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RESEARCH ARTICLE

Liposomes- and ethosomes-associated distamycins: a comparative study

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Abstract

The present article describes a comparative study of the performances of liposomes and ethosomes as specialized delivery systems for distamycin A (DA) and two of its derivatives. Liposomes and ethosomes were prepared by classical methods, extruded through polycarbonate filters, and characterized in terms of dimensions, morphology, and encapsulation efficiency. It was found that DA was associated with vesicles (either liposomes or ethosomes) by around 16.0%, while both derivatives of DA showed a percentage of association around 80% in the case of liposomes and around 50% in the case of ethosomes. *In vitro* antiproliferative activity experiments performed on cultured human and mouse leukemic cells demonstrated that vesicles were able to increase the activity of both derivatives of DA. In addition, it was demonstrated that the aging of both liposomes- and ethosomes-associated distamycin suspensions did not heavily influence the vesicle size, while all samples showed a relevant drug leakage with time. Moreover, according to the different physicochemical characteristics of DA and its derivatives (i.e., log P), vesicle-associated DA showed the highest loss of drug with respect to both its derivatives. In conclusion, the enhancement of drug activity expressed by these specialized delivery systems-associated DD could be interesting to obtain an efficient therapeutic effect aimed at reducing or minimizing toxic effects occurring with distamycins administration.

Keywords: *Distamycins; liposome; ethosomes; specialized delivery systems; antiproliferative activity*

Introduction

Distamycin A (DA) is an antiviral and antiprotozoal drug (Cozzi and Mongelli, 1998; Cozzi, 2000) able to reversibly bind to the minor groove of DNA with a high selectivity for TA-rich sequences. Precisely, DA is a metabolite of *Streptomyces distallicus*, characterized by an oligopeptidic pyrrolocarbamoyl frame ending with an amidino moiety. In addition, DA was used as a DNA sequence selective vector of alkylating functions, leading to a substantial increase of cytotoxicity, in comparison to that—very weak—of distamycin itself (Cozzi, 2001; Marchini et al., 2001).

Chemical modifications of distamycin could result in active molecules to be employed as anticancer drugs (Khalaf et al., 2004; Punt et al., 1996; Anthony et al., 2007), such as tallimustine (Beran et al., 1998; Tagliabue et al., 1997; Weiss et al., 1997) and lexitropsins (Anthony et al., 2007).

Bearing in mind these potential therapeutic properties, the synthesis of new distamycin derivatives (DDs) and the development of controlled delivery strategies could lead to significant advantages for the clinical use of these molecules, such as solubility, specificity, and toxicity problems associated with their use (Agen et al., 1992; Baraldi et al., 2000).

Among delivery strategies to transport efficiently and selectively a drug, vesicular systems have attracted attention for the number of advantages that vesicles show, such as: (1) the increase of solubilization capacity

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of lipophilic compounds; (2) the modulation of both the pharmacokinetic and the bioavailability of drug; (3) the possible enhancement of cellular internalization; and (4) the decrease of unwanted systemic toxic effects. Further, in some cases, the use of phospholipid vesicles to carry a drug to target cells could possess specific release by passive or active targeting strategies (Lian and Ho, 2001).

Liposomes are vesicles in which an aqueous volume is entirely enclosed by a lipid membrane (Vemuri and Rhodes, 1995; Lian and Ho, 2001). The composition of liposomes is complex, with phospholipids (i.e., phosphoglycerides and sphingolipids together with their hydrolysis products) and cholesterol being the main ingredients. A classification of liposomes can be made on the basis of lamellae, composition, and size and number of membranes.

A liposomal drug-delivery system is advantageous in relation to the drug protection, controlled release of active moiety along with targeted delivery, and cellular uptake via endocytosis (Nakamura et al., 2009; Hillaireau and Couvreur 2009). Drawbacks are degradation by hydrolysis (Krickau et al., 2007), oxidation (Watabe et al., 2007), sedimentation, drug leaching, aggregation, or fusion during storage (Casals et al., 2003).

However, liposome encapsulation can overcome various problems commonly encountered in the delivery of biologicals, such as toxicity, solubility, bioavailability, and immunogenicity of the compound to be delivered. Although the amphiphilic properties of the molecules used to prepare vesicles should increase the permeability of the drug through biological membranes, generally resulting in an augmented intracellular drug concentration (Kakemi et al., 1993), classic liposomes are poorly efficient at delivering molecules through membrane bilayer into cells. In this view, many groups devoted their research to modify phospholipid vesicles in order to increase their ability to penetrate biological membranes (Hillaireau and Couvreur, 2009; Karmali and Chaudhuri, 2007; Miller et al., 1998; Jain and Gewirtz, 1998; Kao et al., 1996). Among these modifications in the obtaining of vesicular carriers, such as ethosomes (Touitou et al., 2000a), have led to a good improvement.

Ethosomes are lipid carriers developed by Touitou et al. (1997, 2000a, 2000b, 2001). Ethosomal systems are easy to prepare, nonirritant, and composed mainly of phospholipids and ethanol—compounds commonly found in pharmaceutical preparations. These "soft, malleable" vesicular systems were found to be highly efficient carriers for the delivery of molecules with various lipophilicities into and through skin, *in vitro* and *in vivo*, in animal and clinical studies (Touitou et al., 2000a, 2000b; Dayan and Touitou, 2000).

Ethosomes have a particle size modulable from tens of nanometers to microns. A reported characteristic of ethosomes is their small size relative to liposomes, due to the incorporation of ethanol (Touitou et al., 2000a, 2000b; Dayan and Touitou, 2000): the higher the ethanol concentration (in the range of 20–45%), the lower the size of ethosomal vesicles (Touitou et al., 2000b; Lopez-Pinto et al., 2005). From a morphological point of view, ethosomes are very similar to liposomes; however, ethosomes exhibit high encapsulation efficiency for a wide range of molecules, including lipophilic drugs. This could be explained by the multilamellarity of ethosomal vesicles (Touitou et al., 2000b) as well as by the presence of ethanol, which allows for better solubility of many drugs. In addition, previous studies have demonstrated that ethosomes were not toxic to cultured cells (Touitou et al., 2001) and are able to highly and efficiently deliver biological and chemical compounds both to skin and cultured cells.

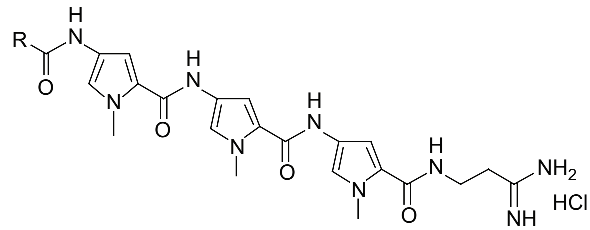
The present study should be considered as an extension of our previous work concerning the influence of the delivery system on the activity of a wide number of DDs characterized by different physicochemical properties (e.g., molecular weight, solubility, interaction with phospholipid, presence of charged groups, hydrophilic/lipophilic balance, etc.) (Cortesi et al., 2004, 2007).

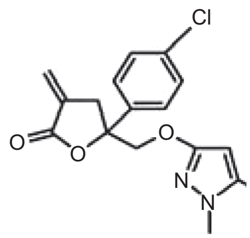
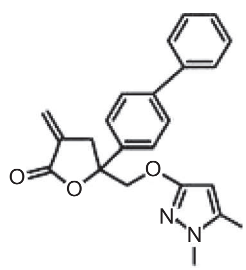
Chemical modifications were introduced in the attempt to obtain more stable compounds, possibly increasing the binding to DNA and selectivity of alkylation, with a concomitant reduction of the adverse effects characterizing the pharmacological profile of DA (Baraldi et al., 2000).

It was demonstrated that the activity of distamycins released by specialized delivery systems is, in many cases, higher with respect to the corresponding drug tested in the free form. The obtained results demonstrated that vesicles did not cause cell-growth inhibition, thus suggesting that the enhanced effect of DD (where it happened) could be reasonably due to the increased solubility and/or bioavailability of the encapsulated compounds. (Cortesi et al., 2004, 2007)

Bearing in mind these considerations, this paper presents (1) the preparation and characterization of liposomes and ethosomes loaded with DA and two DDs, namely DD17 and DD18 (Table 1); (2) a comparative analysis of *in vitro* antiproliferative activity of both liposomes- and ethosomes-associated distamycins; and (3) a preliminary study of the effect of aging on size and drug leakage from these two specialized delivery systems.

Table 1. Chemical structure and some physicochemical characteristics of distamycin A and its derivatives used in the present study.



Drug	R	MW	λ_{max} (nm)	log P
DA	H	518	304	–0.386124
DD17		834.71	309	1.33374
DD18		876.36	306	2.50874

DA, distamycin A; DD17, (5-{5-[5-(2-carbaimmidoyl-ethyl-carbamoyl)-1-methyl-1*H*-pirrol-3-yl-carbammoyl]-1-methyl-1*H*-pyrrol-3yl-carbammoyl}-1-methyl-1*H*-pyrrol-3-yl) amide of 5-[2-(4-chlorophenyl)-4-methylen-5-oxo-tetrahydrofuran-2-yl-methoxy]-2-methyl-2*H*-pyrazol-3-carboxylic acid; DD18, (5-{5-[5-(2-carbaimmidoil-ethyl-carbamoyl)-1-methyl-1*H*-pyrrol-3-yl-carbammoyl]-1-metyl-1*H*-pyrrol-3-yl-carbammoyl}-1-methyl-1*H*-pyrrol-3-yl) amide of 5-(2-diphenyl-4-yl-4-methylen-5-oxo-tetrahydrofuran-2-yl-metoxy)-2-methyl-2*H*-pyrazol-3-carboxylic acid.

Materials and methods

Chemicals

DA was purchased from Sigma-Chemical Co. (St. Louis, Missouri, USA). DDs were synthesized following the procedure reported by Baraldi and colleagues (2004) (see Table 1 for chemical structures). Log P values were calculated by using Advanced Chemistry Development (ACD/Labs 7.0) software. Egg phosphatidyl choline (Egg-PC) was purchased from Lipid Products (Surrey, UK). Cholesterol (CH) was from Sigma-Chemical Co. All other materials and solvents at the high-purity grade were from Fluka (Buchs, Switzerland).

Preparation of liposomes and ethosomes

Liposome-containing distamycins were prepared by reverse-phase evaporation, followed by extrusion through two polycarbonate filters with 200-nm pore size (Nucleopore Corp, Pleasanton, California, USA), as previously described (Cortesi et al., 1994). In particular, 10 mg of the mixture of PC:CH (4:1 mol/mol) and distamycins, in a molar ratio of 1:25 (drug:PC), were dissolved in 1 mL of chloroform-methanol (2:1; v/v) in a 25-mL round-bottomed flask. After removal of solvent by rotary evaporation, the resulting dried lipid-drug mixed film was dissolved in 4 mL of diethyl ether. Then, 3 mL of isotonic Palitzsch buffer (IPB) pH 7.4 (5 mM $Na_2B_4O_7$, 180 mM H_3BO_3, 18 mM NaCl) were added to the solution, and the mixture was sonicated in an ice bath for 10 minutes within a bath sonicator. The ether present was removed by rotary evaporation under reduced pressure until reaching the final liposome suspension.

Ethosomes were prepared by dissolving the mixture of PC:CH (4:1 mol/mol) and distamycins in 0.4 mL of ethanol (20% v/v of the final volume of the formulation). Then, 1.6 mL of IPB were slowly added to the ethanolic solution, continuously stirring at 700 rpm by a Eurostar digital instrument (IKA Labotechnik; Sardo, Torino, Italy). Mechanical stirring was performed for 15 minutes at room temperature.

Both types of vesicle dispersion were then extruded through two stacked, standard 25-mm diameter polycarbonate filters with 200-nm pore size (Nucleopore) at a nitrogen pressure of 10–20 bars, using an extruder (Lipex Biomembranes, Inc., Vancouver, Canada). The vesicles were collected and reinjected three times.

The free compound was separated from vesicle-associated drug by gel filtration on a Sepharose 4B column (Pharmacia, Uppsala, Sweden) (1.5 cm diameter, 50 cm length) preequilibrated and eluted with IPB. The collected fractions from the Sepharose 4B column were checked for drug content by ultraviolet (UV) analysis monitoring at the λ_{max} characteristic of each compound (see Table 1), using a UV/Vis spectrophotometer (Lambda 19; PerkinElmer, Waltham, Massachusetts, USA) after methanol dissolution of the vesicles.

Characterization of liposomes and ethosomes

Vesicle shape was studied by Cryo-TEM (transmission electron microscopy) analyses. Briefly, 2 µL of sample dispersions were placed on a pure, thin-bar 600-mesh TEM grid and blotted with filter paper until it was reduced to a thin film (10–200 nm) spanning the hexagonal holes of the TEM grid. The sample was then vitrified by liquid ethane. The vitrified specimen was transferred to a Zeiss EM922 transmission electron microscope for imaging using a cryoholder (CT3500, Gatan). Specimens were examined with doses of about 1,000–2,000 e/nm^2 at 200 kV. Images were recorded digitally with a charge-coupled device (CCD) camera (Ultrascan 1000; Gatan GmbH, Munich, Germany) interfaced with an image-processing system (GMS 1.4 software; Gatan). Submicron particle-size analysis was performed by using a Zetasizer 3000 PCS (Malvern Instruments, Malvern, UK) equipped with a 5-mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing it with detergent and rinsing it twice with water before injections. Measurements were made at 25°C at an angle of 90 degrees with a run time of 120 seconds. Data were interpreted by using the method of cumulants.

Cell-growth studies

The effect of vesicle dispersions containing distamycins were determined on *in vitro* cultured human and mouse leukemic cells, namely K562 (Lozzio and Lozzio, 1975; Bouffard et al., 1993) and L1210 (Lech-Maranda et al., 2000; Jordheim et al., 2004). Standard conditions for cell growth were α-medium (Gibco, Grand Island, New York, USA), 50 mg/L of streptomycin, and 300 mg/L of penicillin, supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, California, USA) in 5% CO2 at 90% humidity. Cell growth was determined by counting with an automatic blood cell counter (Model LC-550; Horiba Ltd., Kyoto, Japan). Counts of viable cells were performed after a 0.1% trypan blue exclusion test.

Results and discussion

Liposomes- and ethosomes-associated distamycins: production and characterization

As reported above, the present article focuses on liposomes and ethosomes loaded with DA and two of its α-methylen-γ-butyrolactone derivatives, namely the (5-{5-[5-(2-carbaimmidoyl-ethyl-carbamoyl)-1-methyl-1*H*-pirrol-3-yl-carbammoyl]-1-methyl-1*H*-pyrrol-3-yl-carbammoyl}-1-methyl-1*H*-pyrrol-3-yl) amide of 5-[2-(4-chlorophenyl)-4-methylen-5-oxo-tetrahydrofuran-2-yl-methoxy]-2-methyl-2*H*-pyrazol-3-carboxylicacid(DD17) and the (5-{5-[5-(2-carbaimmidoil-ethyl-carbamoyl)-1-methyl-1*H*-pyrrol-3-yl-carbammoyl]-1-metyl-1*H*-pyrrol-3-yl-carbammoyl}-1-methyl-1*H*-pyrrol-3-yl) amide of 5-(2-diphenyl-4-yl-4-methylen-5-oxo-tetrahydrofuran-2-yl-metoxy)-2-methyl-2*H*-pyrazol-3-carboxylic acid (DD18) (Baraldi et al., 2004; Romagnoli et al., 2005). These derivatives belong to a series of conjugates characterized by a distamycin-like moiety able to guarantee the DNA minor groove-binding interaction and a α-methylen-γ-butyrolactone residue to acquire alkylation with biological nucleophiles (Baraldi et al., 2004).

Table 2. Size[a], content and Z potential of vesicle-associated distamycins.

	Liposomes				Ethosomes			
Drug	Mean diameter (nm)	PI	% of drug association	Z potential (mV)	Mean diameter (nm)	PI	% of drug association	Z potential (mV)
None	165.1 ± 1.5	1.0	/	-4.82 ± 0.86	179.5 ± 3.3	0.09	/	-3.12 ± 0.52
DA	142.9 ± 1.5	0.20	16.04 ± 5.10	–5.55 ± 1.15	158.5 ± 0.9	0.18	16.02 ± 2.32	-3.45 ± 0.65
DD17	179.5 ± 0.6	0.23	78.24 ± 8.36	–7.80 ± 0.22	160.6 ± 1.3	0.09	62.52 ± 4.54	-3.30 ± 0.14
DD18	219.7 ± 1.3	0.15	86.80 ± 2.52	–3.92 ± 0.31	167.6 ± 0.7	0.16	41.26 ± 5.23	-4.75 ± 0.71

Data are the mean of five independent experiments.

PI, polydispersity index.

[a]Analyses were performed after extrusion. Vesicles were extruded 3-fold through two stacked, standard 25-mm-diameter polycarbonate filters with 200-nm pore size.

Liposome-associated distamycins were prepared by reverse-phase evaporation and extrusion through polycarbonate membranes filters with 200-nm pores, as described by ourselves (Cortesi et al., 1994, 2004, 2007).

Ethosomes were spontaneously produced by dissolution of PC, CH, and distamycins in ethanol, followed by the slow addition of an aqueous buffer under continuous stirring at room temperature (Touitou et al., 2001; Esposito et al., 2004). During preparation, dispersions displayed initial optical transparency due to the high ethanol concentration able to maintain PC in solution. By adding increasing concentrations of IPB buffer, dispersions became turbid because of the reorganization of PC molecules within the system that results in ethosomal vesicles formation.

Table 2 reports mean diameters of liposomal and ethosomal vesicles after extrusion, as determined by PCS and expressed as Z average diameter. Before extrusion, the mean diameter of liposomes are larger (ranging from 385 to 832 nm) with respect to ethosomes (ranging from 196 to 226 nm). Moreover, the high polydispersity index (PI) of liposomes (PI = 1.0) reflects a broad dimensional distribution; conversely, ethosome PIs do not exceed 0.37, indicating a narrow size distribution.

In order to size the vesicles, distamycins-containing vesicles were extruded through calibrated membrane. Extrusion was, first, performed by one cycle through two stacked membranes with 400-nm pore size and, second, by three cycles through two stacked membranes with 200-nm pore size. As it is clearly appreciable from the obtained data, after extrusion, vesicle populations showed a monomodal distribution, with lower PIs with respect to not-extruded preparations, namely in the range between 0.15 and 0.23 for liposomes and between 0.09 and 0.18 for ethosomes. This result can be attributed to the improving effect of the extrusion step on size distribution.

As previously stated, a gel-permeation chromatography was performed in order to separate the liposome-associated from the free drug. In Table 2 are also reported the percentage of DD association to the produced vesicular systems (i.e., liposomes and ethosomes). It should be noticed that, due to the lipophilic characteristics of DA and its derivatives, these drugs are reasonably expected to be associated (i.e., interpolated) within the vesicle phospholipid bilayer, thus the term "liposome- or ethosomes-associated distamycins" is used to describe vesicle-carrying distamycins. Both types of vesicles showed a DA association of around 16.0%, while, as expected, DD association was higher. Particularly, DD17 displayed an association yield of 78.24% for liposomes and 62.25% for ethosomes, while DD18 showed an association yield of 86.80 and 41.26% for liposomes and ethosomes, respectively. This behavior could be ascribed to the different degree of lipophilicity of the three molecules (Table 1). In particular, considering the log P of the compounds, it is evident that DA (log P = – 0.386) is more hydrophilic than DD17 (log P = 1.333) and DD18 (log P = 2.509), thus, reasonably, its association to both types of vesicles turns out to be lower with respect to its derivatives (Gruner et al., 1985; Mayer et al., 1986).

Moreover, it should be noted that liposomes are able to associate higher amounts of DD, as compared to ethosomes. Particularly, concerning ethosomes, the association yield of the more lipophilic compound (DD18) is lower (41.26%) with respect to that of DD17 (62.52%). The reasons of these results can be ascribed to the partition coefficient of each drug and also to the presence of embedded ethanol within ethosomes able to influence the hydrophobic environment of vesicle bilayer.

In fact, considering the log P of each DD, the higher the log P, the higher the association to liposomes and the lower the association to ethosomes. Moreover, knowing that ethosomes are lipid vesicular systems embodying ethanol in relatively high concentrations, the presence of ethanol intercalating within the phospholipid bilayer is able to enhance lipid fluidity and to disorganize the tight interchain hydrophobic interactions between phospholipid tails. In this respect, DA derivatives are subjected to a reduction of the ability in forming hydrophobic interactions with phospholipid tails, and thus, the association proves to be lower for ethosomes with respect to liposomes.

Further, the polarity of the ethanol molecule could be responsible for the reduced solubility of DD18, as compared to that of DD17 within ethosomes.

Table 3. Effect of free or delivered distamycins on proliferation of murine (L1210) and human (K562) leukemic cells.

		IC_{50}		
Drug	Cell line	Free drug(μM)	Liposome- associated drug(nM)	Ethosome- associated drug(nM)
DA	L1210	74.12 ± 0.92	2032 ± 356	1.02 ± 0.35
	K562	15.36 ± 0.24	1020 ± 614	4.20 ± 0.61
DD17	L1210	22.24 ± 1.11	49.12 ± 2.31	1.3 ± 0.1
	K562	8.70 ± 0.71	24.18 ± 1.16	3.5 ± 0.3
DD18	L1210	31.47 ± 2.33	58.26 ± 3.62	1.6 ± 0.2
	K562	11.41 ± 2.01	40.32 ± 2.44	2.5 ± 0.1

Data are the mean of five independent experiments ± standard deviation.

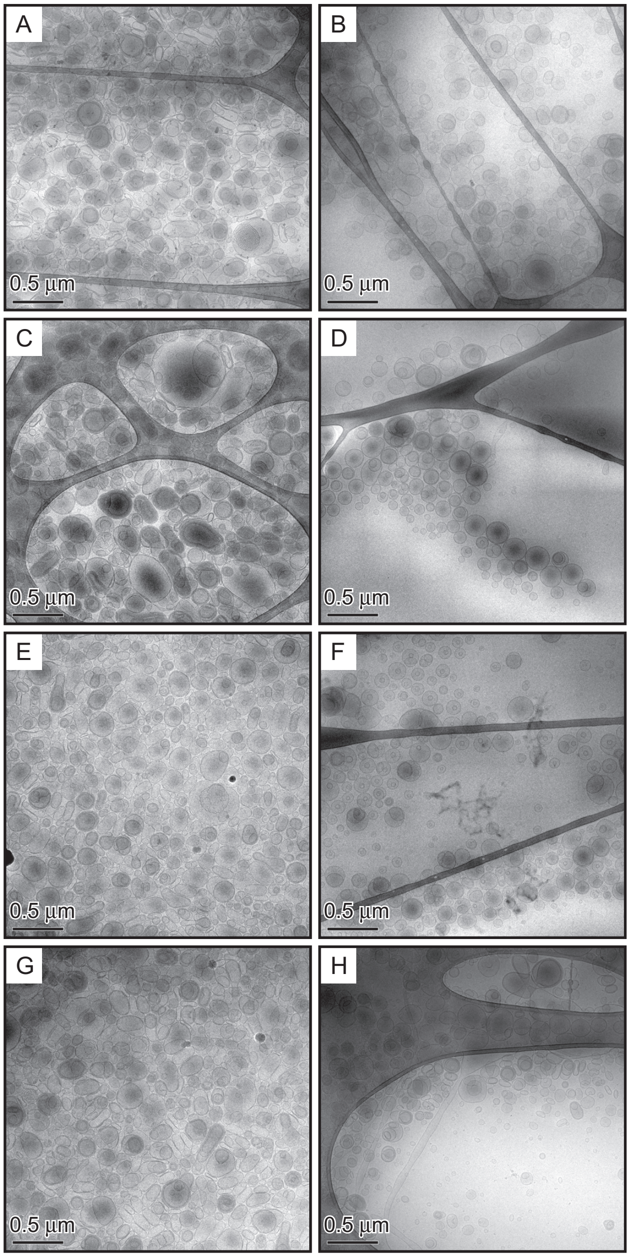


Figure 1. Cryo-TEM microphotographs of liposomes- (A, C, E, G) and ethosomes-associated distamycins (B, D, F, H). Panels A and B: empty vesicles. Panels C and D: DA. Panels E and F: DD17. Panels G and H: DD18.

Shape and morphology of extruded distamycins-associated vesicles were observed by cryo-TEM (Figure 1). One can observe the presence of oligolamellar vesicles, both in liposome- and ethosome-based dispersions. Vesicles are characterized by spheroidal morphology, the same dimensional range, and an average diameter reflecting the pore size of the membrane used for extrusion (Table 2).

From the analysis of the cryo-TEM photographs, no appreciable alteration of the bilayer structure was detectable, due to a possible interaction with the drug.

In vitro *activity of liposomes- and ethosomes-associated distamycins*

As previously reported by Baraldi et al. (2004), among the synthesized α-methylen-γ-butyrolactone derivatives, DD17 and DD18 showed the lower degree of inhibition toward cell proliferation. In order to possibly increase their activity, the effect of vesicular carrier on their *in vitro* performance was investigated.

In vitro antiproliferative activity of liposomes- or ethosomes-associated distamycins, namely DA, DD17, and DD18, was determined and compared with that of the corresponding free compound. Human erythroleukaemic K562 and murine lymphocyte L1210 leukemic cells were treated with the same molar concentrations of free or vesicle-associated distamycins. After 6 days of cell culture, cells were electronically counted and the number of cells/mL was compared with the values obtained in the case of untreated cells. As is clearly evident from the results of these experiments reported in Table 3, in general, the antiproliferative effect of DA, and both DDs delivered by vesicles, is higher with respect to the corresponding free drug.

In particular, an enhancement of drug activity is highly evident for DD17 and DD18, as compared to DA toward both cell lines. Moreover, in general, the used murine cell line showed a high sensitivity to vesicle-associated DD, with respect to the human one. In fact, DA increased its activity against L1210 cells by 36-fold for liposomes and 74-fold for ethosomes, while K562 cells showed an increase of 15-fold for liposomes and 3.7-fold for ethosomes. On the other hand, the DD activity increase toward L1210 was around 500-fold in the case of liposomes and around 18,000-fold in the case of ethosomes. Against K562, the activity was in the range of 300-fold for liposomes and comprised between 2,400 and 4,500 for ethosomes.

These results suggest that the inclusions of DD inside phospholipid vesicles could influence the mechanisms of drug uptake and triggering within cells, leading to an increase in their activity.

It should be underlined that the antiproliferative effect of empty delivery systems on both cell lines was evaluated as control. The obtained results demonstrated that the vehicles do not cause inhibition of cell growth, thus confirming that the enhanced effect of DD is reasonably due to the increased solubility and/or bioavailability of the encapsulated compounds.

Effect of aging on dimensions and drug release of liposomes- and ethosomes-associated distamycins

In order to evaluate the effect of storage on liposome and ethosome size and the possible drug release from distamycins-associated vesicles, the preparations underwent

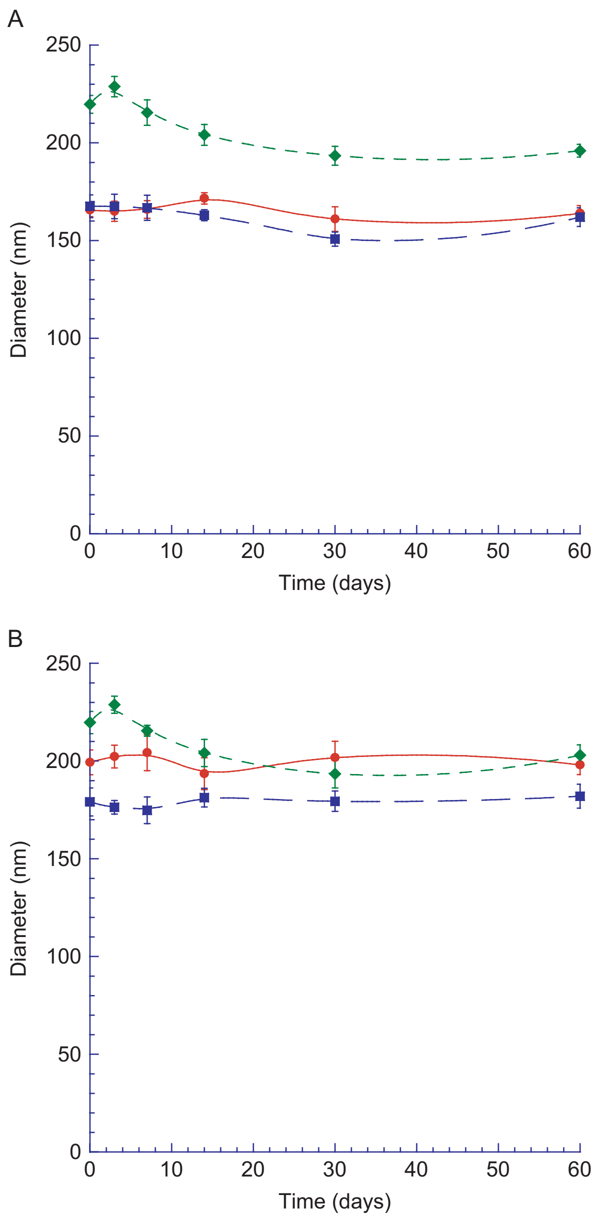


Figure 2. Effect of aging on dimensions of liposome- (panel **A**) and ethosomes-associated (panel **B**) distamycins. (◆): DA; (■): DD17; (◆): DD18. Data represent the mean of three independent experiments ± standard deviation.

dimensional and drug-content analyses. Analyses were performed after different periods of time, namely 7, 14, 21, 30, and 60 days from the preparation.

As can be seen from data reported in Figure 2, aging does not heavily influence the vesicle size of both liposomes- and ethosomes-associated distamycins. Indeed, during the first 2 months, vesicles maintain their mean diameters quite stably, showing at least a variation of around 8–10% with respect to the size of vesicles measured just after production.

The effect of aging was evaluated by taking into consideration the leakage of drug from vesicles. At determined days, the vesicles, previously stored at 4°C, were eluted on a gel-permeation column, in order to evaluate the amount of distamycins released. The results of these experiments, reported in Table 4, indicate that all the preparations showed a relevant drug leakage, as expected. In particular, liposome-associated DA displayed a drug loss of 93.3% after 2 months from preparation. On the other hand, within 2 months from production, the preparations containing compounds of DDs showed a more limited drug leakage with respect to DA, being 31.5% for DD17 and 28.5% for DD18, respectively.

In the case of ethosomes, after 2 months, DA was released for the 65.4%, while DD17 and DD18 were released for the 31.0 and 37.7%, respectively.

The highest loss of drug displayed by DA associated with the vesicles, as compared to that of DD, could be ascribed to the different log P values of the compounds. Moreover, the hydrophobic environment of vesicle bilayer characterized by the presence of embedded ethanol could explain the different extent of DA leakage expressed by ethosomes with respect to liposomes (almost 30% lower for ethosomes).

Conclusions

In the last decade, a large number of studies, performed on the synthesis of new compounds and on the production of drug-delivery systems for antitumor drugs, was aimed at increasing the efficacy of these drugs, limiting their dose. Indeed, the development of an efficient drug-

Table 4. Release of distamycins from liposomes and ethosomes during time.

	Percentage of drug released					
	Liposomes			Ethosomes		
Day	DA	DD17	DD18	DA	DD17	DD18
0	0	0	0	0	0	0
7	19.6±1.2	9.3±2.1	7.8±1.3	18.5±1.6	7.9±1.8	8.3±2.3
14	22.4±1.4	11.7±1.6	9.5±1.6	28.3±2.3	10.3±2.2	19.5±2.5
21	31.7±2.3	13.6±1.8	11.2±2.6	35.7±3.2	17.1±2.8	25.8±3.1
30	40.3±3.1	19.3±2.4	14.9±2.1	44.6±1.9	21.4±1.6	27.4±2.4
60	93.3±3.6	31.5±3.1	28.5±2.9	65.4±2.6	31.2±3.4	37.7±1.9

Data are the mean of five independent experiments ± standard deviation.

entrapment protocol and studies on *in vitro* activity are essential prerequisites to any reproducible clinical trial.

The encouraging results reported above suggest that the enhancement of drug activity expressed by these delivery systems-associated DD and the different extent of DA leakage expressed by ethosomes, as compared to liposomes, could be interesting to obtain efficient therapeutic activities able to reduce or minimize the toxic effects associated with a high dose of distamycins administration.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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