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Influence of lipid composition on the ability of liposome loaded voacamine to improve the reversion of doxorubicin resistant osteosarcoma cells

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ABSTRACT

The plant alkaloid voacamine (VOA) displays many interesting pharmacological activities thus, considering its scarce solubility in water, its encapsulation into liposome formulations for its delivery is an important goal. Different cationic liposome formulations containing a phospholipid, cholesterol and one of two diasteromeric cationic surfactants resulted able to maintain a stable transmembrane difference in ammonium sulfate concentration and/or pH gradient and to accumulate VOA in their internal aqueous bulk. The fluidity of the lipid bilayer affects both the ability to maintain a stable imbalance of protons and/or ammonium ions across the membrane and the entrapment efficiency. It was shown that VOA loaded into liposomes is more efficient than the free alkaloid to revert resistance of osteosarcoma cells resistant to doxorubicin to an extent depending on their composition.

1. Introduction

The plant alkaloid voacamine (methyl-12-methoxy-13-(17-methoxy-17-oxovobasan-3alpha-yl)ibogamine-18-carboxylate, VOA, Chart 1), can be described as a bisindole alkaloid being constituted from a 2-acyl indole unit (vobasine) directly linked to an Iboga skeleton (voacangine).

The biological activity of VOA attracts increasing interest and could lead to the application of this substance in the treatment of many diseases (Amaral et al., 2007; Ramanitrahasimbola et al., 2001; Currais et al., 2014; Chowdhury et al., 2017; Wang et al., 2018). In particular, VOA, due to its inhibitory action on P-glycoprotein (P-gp), could allow bypassing the serious barrier of multidrug resistance (MDR) that is responsible of the failure of the treatment of many human cancers (Condello et al., 2014). In fact, it is known that the administration of VOA in combination with doxorubicin (DOX) increases the cytotoxic effect of DOX on some multidrug resistant tumor cells by inhibiting P-gp action in a competitive way thus inducing an increase of DOX retention and intranuclear location in resistant cells (Meschini et al., 2007, 2005). However, VOA low solubility in water hampers its clinical application. In a previous investigation we included VOA in cationic liposomes increasing its efficiency with respect to the free alkaloid to revert resistance of human osteosarcoma resistant cell line, U-2 OS/DX, to DOX (Altieri et al., 2014). The liposomal carrier not only allowed to circumvent the limitation of the scarce solubility of this alkaloid in water, but also increased its accumulation in target tissues. The cationic component of the formulation, gemini amphiphile (*2S,3S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonium) butane bromide, **1** (Chart 2), had previously shown low toxicity and high efficiency in the delivery of a photosensitizer (Molinari et al., 2007a,b) and in DNA condensation (Bombelli et al., 2005a) and transfection (Bombelli et al., 2005b).

In this work we report on the investigation on the physicochemical properties of liposomes formulated with a phospholipid (PC), namely 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), cholesterol (chol), and diasteromeric amphiphiles **1** or (*S,R*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonio)butane bromide, **2** (Chart 2) and on their ability to deliver VOA to U-2 OS/DX osteosarcoma cells. In fact, it is known that the stereochemistry of liposome components can affect the stability (Morigaki et al., 1997), the morphology (Fuhrhop and Helfrich, 1993) and the physicochemical properties of the aggregates they form alone

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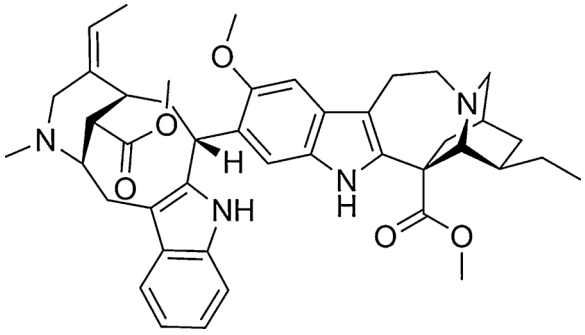

Chart 1. Molecular structure of voacamine.

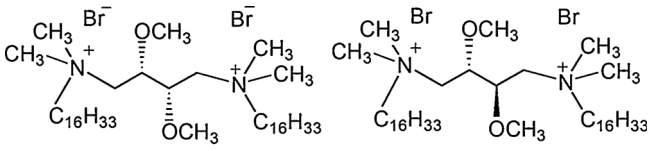

Chart 2. Cationic gemini amphiphile **1** and **2.**

or in formulation with other lipids (Aleandri et al., 2012; Tsuchiya and Mizogami, 2018), and hence their biological activity (Molinari et al., 2007a,b; Bombelli et al., 2005a,b; Touitou et al., 2004; Reddy et al., 2000; Bozzuto and Molinari, 2015). Moreover, also the nature of phospholipid component can play a pivotal role in controlling features and functionality of lipid bilayers (Som and Tew, 2008; Gradella Villalva et al., 2017; Scindia et al., 2007). We also evaluated the enhancement of DOX uptake induced by liposome loaded VOA and eventual cytotoxicity and morphological modifications induced by liposome loaded VOA plus DOX.

The presence of two tertiary amine groups (pKa = 5.19 and 6.78) (Buchi et al., 1964) in VOA molecule allowed us to load it actively in the internal aqueous compartment of the liposomes by ammonium sulphate transmembrane pH gradient (Haran et al., 1993).

2. Methods

2.1. Materials

DPPC and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification (purity > 99%). Gemini **1** and **2** were prepared and purified as previously described.[25] Chol (purity 99%), Sephadex G-50, 4-heptadecyl-7-hydroxycoumarin (C17-HC), dialysis tubing cellulose membrane D 9527, ammonium sulfate, MTT solution, trypan blue and PBS (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4 at 25 °C) were purchased by Sigma Aldrich (Milano, Italy). VOA was purchased from THC Pharm GmbH The Health Concept (Frankfurt am Main, Germany). Staurosporin (STS) Cyclosporine A (CsA) and DOX were purchased by Sigma Chemical Co. (St Louis, MO, USA).

2.2. Liposomes preparation

Lipid films were prepared on the inside wall of a round bottom flask by evaporation of $CHCl_3$ solutions containing the proper amount of lipids (PC/chol/**1**(**2**) at 1.6:0.4:1 M ratio). The obtained films were stored overnight under reduced pressure (0.4 mbar) and then hydrated using 120 mM ammonium sulfate solution at pH = 5 to obtain a lipid dispersion of the desired concentration. The aqueous suspensions were vortex-mixed and then freeze-thawed six times from liquid nitrogen to 50 °C. Lipid suspensions were then extruded (10 times) through a 200 nm polycarbonate membrane. Extrusions were carried out at 50 °C using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

2.3. Inclusion of VOA into liposome internal aqueous compartment by remote loading

Mixed PC/chol and PC/chol/gemini formulations were prepared as described above at 10 mM total lipid concentration. The ammonium salt in the bulk was removed by exchanging 4 times the external medium with 5% glucose solution (25 fold the liposome dispersion volume) by dialysis. The external concentration of ammonium ions was monitored by conductivity measurements in order to evaluate the removal of ammonium ion from the solution. A proper amount of VOA dissolved in DMSO (the final volume of DMSO being < 1% of aqueous volume) was added over 5 min to preformed liposomes after the formation of the ammonium sulfate gradient to obtain a 1:3.5 VOA/lipid molar ratio. Liposomes were incubated with VOA for 1 h, at 60 °C in the case of the DPPC containing formulations, and at 30 °C in the case of DOPC and DMPC containing formulations. The removal of unentrapped VOA was performed by filtration on Sephadex G-50 minicolumns, preequilibrated with PBS at pH = 7.4 using the dry filtration protocol. 200 µL of liposomes suspension were loaded dropwise on the top of the gel bed and the minicolumn was centrifuged at 1000*g* for 3 min to expel the void volume. Clean buffer (250 µL) was added on top of the column and the column was centrifuged as above. Other 100 µL of buffer solution were loaded on the column that was then centrifuged again as above. This step was repeated until all liposome/entrapped VOA come off the column. The eluted fractions containing VOA entrapped in liposomes were identified by fluorescence measurements. Conductivity was measured at constant temperature (25 °C) using a conductimeter (Hanna Instruments 9932 Microprocessor Conductivity Meter). Formulations were used for physicochemical characterization and biological evaluation within 24 h, though stable for at least one week.

2.4. Liposome size determination

The size and the size distribution of the lipid aggregates were characterized by dynamic light scattering (DLS) measurements. A Malvern NanoZetasizer apparatus, equipped with a 4 mW HeNe laser source (632.8 nm) was used. In this apparatus, the light scattered by the sample, placed in a thermostated cell-holder, is collected at an angle of 173°. To obtain the size distribution the measured autocorrelation functions were analyzed by means of the CONTIN algorithm, NNLS and exponential sampling in order to obtain the decay time distribution. Decay times are used to determine the distribution of the diffusion coefficients D of the particles, which in turn are converted in a distribution of apparent hydrodynamic radii RH using the Stokes-Einstein relation $R_H = K_BT/6\pi\eta D$ where K_BT is the thermal energy and η the solvent viscosity. Monomodal distribution were obtained for all the samples, with peaks at values very close to the hydrodynamic radii inferred from cumulant analysis. Therefore the cumulant analysis radii were reported in this work along with the corresponding polydispersity index (PDI). The data were averaged over several measurements.

2.5. Evaluation of the entrapment efficiency (E.E)

Equal amounts (20 µL) of the liposome suspension before and after removal of free VOA were dissolved in 3 mL of isopropanol. The E.E. was evaluated by the ratio of the fluorescence emission at 365 nm (λ_{exc} = 280 nm) of the samples before (Ib) and after (Ia) removal of free VOA, *i.e.* (Ia /Ib)·100 after the correction of Ia for the dilution factor.

2.6. Surface potential measurements

C17-HC-containing liposomes were prepared by adding the proper amount of C17-HC in CHCl3 to the lipid chloroform solution to obtain,

after hydration 50 μM HC, at a lipids/C17-HC molar ratio 250:1 (0.5 mM total lipids). The preparation of C17-HC-containing liposomes was performed using the dry lipid film procedure described above in the dark to avoid C17-HC photodegradation. The fluorescence measurements were performed under stirring at 25 °C on a Fluoromax-4 Horiba-Jobin Yvon spectrofluorometer. Fluorescence of C17-HC was measured by scanning at the excitation wavelength between 300 and 400 nm at an emission wavelength of 450 nm, varying the pH of the solution between 2 and 12 by addition of concentrated aqueous sodium hydroxide or hydrochloric acid. The extent of dissociation of C17-HC included in liposome bilayer was evaluated by plotting the ratio of the excitation fluorescence intensities at 380 and 330 nm (pH-independent isosbestic point) as a function of pH. pKa of HC associated with the cationic liposome bilayer ($pKa^{charged}$) corresponds to the inflection point of the plot. The surface potential (ψ°) was obtained by a conversion and rearrangement of the Boltzmann equation:

$$\psi^{\circ} = -e^{-1} (pKa^{charged} - pKa^{neutral}) k_B T \ln 10$$

where k_B is the Boltzmann constant, T is the absolute temperature, *e* is the electron charge, and $pKa^{neutral}$ is the pKa of HC associated with neutral lipid bilayers.

2.7. Cell cultures

The human multidrug resistant (MDR) osteosarcoma cell line (U-2 OS/DX) was kindly provided by Dr. K. Scotlandi, Istituto Ortopedico Rizzoli, Bologna, Italy. U-2 OS/DX cell line was obtained by exposing the parental sensitive cell line to increasing sublethal concentration of DOX up to 580 ng/mL (Serra et al., 1993). U-2 OS/DX cell line was grown as monolayer in Iscove's modified Dulbecco's medium (IMDM Euroclone Carmlington UK) supplemented with 10% fetal bovine serum (FBS, Euroclone), 1% penicillin (50 U/mL) and streptomycin (50 μg/mL) (Euroclone), and 1% non essential amino acids (Euroclone), in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C. As not specified, 1.5×10^5 cells were seeded in 6-well plates for 24 h, and then treated.

2.8. Flow cytometry

All flow cytometric analyses were carried out on cell suspensions (10^6 cells/mL) by incubating monolayer cell cultures with EDTA and trypsin. The analysis of DOX accumulation was performed on U-2 OS/DX cells treated with DOX alone (1.7 μM) or in combination with CsA (5 μM), or VOA (1.4 μM) or empty PC and PC/chol liposomes with or without **1** or **2** surfactant (at the same concentration of VOA loaded liposomes), or the same formulations loaded with VOA (1.4 μM) for 4 h at 37 °C. All liposomes used in these experiments were freshly prepared. Then, cells were detached, resuspended in ice-cold PBS, and analyzed for DOX content. Dead cells were excluded from the analysis by adding trypan blue to the cell suspension before the acquisition. The fluorescent signals were analyzed by a BDLSRII flow cytometer (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) equipped with a 15 mW, 488 nm, air- cooled argon ion laser and a Kimmon HeCd 325 nm laser. The fluorescent emissions were collected through a 575 nm band pass filter for doxorubicin. At least 10,000 events were acquired in log mode. Quantitative analysis of DOX content was carried out on population of living cells identified by selecting cell population from dot plot FSC towards SSC parameters and excluding cell death by trypan blue staining. ACS Diva Software was used to calculate mean fluorescence channel (MFC). Then, arbitrary units: MFC of each sample/ MFC of control cells.

2.9. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

U-2 OS/DX cells were seeded for 24 h in 96-well plates (1×10^4 U-2 OS/DX cells) and then treated for 24 h with DOX (1 μg/mL, 1.7 μM), or VOA (1 μg/mL, 1.4 μM), or empty DOPC/**1**/chol or DPPC/**1**/chol liposomes (at the same concentration of VOA loaded liposomes), or VOA loaded DOPC/**1**/chol liposomes or VOA loaded DPPC/**1**/chol liposomes or free VOA plus DOX, or empty liposomes plus DOX. VOA, either free or loaded in liposomes was administrated 1 h before DOX. All liposomes used in these experiments were freshly prepared. As positive control, cells were treated with 1 μM STS for 24 h. Cell viability was then assessed by MTT assay (Berridge et al., 2005). After washing with PBS, cells were incubated with 2 mg/mL MTT solution for 3 h at 37 °C. All samples were lysed by DMSO and analyzed by a microplate reader (BioRad, California) at 570 nm. Cell viability (%) was calculated as follow: (absorbance mean value of the treated sample/absorbance mean value of the control sample) x 100.

2.10. Phase contrast microscopy

After treatment with DOX alone (1.7 μM) or in combination with VOA (1.4 μM), or empty DOPC/**1**/Chol or empty DPPC/**1**/Chol liposomes (at the same concentration of VOA loaded liposomes), or VOA loaded DOPC/**1**/Chol or DPPC/**1**/Chol liposomes (1.4 μM) freshly prepared for 24 h, cells were washed with medium and re-incubated at 37 °C in drug free medium for 24 h, and then observed by phase contrast microscopy (Zeiss, Axiovert200, Gottingen, Germany).

2.11. Statistical analysis

The values shown in Figs. 1–3 represents the averages ± standard deviations of three independent experiments. Student's *t*-test was used

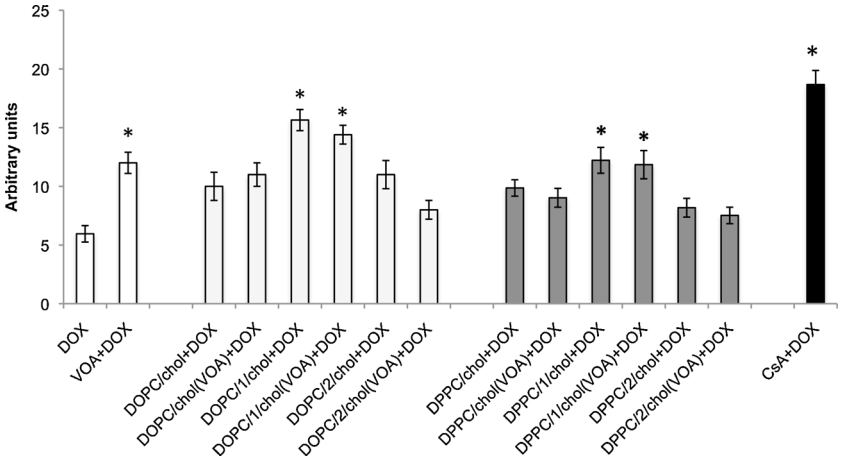

Fig. 1. The effect of VOA loaded neutral liposomes and VOA loaded cationic liposomes on DOX uptake evaluated by flow cytometry. U-2 OS/DX cells were treated with VOA, or VOA plus DOX, or empty liposomes (DOPC/chol, DOPC/**1**/chol, DOPC/**2**/chol or DPPC/chol, DPPC/**1**/chol, or DPPC/**2**/chol) plus DOX, or VOA loaded liposomes (DOPC/chol, DOPC/**1**/chol, DOPC/**2**/chol or DPPC/chol, DPPC/**1**/chol, or DPPC/**2**/chol) plus DOX. CsA was used as positive control. The values are averages ± SD (*, statistically significant when compared to doxorubicin-treated cells).

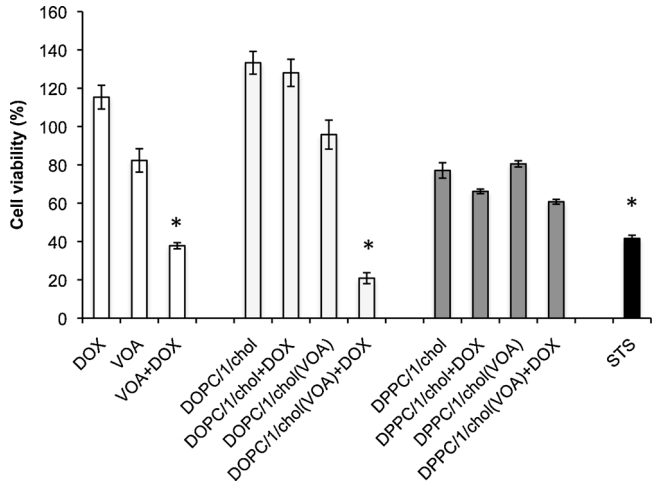

Fig. 2. U-2 OS/DX cell viability evaluated by MTT assay upon different treatments for 24 h: DOX alone, VOA alone, VOA plus DOX, empty liposomes (DOPC/**1**/chol or DPPC/**1**/chol), before empty liposomes then doxorubicin (DOPC/**1**/chol + DOX or DPPC/**1**/chol + DOX), VOA loaded liposomes alone, DOPC/**1**/chol(VOA) or DPPC/**1**/chol(VOA), and VOA loaded liposomes, DOPC/**1**/chol(VOA) or DPPC/**1**/chol(VOA), plus DOX. STS was used as positive control. The values are averages ± SD (*, statistically significant when compared to voacamine plus doxorubicin-treated cells).

for statistical analysis. Differences were considered significant at p values of ≤ 0.05.

3. Results

3.1. Liposome size determination

The size and the size distributions of the liposomes were measured by DLS. Results reported in Table 1 showed that all liposome formulations could be considered as monodisperse and characterized by a ~ 200 nm hydrodynamic diameter, $2R_H$, in agreement with the size imposed by the extrusion procedure. The inclusion of VOA in liposomes does not affect their size and size distribution (data not shown).

Table 1
Size[a] of different mixed liposomes containing DPPC or DOPC, chol and one of the two the cationic surfactants **1** or **2** in different percentages determined by DLS. The reported R_H values were averaged values over several measurements.

Liposomes composition	$2R_H$ (nm)	PDI
DPPC/chol (2:1)	210 ± 13	0.15 ± 0.03
DPPC/**1**/chol (1.6:0.4:1)	220 ± 21	0.20 ± 0.07
DPPC/**2**/chol (1.6:0.4:1)	215 ± 15	0.26 ± 0.04
DOPC/chol (2:1)	203 ± 16	0.19 ± 0.05
DOPC/**1**/chol (1.6:0.4:1)	201 ± 18	0.21 ± 0.02
DOPC/**2**/chol (1.6:0.4:1)	212 ± 26	0.18 ± 0.07

3.2. Surface potential

The ψ° of the cationic liposome formulations was evaluated by measuring the pKa of C17-HC included in the lipid bilayers of charged and neutral liposomes according to a described procedure (Zuidam and Barenholz, 1997). The obtained pKa and the corresponding ψ° values are reported in Table 2.

All the investigated cationic formulations feature as expected a positive ψ°. Liposomes formulated with unsaturated lipids show values of ψ° lower with respect to liposomes formulated with saturated lipids, whereas the nature of gemini component does not affect surface potential.

3.3. Inclusion of VOA into liposome bilayer

Due to the hydrophobic nature of VOA, it could be included into liposome lipid bilayer by passive loading by either adding it to the chloroform solution of lipids before film formation and hydration, or by adding its DMSO solution to preformed liposomes (both in the presence and in the absence of chol). The experiments were carried on 1.4:0.6 PC/**1**(**2**) liposomes devoid of chol, at 1:500 VOA/lipid ratio. Results

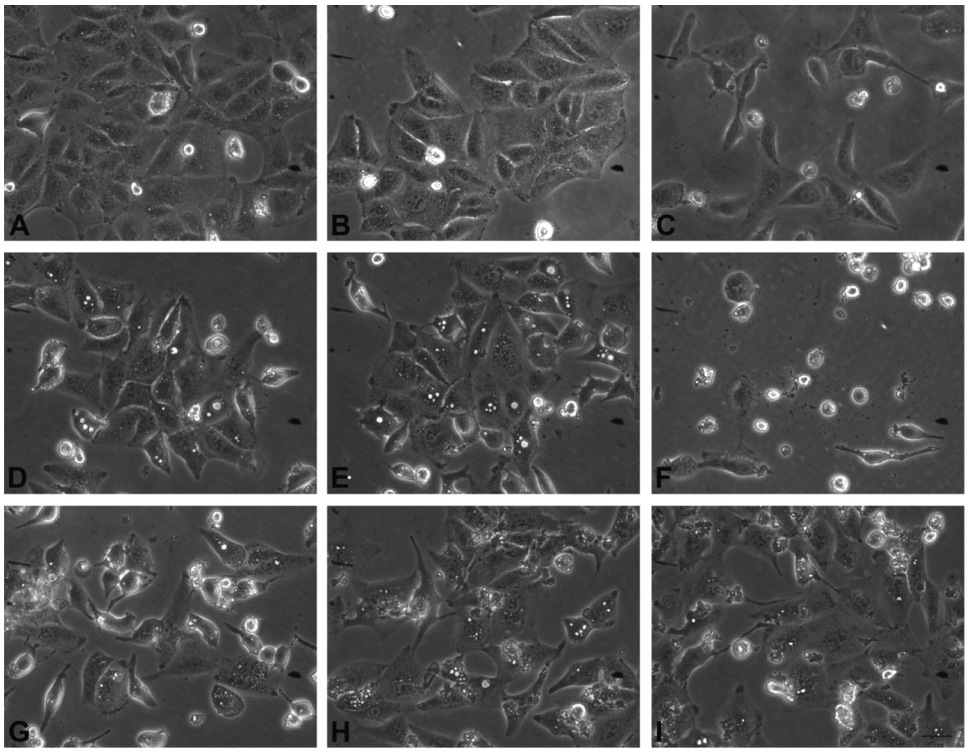

Fig. 3. Phase contrast microscopy observations of U-2OS/DX cells untreated (A) and 24 h treated with DOX alone (B), VOA plus DOX (C), empty DOPC/**1**/chol liposomes (D), empty DOPC/**1**/chol liposomes plus DOX (E), VOA loaded DOPC/**1**/chol liposomes plus DOX (F) empty DPPC/**1**/chol liposomes (G), empty DPPC/**1**/chol liposomes plus DOX (H), VOA loaded DPPC/**1**/chol liposomes plus DOX (I) and then left to recover in drug free medium for 24 h. Scale bar: 10 μm.

Table 2
Surface potential[a] of different mixed liposomes containing DPPC, DOPC, chol and one of the two the cationic surfactants **1** or **2** in different percentages determined by fluorescence measurements.

Liposomes composition	pKa	ψ° (mV)
DPPC/chol (2:1)	9.60	–
DPPC/**1**/chol (1.6:0.4:1)	7.40	130
DPPC/**2**/chol (1.6:0.4:1)	7.40	130
DOPC/chol (2:1)	9.10	–
DOPC/**1**/chol (1.6:0.4:1)	7.82	94
DOPC/**2**/chol (1.6:0.4:1)	7.82	94

[a] The reported values were averaged values over several measurements (error in determination within 5%).

Table 3
Entrapment efficiency[a] of VOA (initial analytical ratio VOA/total lipid 1:3.5) into liposome formulations using the ammonium sulfate gradient method.

Liposomes composition	E.E. (VOA/lipid ratio)
DPPC/chol (2:1)	23 (1:10)
DPPC/**1**/chol (1.6:0.4:1)	27 (1:9)
DPPC/**2**/chol (1.6:0.4:1)	35 (1:7)
DOPC/chol (2:1)	47 (1:5)
DOPC/**1**/chol (1.6:0.4:1)	30 (1:12)
DOPC/**2**/chol (1.6:0.4:1)	37 (1:7)

[a] Results are the average of three experiments and the error in determination is within ± 5% of the value.

from fluorescence measurements before and after gel filtration showed that VOA did not remain included in the lipid bilayer.

3.4. Inclusion of VOA in the internal aqueous compartment of liposomes by remote loading

The E.E. obtained by fluorescence measurements are summarized in Table 3. Care was taken to avoid the precipitation of the uncharged VOA outside liposomes by adding few microliters of a concentrated VOA solution in DMSO to the liposome suspension before incubation. It can be observed that in the formulations devoid of gemini component, the presence of unsaturated phospholipid, DOPC, involves a higher E.E. However the presence of the gemini component levels off the differences due to PC component, with E.E. values slightly higher in the presence of gemini surfactant **2**.

3.5. Effect of VOA encapsulation on DOX accumulation

The effect of VOA loaded in neutral or cationic liposomes on DOX accumulation in resistant cells was evaluated by flow cytometry taking advantage of DOX fluorescence (Fig. 1).

Results, reported in Fig. 1, indicated a low DOX accumulation when resistant cells were treated only with DOX (confirming the resistant phenotype of osteosarcoma cells). Treatment with free VOA before administration of DOX increased DOX accumulation by twice. As positive control, cells were treated with CsA (a known P-gp inhibitor) before DOX administration, obtaining a noticeable increase of DOX utpake. When cells were treated with DOPC/chol or DPPC/chol liposomes either empty or loaded with VOA no significant variation of DOX uptake was observed with respect to the experiment concerning treatment with free VOA plus DOX.

We also evaluated DOX uptake in cells previously treated with VOA loaded in cationic liposomes formulated with gemini **1** or **2** and, in the experiment with liposomes formulated with gemini **1** (either empty or loaded with VOA) we observed (Fig. 1) an increment of DOX uptake with respect to the experiment involving the treatment with free VOA plus DOX. In particular, DOPC/**1**/chol formulation was more effective than DPPC/**1**/chol formulation. On the other hand, DOPC and DPPC liposomal formulations containing **2** and chol did not promote DOX accumulation, with respect to free VOA.

3.6. MTT viability assay of osteosarcoma cells treated with DOPC liposome formulation

MTT assay was carried out on both control and treated U-2 OS/DX cells to verify if the increased accumulation of DOX induced by DOPC/**1**/chol liposomes also involved reduction of osteosarcoma cell viability. The results reported in Fig. 2 show that treatment with 1.7 μM DOX for 24 h did not reduce cell viability. 1.4 μM free VOA induced a slight decrease of cell viability (90%); on the other hand when cells were treated with VOA plus DOX, cell viability decreased up to 40%, a result comparable with treatment with 1 μM STS (positive control) for 24 h. The treatment with empty DOPC/**1**/chol liposomes, empty DOPC/**1**/chol liposomes plus DOX, or VOA loaded DOPC/**1**/chol liposomes did not significantly affect cell viability. However, when cells were treated with DOX after treatment with VOA loaded DOPC/**1**/chol liposomes their viability decreased up to 20%. A slightly toxicity was observed in the case of empty DPPC/**1**/chol liposomes as such or followed by DOX treatment, and in the case of VOA loaded DPPC/**1**/chol liposomes. On the other hand, when cells were treated with VOA loaded DPPC/**1**/chol liposomes plus DOX, their viability did not significantly decreased compared to the experiment involving the treatment with free VOA plus DOX. Therefore, these results indicate that VOA loaded DOPC/**1**/chol liposomes were indeed the most effective formulation to sensitize resistant cells to DOX.

3.7. Assessment of cell morphological changes by phase contrast microscopy

Optical microscopic observations were performed on U-2 OS/DX cells treated for 24 h and then left to recover in drug free medium for additional 24 h to investigate the extent and reversibility of cellular damage. The morphology of cells treated with DOX (Fig. 3B) was not altered and actually cell treated appeared like control cells (Fig. 3A). The treatment with VOA plus DOX induced a reduction of cell population density and some morphological changes (Fig. 3C) compared to the treatment with mere VOA (data not shown) or with DOX alone (Fig. 3B)because cells appeared small and spindly (Fig. 3C). The treatment either with empty DOPC/**1**/chol liposomes (Fig. 3D) or empty DPPC/**1**/chol liposomes (Fig. 3G) induced a negligible cell vacuolization that increased when cells were treated with empty liposomes plus DOX (Fig. 3E and 3 H). The cells treated with VOA loaded DOPC/**1**/chol-liposome plus DOX (Fig. 3F) showed an evident irreversible damage. In fact, most of resistant cells showed rounded and shrinkage shape when compared with cells treated with VOA plus DOX (Fig. 3C). After treatment with VOA loaded DPPC/**1**/chol-liposome plus DOX some cells were vacuolized but alive, their morphology result modified suggesting the recovery of cell damage (Fig. 3I). These evidences, in agreement with MTT test (Fig. 2), confirmed that osterosarcoma resistant cells resulted more sensitive to DOX treatment when VOA was delivered by DOPC/**1**/chol liposomes rather than free or delivered by DPPC/**1**/chol liposomes.

4. Discussion

In a previous investigation we showed that the inclusion of gemini amphiphile **1** in DOPC liposomes increased VOA uptake to U-2 OS/DX osteosarcoma cells (Altieri et al., 2014), therefore we evaluated the influence of lipid composition, by changing both the gemini and the phospholipid component, on physicochemical properties of liposomes and on their ability to deliver their VOA cargo to cells. The results of physicochemical characterization show that all the investigated liposomes feature the same dimension independently of lipid components. The evidence that positive surface potential, ψ°, of cationic liposomes is

not affected by the stereochemistry of the gemini component is in contrast with what observed previously in the case of DMPC/**1** and DMPC/**2** liposomes devoid of chol where ψ° value was strictly dependent on the stereochemistry of gemini surfactants (Bombelli et al., 2010). It is reasonable to hypothesize that the presence of chol, besides reducing permeability of lipid bilayer, also affects lipid organization by leveling off the exposure of ammonium groups of gemini component at the lipid/water interface. On the other hand, ψ° is affected by the nature of phospholipid, DPPC based cationic liposomes featuring the highest surface potential; this result is probably due the higher rigidity of DPPC with respect to DOPC membrane that do not allow a deep embedding of cationic headgroups.

VOA was loaded in the internal aqueous compartment of liposomes at a satisfactory drug/lipid ratio by exploiting both a pH imbalance between the internal compartment of lipid vesicles and the bulk and the weak alkaline nature of VOA. Chol was included in lipid formulation to guarantee the maintenance of pH gradient across the liposome membrane. The capability of 1.6:0.4:1 PC/**1**(**2**)/chol liposomes of maintaining a stable pH gradient (and a stable ammonium sulfate gradient) across liposome membrane and of encapsulating VOA was investigated and compared with that of the 2:1 PC/chol formulation (Table 3). It is known that 2:1 DPPC/Chol liposomes are capable of maintaining a pH gradient, due to an ammonium sulfate concentration gradient, across the liposome membrane, and of loading weak base species due to pH imbalance (Haran et al., 1993). Therefore a pH imbalance across liposomes membrane was created by a transmembrane difference in ammonium sulfate concentration as described in the experimental part. It is worth of note that the impermeability of the liposome membrane to ammonium ions is a crucial parameter to obtain the formation of the transmembrane gradient by the removal of ammonium sulfate from the bulk. An efficient loading of VOA into liposomes requires a pH of the internal aqueous compartment lower than the pKa of its amine functions (pKa 5.19 and 6.78). Usually the acidity of the internal aqueous compartment of liposomes is measured by using pH sensitive probes such as pyranine or acridine orange (Clerc and Barenholz, 1998). In the case of remote loading of VOA, this general procedure is not suitable. In fact, both pyranine and acridine orange are completely protonated in the required internal pH conditions (pH ˜ 4). Hence, after dialysis, different liposomes suspensions were directly incubated for 1 h in the presence of VOA (VOA/lipid 1:3.5) and the efficiency of the entrapment was considered as a proof of the formation and maintenance of the transmembrane pH gradient. Liposomes suspensions were incubated at 60 °C in the case of DPPC containing and at 30 °C in the case of DOPC containing liposomes, because loading has to be performed in conditions in which the lipid bilayer is in its fluid and permeable liquid-crystal state to facilitate its crossing.

The nature of both components affects the E.E. of VOA. Though the rigidity ascribed by chol is necessary to maintain the transmembrane gradient, results clearly show that the disorder attributed to lipid membrane organization by lipid unsaturations better allows membrane crossing by bulky VOA. The presence of gemini component increases the E.E. in the case of DPPC based liposomes, while it reduces it in the case of the DOPC ones. In the first case the effect has probably to be ascribed to the gemini structure that, due to the spacer between head groups, might create gaps in the tight lipid organization of DPPC, thus better allowing bulky VOA to cross lipid membrane. In the case of DOPC the effect has to be ascribed to the saturated chains of gemini that might interfere with the voids created by unsaturated chains of DOPC. The different stereochemistry of **1** and **2** has also a role in E.E. due to the different exposure of gemini methoxy groups (Bello et al., 2006).

Therefore loading of bulky drugs depends on a fine balance between membrane fluidity and organization (compaction). However though characterized by low compaction the membrane of DOPC liposomes showed a scarce affinity toward VOA (even in the absence of the rigidifying component, chol), since they were unable, as well as DPPC liposomes, to bind VOA in the passive loading experiments.

Experiments on an osteosarcoma resistant cell line, U-2 OS/DX, were carried out to evaluate how and to what extent the stereochemistry of the gemini component and the nature of the phospholipid, modify the interaction with cells and the delivery of their cargo. The main outcome of the biological evaluation reported here involved the higher efficiency of VOA delivery of DOPC/**1** liposomes with respect to free VOA and to the other investigated formulations suggesting a different mode of interaction with cell membranes as a function of lipid bilayer fluidity and of the stereochemistry of the gemini component. DOPC/**1**/chol formulation was more effective than DPPC/**1**/chol formulation in favouring DOX cell accumulation (Fig. 1) and, consequently, osteosarcoma resistant cells were more sensitive to DOX cytotoxicity when VOA was delivered by DOPC/**1**/chol liposomes (as indicated by MTT test, Fig. 2, and phase contrast microscopy observations, Fig. 3). These results demonstrated that the fluidity of lipid bilayer is a crucial parameter in controlling cell internalization and/or the release of liposomal drugs, as reported in literature (Kawano et al., 2009). Besides lipid packing, also stereochemistry strongly affects the biological behavior of the formulations. This is not surprising because stereochemistry affects molecular interactions, but it is interesting that in this case **1** containing liposomes interact more efficiently with cells with respect to **2** containing ones, differently from what previously observed studying the delivery efficiency to tumor cells of mixed liposomes formulations containing **1** or **2** devoid of chol (Bombelli et al., 2010). This evidence indicates that the presence of chol not only rigidify lipid bilayer, but also alters the overall organization of lipid and the exposure of polar headgroup; it is possible that the combined presence of chol and **1** or **2** induces a different intracellular drug distribution (as previously observed) (Bombelli et al., 2010) and/or a different internalization pathways thus influencing the efficiency of the treatment with respect to the presence of **1** or **2** without chol.

5. Conclusions

The correlation between the physico-chemical properties of liposomes containing diastereoisomeric surfactant **1** or **2** and their ability to efficiently deliver VOA to U-2 OS/DX cancer cells confirmed that VOA loaded into our cationic liposomes is more efficient than the free alkaloid to revert resistance of osteosarcoma cells resistant to DOX. The stereochemistry of the components seems to modify the interaction with cells, and therefore the efficiency of the treatment. Moreover, our results demonstrate that lipid organization, in particular fluidity of lipid bilayer, affects the efficiency of delivery and thus the ability of VOA to exert its pharmacological effect. As a whole, the results reported in this investigation show that also subtle variations of the molecular structure of liposomes components can control the organization of lipids in the vesicle membrane and, as a consequence, their physicochemical and biological behavior.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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