, OCH_3); 4.25 (q, 1H, $^{5'}CH$); 4.87 (bs, 1H, $^{4'}COH$); 5.25 (m, 3H, ^{7}CH + $^{14}CH_2$); 5.45 (bs, 1H, ^{9}COH); 5.73 (s, 1H, $^{1'}CH$); 7.55 (m, 1H), 7.79 (m, 2H) (3C*H* Ar); 8.02 (bs, 3H, NH_3^+); 13.13 (s, 1H, PhO*H*); 13.94 (s, 1H, PhO*H*).

ESI-MS m/z = 726.1 $[M+H]^+$.

C_{12}-DOXO amide: to synthesize lauroyl chloride, lauric acid (0.50 g, 2.5 mmol) was added to freshly distilled thionyl chloride (10 ml) and stirred under reflux for 8 hours. Thionyl chloride was then evaporated and product was used in the next step without further purification.

To synthesize DOXO lauroyl amide, DOXO hydrochloride (0.23 g, 0.40 mmol) was dissolved in water/acetonitrile mixture (10 ml/10 ml) and pH was adjusted to 7 by addition of diluted NaOH solution. A solution of lauroyl chloride (0.36 g, 1.64 mmol) in acetonitrile (15 ml) was added dropwise at room temperature. During the addition pH was monitored and maintained to 7 by addition of diluted NaOH aqueous solution. Reaction was monitored by TLC (silica gel, dichloromethane/methanol = 6/4) and when it was finished acetonitrile was removed under vacuum and aqueous phase was extracted with dichloromethane (3×15 ml). Organic layer was dried with Na_2SO_4 and solvent was removed under vacuum. Crude product was then purified by flash chromatography (eluent: 8/2 dichloromethane/methanol) to obtain a red solid (yield 80% w/w).

TLC: silica gel, dichloromethane/methanol = 6/4, Rf=0.9; HPLC (Waters 1525EF pump, W717 autosampler and Waters 2996 PDA detector, column Xterra C8 4.6×150, 5 µm; flow rate =1 ml/min; A = 0.1% v/v trifluoroacetic acid in water, B = 0.1% v/v trifluoroacetic acid in methanol, gradient (B%,time (min)): 0,30; 7.5,30; 25.0,100; 35.0,100): Rt = 29.3 min, 85% (λ 480 nm and 254 nm); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 0.64-0.53 (m, 3H, CH_3); 0.76 (m, 16H, 8CH_2); 0.95 (d, 3H, $^{6'}CH_3$); 1.41 (d, 2H, NHC(O)CH_2CH_2); 1.81-1.70 (m, 1H, ^{7eq}H); 2.04 (dd, J=9.35 Hz, J=6.31 Hz, 1H, ^{7ax}H); 2.08-2.13 (m, 2H, NHC(O)CH_2); 2.46 (d, 1H, $^{3'ax}H$); 2.55 (d, J=4.11, 1H, $^{3'eq}H$); 2.96 (d, J=19.8 Hz,

^{9ax}H); 3.22 (d, J=19.2 Hz, ^{9eq}*H*); 3.70 (s, 1H, $^{6'}$*H*); 3.99 (s, 3H, OC*H*$_3$); 4.13-4.04 (m, 2H, ^{10}H + $^{4'}$H); 4.67 (s, 2H, ^{14}C*H*$_2$); 5.20 (s, 1H, $^{5'}$*H*'); 5.41 (m, 1H, $^{2'}$*H*); 5.76 (d, J=6.3, 1H, O*H*); 6.80 (s, 1H, N*H*); 7.31 (d, *J* = 8.6 Hz, 1H, Ar ^{4}C*H*); 7.70 (t, *J* = 8.2 Hz, 1H, Ar ^{3}C*H*); 7.95 (d, *J* = 7.2 Hz, 1H, Ar ^{2}C*H*); 13.18 (s, 1H, PhO*H*).

ESI-MS m/z = 726.73 $[M+H]^+$, 748.64 $[M+Na]^+$.

Determination of partition coefficient of C_{12}-DOXO derivatives

Octanol/water partition coefficient (P) of C_{12}-DOXO derivatives was determined using biphasic n-octanol/water system (octanol and water were mutually saturated). 2 ml C_{12}-DOXO ester or amide-saturated water solution, obtained centrifuging an oversaturated suspension of both compounds, was added to 2 ml n-octanol in a separating funnel. The aqueous phase concentration of both C_{12}-DOXO derivatives was determined by HPLC. The mixture was then shaken for 10 minutes and left to rest overnight; after phase separation, C_{12}-DOXO derivative aqueous phase concentration was determined by HPLC. The C_{12}-DOXO derivative concentration in the organic phase was determined by the difference between its aqueous phase concentrations before and after phase separation. P was calculated as n-octanol/water C_{12}-DOXO derivatives molar concentration ratio at the equilibrium. The results were expressed as log P. Each experiment was repeated thrice.

C_{12}- DOXO derivatives loaded SLN preparation

SLNs were prepared using the coacervation method (Battaglia et al., 2010) which uses hydrophilic polymeric as stabilizers able to confer hydrophilicity to SLN surface. Further to a preliminary cytotoxicity screening against hCMEC/D3 cells relating to different fatty acids, which evidenced that BA-SLNs didn't cause any detectable cytotoxicity against hCMEC/D3 cells (Gallarate et al., 2014), BA was chosen as lipid matrix.

Briefly, appropriate amounts of Na-BA (1% w/w) and PVA 9000 (2% or 4% w/w), as steric stabilizer (Scholes et al. 1999), were dispersed in 5 ml deionized water and the mixture was then heated under stirring (300 rpm) just above the Krafft point of Na-BA (75 °C) to obtain a clear micellar solution.
A selected acidifying solution (100 µl 1M NaH_2PO_4 + 160 µl 1M HCl) was then added drop-wise until pH 3.5-4.0 was reached. The obtained suspension was then cooled in a water bath under stirring at 300 rpm up to complete precipitation of SLNs. SLNs were stored at 4 °C before characterization.

Drug-loaded SLNs were prepared as described for blank SLNs (empty SLNs), introducing C_{12}-DOXO derivatives as follows:
- 400 µl of C_{12}-DOXO ester methanolic solution (10 mg/ml) was added:
a) in the micellar hot solution before acidification and SLN formation;
b) directly into the SLN suspension heated just above the melting point of BA (80 °C);
in both methods a) and b) the sample was then cooled in a water bath under stirring at 300 rpm until the temperature reached 15 °C.
- 100 µl of C_{12}-DOXO amide ethanolic solution (40 mg/ml) was added in the same way as the ester derivative.
SLN samples were prepared under sterile conditions working in a horizontal laminar flow hood to perform *in vitro* studies on cell lines.

SLN characterization

C_{12}-DOXO derivatives localization into SLN dispersion was determined using optical microscopy equipped with a fluorescent lamp (Leica DM 2500, Solms, Germany) at 630x magnification.
SLN particle sizes, polydispersion indexes (PDI) and Zeta potential were determined 1 hour after preparation using dynamic light scattering technique-DLS (Brookhaven, New York, USA). Size measurements were obtained at an angle of 90° at 25 °C. The dispersions were diluted with water or

with the different growth media described later in cytotoxicity studies for size determination, or with 0.01 M KCl for Zeta potential determination, in order to achieve the prescribed conductibility.

For size stability studies *vs* time, the samples were stored at 4 °C. All data were determined in triplicate.

%EE determination was performed as follows: 1 ml SLN suspension was centrifuged for 15 min at 62,000 g, the precipitate was washed twice with 1 ml 30:70 v/v ethanol:water to eliminate adsorbed drug. The solid residue was dissolved in 1:1 v/v DMSO:dichloromethane; 0.1 ml water was then added to precipitate the lipid matrix and the supernatant was injected in HPLC for C_{12}-DOXO derivative determination.

C_{12}-DOXO derivative %EE was calculated as the ratio between drug amount in SLNs and that in the starting micellar solution × 100.

%EE was also determined after 1:100 dilution in water. An aliquot of suspension was stirred for 1 hour, centrifuged for 15 min at 62,000 g and then treated as previously described.

The amount of unentrapped drug was eliminated from drug-loaded SLN formulation by gel centrifugation on Bio-gel P-6DG (pore size 90-180 μm, nominal exclusion limit 6000 Da).

HPLC analysis

C_{12}-DOXO amide: HPLC analysis was performed using a LC-6A pump (Shimadzu, Tokyo, Japan) with a reversed-phase column (Chromsystem™ ODS, 125×4.6mm, 2.5 μm particle size) and a C-R6A integrator (Shimadzu, Tokyo, Japan). HPLC grade methanol/water (85/15 v/v) with pH 7 adjusted by NaOH 0.01 N was used as a mobile phase with a flow rate of 1ml/min. UV-Vis detector was set at λ=480 nm. Rt was 5 min.

C_{12}-DOXO ester: HPLC analysis was performed with YL9100 HPLC system equipped with a YL9110 quaternary pump, a YL 9101 vacuum degasser and a YL 9160 PDA detector linked to YL-Clarity software for data analysis (Young Lin, Hogye-dong, 258 Anyang, Korea) and with MERK 50986 LiChrospher 100 RP8 5 μm 80×4.6mm column. Acetonitrile (A)-phosphate buffer (50 mM at

pH=2 adjusted by HCl 0.01 N) (B) with gradient system (30%A/70%B to 90% A/10% B in 15 min at flow rate: 1ml/min; total run 25 min) was used as mobile phase. Rt was 14 min.

In vitro cytotoxicity studies

MTT assay

The following human tumor cells were used: CFPAC-1 (pancreatic adenocarcinoma cell line), A459 (lung cancer cell line), A2780 (ovarian cancer cell line) and A2780 res (A2780 resistant to DOXO cancer cell line). All of them were obtained from the American Type Culture Collection (ATCC; Manassas, VA). A2780 and A2780 res were grown as a monolayer culture in RPMI 1640 medium, CFPAC-1 in DMEM, A549 in DMEM+F12, all of them supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l L-glutamine and penicillin/streptomycin (100 units/ml), at 37 °C in 5% CO_2 humidified atmosphere.

A2780 and A2780 res cell lines ($2x10^3$/well), CFPAC-1 and A549 (800/well) were seeded in 96-well plates and incubated at 37 °C, 5% CO_2, for 24 h. Then, they were treated with C_{12}-DOXO amide and C_{12}-DOXO ester loaded SLNs prepared as reported above and diluted to obtain C_{12}-DOXO derivatives in the 10^{-2}-10^{-5} mM range, based on %EE. After 72 h incubation, the amount of viable cells was evaluated by MTT (Sigma-Aldrich) inner salt reagent at 570 nm, as described by the manufacturer's protocol. The controls (i.e. cells that had received no drug) were normalized to 100%, and the readings from treated cells were expressed as % viability. Eight replicates were used to determine each data point and five different experiments were performed.

Colony-Forming assay

A2780 and A2780 res cell lines ($2x10^3$/well), CFPAC-1 and A549 (800/well, determined according to cell growth rate), were seeded into six-well plates and the day after they were treated with the compounds diluted to obtain DOXO derivatives concentrations in the 10^{-2}-10^{-6} mM range (based on %EE). The medium was changed after 72 h and cells were cultured for additional 7 days in a drug-

free medium. Subsequently, cells were fixed and stained with a solution of 80% crystal violet (Sigma-Aldrich) and 20% methanol. Colonies were then photographed and counted with a Gel Doc equipment (Bio-Rad Laboratories, Milan, Italy). Then the cells were perfectly washed and 30% v/v acetic acid was added to induce a completely dissolution of the crystal violet. Absorbance was recorded at 595 nm by a 96-well-plate ELISA reader. Five different experiments were performed.

Data analysis

Data are shown as mean ± SEM (standard error mean = standard deviation/√number of replicates). Statistical analyses were performed with GraphPad Prism 3.0 software (La Jolla, CA, USA) using one-way ANOVA and Dunnett's test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Water solubilities of C_{12}-DOXO amide and C_{12}-DOXO ester were respectively 64.49 µg/ml and 38.47 µg/ml.

Log P values of C_{12}-DOXO amide and C_{12}-DOXO ester, 2.58 and 3.47 respectively, were significantly higher than that of DOXO (log P = 0.6 reported by Chhikara et al., 2011), showing an increase in lipophilicity compared to the parent drug.

Mean diameters (±standard error, S.E.), PDI and Zeta potential of SLNs, determined by DLS, are reported in Table 1.

[TABLE 1 HERE]

Mean diameters are in the 200-300 nm range. Zeta potential of SLNs prepared by coacervation is around 0 mV (Chirio et al., 2011). Probably, PVA 9000 used as stabilizer locates itself externally, thus partially screening the surface charge. Zeta potential indicates the surface charge of SLNs: Zeta potential values of empty SLNs confirmed the external positioning of the stabilizing polymer. Being Zeta potential also related to drug arrangement in the lipid matrix of these systems (Kushwaha et al., 2013), probably, the slightly positive Zeta potential of SLN C_{12}-DOXO ester is due to partial arrangement of the drug to the outside of SLN surface, conferring them a positive charge.

Although a high value of Zeta potential is generally required to improve the stability of disperse systems, in the present case SLNs are stable in spite of 0 mV Zeta potential, due to the surface stabilization of polymer that avoids aggregation.

Size measurements were performed also on samples diluted with the different growth media used in *in vitro* experiments in order to evaluate the possible changes of SLN sizes when tested *in vitro.* The growth media do not seem to influence SLN dimensions, as all SLNs maintained their size (± 5%).

The formulations were analyzed to verify their overtime size stability. Particles diameters measured at different times up to 70 days were practically unmodified (Figure 1).

[FIGURE 1 HERE]

In Figure 2a and 2b SLN microphotographs are reported. The observation of SLN suspensions under optical microscope confirmed the spherical shape of the particles. The use of fluorescent light allowed to locate C_{12}-DOXO derivatives within the SLNs: the dispersions of fluorescent SLNs were observed in both suspensions.

[FIGURE 2 HERE]

In Table 2 the amount of C_{12}-DOXO derivatives in SLN, prepared by dispersion in 2% w/w PVA9000 aqueous solution, is expressed as %EE and as drug loading (µg C_{12}-DOXO derivative/mg BA). %EE was calculated after SLN centrifugation and washing with ethanol:water 30:70 v/v.

[TABLE 2 HERE]

SLNs showed a good C_{12}-DOXO derivatives loading capacity. In particular, %EE did not significantly vary by increasing the drug amount, while it was significantly influenced by PVA 9000 content. When the stabilizer percentage was increased from 2% w/w to 4% w/w in SLN loaded with C_{12}-DOXO ester (added after re-melting) a decrease in drug %EE from 80 to 70 ± 5% (w/w) was noted; probably PVA 9000 formed micelles in aqueous phase able to solubilize the ester derivative.

When SLNs carrying both derivatives (added after SLN re-melting) were 1:100 diluted in physiological fluids, drug %EE remained almost unchanged (±5%). This is a successful improvement compared with the almost complete loss of %EE registered when DOXO-AOT ion pair was entrapped in SLNs (Battaglia et al., 2014).

On the contrary, when both derivatives were added to the micellar solution, after 1:100 dilution %EE decreased up to 65% w/w. Probably, when added to the micellar solution, derivatives are partially entrapped in PVA chains located on the surface of SLNs and therefore they might be released upon high dilutions.

After gel centrifugation of SLNs, in which the drug was added in the melted lipid, resulting %EE of C_{12}-DOXO ester was 73% w/w and that of C_{12}-DOXO amide was 65% w/w. The gel centrifugation method allows to eliminate the not-completely entrapped drug, that cannot be extracted by simple centrifugation and washing.

In vitro cytotoxicity studies

The effect of C_{12}-DOXO amide loaded SLNs, C_{12}-DOXO ester loaded SLNs and free DOXO on cell growth of three different tumor cell lines was examined. Since, among different carcinoma types, ovarian, pancreatic and lung carcinoma are those with the worse prognosis, any therapeutic improvement is imperative: therefore, their cell types were selected as a model of tumor with high aggressive behavior (Szepeshazi et al., 2013; Giovinazzo et al., 2016; Lixia et al., 2016). Initially, the ability of SLNs loaded with C_{12}-DOXO derivatives and of free DOXO to inhibit the growth of CFPAC-1, A549, A2780 and A2780res cells was compared. Cells were cultured in the presence and absence of titrated amounts (10^{-5}-10^{-2} mM) of DOXO or both C_{12}-DOXO derivative loaded SLNs for 72h, that is the same time point used for the subsequent clonogenic assay. 72 h was chosen as most significant time to detect cell growth inhibition, as preliminary *in vitro* release studies showed a very slow release of both DOXO derivatives from SLNs (data not reported). The amount of viable cells was then assessed by the MTT assay. MTT, a yellow tetrazole, is reduced to purple formazan in living

cells. Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell. Therefore, reduction of MTT depends on the cellular metabolic activity due to NAD(P)H flux. Cells with a low metabolism such as thymocytes and splenocytes reduce very little MTT. In contrast, rapidly dividing cells exhibit high rates of MTT reduction.

The obtained results showed that DOXO displayed an inhibition of cell proliferation greater or similar to C_{12}-DOXO amide loaded SLNs on all cell types (Figure 3). It has been revealed that the presence of the amide bond reduced the anticancer activity on A2780, A549 and CFPAC-1 cell lines, in accordance with previous literature data (Chhikara et al., 2011) suggesting that the presence of free amino group is required for anticancer activity of DOXO. Otherwise C_{12}-DOXO ester loaded SLNs showed a stronger inhibitory effect than DOXO. The effect was concentration-dependent with some differences among the four cell lines. The most responsive cell line was A549, where C_{12}-DOXO ester loaded SLNs were always more efficient than the free drug (Figure 3B; inhibition rate: 90% vs 60% at 10^{-3} mM, 65% vs 40% at 10^{-4} mM). The two formulations displayed a similar efficacy on CFPAC-1 in the range of concentrations 10^{-4}-10^{-2} mM, but C_{12}-DOXO ester loaded SLNs retained their activity at 10^{-5} mM (50% of inhibition) and became inefficient at 10^{-6} mM (data not reported), while the free drug was completely ineffective at 10^{-5} mM (figure 3A). A2780 cell line was sensitive to both compound, but at 10^{-5} mM, only C_{12}-DOXO ester loaded SLNs retained a weak inhibitory effect (Figure 3C; 38% inhibition).

[FIGURE 3]

In order to deepen the evaluation on the efficacy of C_{12}-DOXO derivatives loaded SLNs, we decided to test their ability to exert an antitumor activity also on a particular ovarian cancer cell line resistant to DOXO (A2780res).

As expected, free DOXO was effective only at the highest concentration (10^{-2} mM; 40% inhibition), while C_{12}-DOXO ester loaded SLN was more efficient since it was able to induce the same inhibitory effect (40%) also in the range of concentrations 10^{-2}-10^{-4} mM (Figure 3D).

To investigate the possibility that empty SLNs may exert cell toxicity (not related to DOXO), the effects of empty SLNs (diluted as to obtain 10^{-5}-10^{-2} mM DOXO-derivative concentration range) on cell growth of the four tumor cell lines were evaluated using the MTT assay. Results showed that SLNs did not affect cell growth even at the highest concentrations.

Table 3 shows the IC_{50} for all tested cell lines, revealing that C_{12}-DOXO ester loaded SLNs were always more effective than the free drug except for A2780 cell line, as confirmed by MTT assay, where C_{12}-DOXO ester and free DOXO were similarly efficient at concentrations higher than 10^{-5} mM.

MTT test allows the evaluation of cell enzymatic activity after 72 h of incubation, but it does not give any information on cell ability to further proliferate even after the removal of the drug from culture medium.

Colony-forming (clonogenic) assay is the "gold standard" cellular-sensitivity assay and it is more representative of the *in vivo* condition (Langdon and Macleod, 2004).

In order to validate the previous findings, clonogenic survival assays were performed. Clonogenic assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. After plating at very low density (800 or 2 x 10^3 cell per well), cells were treated with the samples for 72 h, then samples were removed by washing the cells with the cell medium and the cells were allowed to growth over extended period of time (7 days). Only a fraction of seeded cells retained the capacity to produce colonies.

The results shown in figure 4 were similar to those obtained with the MTT assay, but the inhibitory effect was more prominent, since noticeable even at 10^{-6} mM, in A549 and A2780. In fact, C_{12}-DOXO ester loaded SLNs retained their ability to inhibit CFPAC-1 strongly, being able to induce the growth

of only 20% of colony at 10^{-5} mM. DOXO was almost inefficient at the same concentration. A549 were also more sensitive to C_{12}-DOXO ester loaded SLN than to DOXO, since it was still active at 10^{-6} mM, when only 60% of the colony were able to grow. A2780 resulted particularly sensitive to DOXO also in the clonogenic assay, but C_{12}-DOXO ester loaded SLNs demonstrated a stronger efficacy at 10^{-5}-10^{-6} mM. Finally, A2780 res showed a better response to C_{12}-DOXO ester loaded SLN than to DOXO to the concentration 10^{-3} mM, even if they were less responsive than the other cell types.

[FIGURE 4 HERE]

In Figure 5 representative photos of clonogenic assay carried out on the four cell lines are reported. In each non-resistant cell line, C_{12}-DOXO ester loaded SLNs maintained their anti-proliferative effect up to 10^{-5} mM concentration, whereas, samples treated with free DOXO began to grow.

[FIGURE 5 HERE]

DISCUSSION

BA SLNs, loaded with DOXO lauroyl ester or lauroyl amide, were prepared with the coacervation technique.

Spherical shaped SLNs with mean diameters in the 200-300 nm range were obtained.

SLNs showed a good C_{12}-DOXO derivatives loading capacity, but by introducing C_{12}-DOXO derivatives after SLN re-melting instead of adding them to the micellar solution allowed to obtain higher drug %EE, as reported in previous work (Chirio et al., 2014). Probably, the high drug lipophilicity does not allow its distribution within micelles, while promoting its solubilization in the molten lipid.

Therefore, the preparation method consisting in the addition of both lipophilic DOXO derivatives in the re-molten lipid resulted to be the most suitable for our purposes, especially for *in vitro* cell toxicity studies, as well as for a possible future *in vivo* administration.

C_{12}-DOXO amide loaded SLNs were not more effective than DOXO in inhibiting cell growth. Conjugation of 4'-amino group with fatty acid through an amide bond reduced or maintained almost unmodified the anticancer activity of the free drug in tested cancer cell lines. This suggests, as already demonstrated by Chhikara et al. (2011) that the presence of free amino group is required for anticancer activity of DOXO.

DOXO hinders the resealing of DNA double helix strands inhibiting the enzyme topoisomerase II during the replication and thereby arresting the reproduction of the cells. Replication inhibition is due to the structure of DOXO: the planar aromatic portion of the molecule intercalates between two base pairs of the DNA helix. The carbohydrate part of DOXO is involved in the binding to replicating DNA bases during its mechanism of action, and amine group should be freely available for interaction with the flanking base pairs.

Also in this study, the requirement for availability of the sugar moiety in DOXO was confirmed. SLN-loaded C_{12}-DOXO amide was probably only partially hydrolyzed even after long incubation time providing only a slow anti-proliferative activity in these specific cell lines.

The effect on inhibition of cell proliferation induced by C_{12}-DOXO ester loaded SLNs was concentration-dependent, with some differences among the four cell lines and between the SLN-loaded pro-drug and the free drug. The results obtained with clonogenic assay were similar to those obtained with the MTT assay. In fact, C_{12}-DOXO ester loaded SLNs retained the ability of the drug to inhibit CFPAC-1, A2780 and A549 strongly at 10^{-5} mM, while free DOXO was almost inefficient or less efficient at the same concentration. The observation that the inhibition detected by the clonogenic assay was substantially higher than that detected by the MTT assay suggests that cells, which were still viable in the MTT assay, after 72 h of treatment, were severely damaged and unable to proliferate in the clonogenic assay, partially confirming the slow release of DOXO-ester from SLNs. The positive result obtained also on A2780 cell lines, on which C_{12}-DOXO ester loaded SLN and free DOXO were similarly efficient in MTT test, reinforces this hypothesis.

Even A2780 res, less responsive than the other cell types, showed a better response to C_{12}-DOXO ester loaded SLNs than to free DOXO. Probably, the capacity of C_{12}-DOXO ester loaded SLNs to partially inhibit the proliferation also of a resistant cell line might be ascribed to the overcoming of MDR due to the protection of the entrapped drug from the P-gp efflux mechanism of the cell. Further investigation on different resistant cancer cell lines are therefore needed to confirm such hypothesis.

From a formulative point of view, SLNs described in this paper proved to be able to incorporate lipophilic pro-drugs of DOXO with good %EE, which was maintained also after 1:100 dilution, other than what was noted in a previous work (Battaglia et al., 2014) for DOXO-AOT ion pairs.

The relevance of using DOXO derivatives strongly entrapped within the lipid matrix could be an important prerequisite for a future *in vivo* administration. Many problems are related to *in vivo* administration of nanoparticulate systems, such as the quickly binding to opsonin proteins that allows macrophages to easily recognize and remove them before they can perform their designed therapeutic

function. Therefore, the development of stealth SLNs as well as the modification of SLN surface with specific ligands to specific receptor overexpressed on different tumor cells are the mandatory following steps to begin *in vivo* studies. Moreover the effect on MDR of SLNs will be evaluated measuring the expression (by immunoblotting) and the activities (evaluating efflux of fluorescent substrates, ATPase activity) of Pgp and MRP. If the hypothesis that SLNs can overcome MDR will be confirmed, the effective concentration in the targeted cells will be increased, indirectly reducing the required administered drugs.

The results obtained *in vitro*, if supported by satisfactory pharmacokinetic and biodistribution patterns, with a possible targeting to the tumor site, might suggest the potential use of SLNs as DOXO delivery systems.

As one of the major DOXO adverse effect is cardiotoxicity, which may limit its use, the perspective to reduce its therapeutic dose by employing promising vehicles such as C_{12}-DOXO ester loaded SLNs is an important goal of our future research.

CONCLUSION

SLNs are able to incorporate lipophilic pro-drugs of hydrophilic agents with unsatisfactory biodistribution patterns, and to increase cytotoxicity of parent drug.

Although it is still necessary to assess the ability of SLN to revert MDR by in vitro studies, and to optimize SLN loading capacity, C_{12}-DOXO ester loaded SLNs are promising vehicles to increase DOXO cytotoxicity in perspective of reducing its therapeutic dose and consequently its systemic toxic side effect.

Declaration of Interest section

The authors report no declarations of interest

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FIGURE CAPTIONS

Figure 1 *SLN diameter variation ± S.E. (n=3) vs time*

Figure 2 *Optical microscopy images of C_{12}-DOXO amide loaded SLNs a) and C_{12}-DOXO ester loaded SLNs b)*

Figure 3 *Inhibition of CFPAC-1 cells (A), A549 cells (B), A2780 cells (C), A2780res cells (D) proliferation following C_{12}-DOXO ester loaded SLNs, C_{12}-DOXO amide loaded SLNs and DOXO treatment.* Cells were treated with increasing concentrations (10^{-5}-10^{-2} mM) of C_{12}-DOXO ester loaded SLNs, C_{12}-DOXO amide loaded SLNs, empty SLNs, and DOXO for 72 h; the result was expressed as the percentage of viable cells versus the control expressed as mean ± SEM (n=5). One-way ANOVA and the Dunnett's test revealed statistically significance differences (* $p<0.05$; ** $p<0.01$) of DOXO versus C_{12}-DOXO amide loaded SLNs treated cells and (§ $p<0.05$; §§$p<0.01$) of C_{12}-DOXO ester versus DOXO treated cells

Figure 4 *Effect of C_{12}-DOXO ester loaded SLNs and DOXO treatment on cell clonogenicity was tested by colony forming assay.* Cells (800 or 2 x 10^3 per well) were seeded in 6 well plates and treated with each sample at the indicated drug concentrations (10^{-6}-10^{-2} mM) for 72 h. The medium was then changed and cells were cultured for additional 7 days and subsequently fixed and stained with crystal violet (n=5). One-way ANOVA and the Dunnett's tests revealed statistically significance differences (* $p<0.05$; ** $p<0.01$) of C_{12}-DOXO ester loaded SLNs versus DOXO treated cells.

Figure 5 *Effect of C_{12}-DOXO ester loaded SLNs and DOXO treatment on cell clonogenicity was tested by colony forming assay. Clonogenic assay photos from a representative experiment.* The amount of lipid in empty SLN sample corresponds to the dilution required to obtain 10^{-3} mM C_{12}-DOXO ester.

TABLE CAPTIONS

Table 1 *Mean particle size, PDI and Zeta potential ± S.E. (n=3) of empty SLNs and of SLNs loaded with C_{12}-DOXO derivatives*

Table 2 *%EE (w/w) and drug loading (µg/mg) ± S.E. (n=3) of C_{12}-DOXO derivatives loaded SLNs obtained using two different methods of preparation*

Table 3 *IC_{50} ± SEM (n=5) of C_{12}-DOXO derivatives loaded SLNs and DOXO solution.* One-way ANOVA and the Dunnett's tests revealed statistically significance differences (* $p<0.05$; ** $p<0.01$) of C_{12}-DOXO ester loaded SLNs versus DOXO treated cells