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LIPOSOMES AS A POTENTIAL OCULAR DELIVERY SYSTEM OF DISTAMYCIN A

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Graphical abstract

Abstract

Liposomes containing Distamycin A (DA) may be clinically useful in the treatment of ocular HSV infections, especially in acyclovir-resistant HSV keratitis. This study evaluated the *in vitro* and *in vivo* performance of a topical controlled release liposomal formulation containing DA (DA-Lipo) aimed at reducing the toxicity of the encapsulated active agent and improving drug uptake by ocular tissues. The bioavailability of DA in the tear fluid and the DA uptake into the cornea were increased after instillation of DA-Lipo in rabbits, reaching the DA corneal concentration corresponding to IC_{50} values against HSV without any sign of transcorneal

permeation of drug. DA-Lipo was definitely less cytotoxic then plain DA in rabbit corneal epithelial cells. These results provide new insights into the correlation between the in vitro data and the drug kinetics following ocular applications of liposomal vesicles.

Keywords

Liposome, Distamycin A, antiviral, pharmacokinetic, cytotoxicity, rabbit

1. Introduction

Distamycin A (DA), an antibiotic produced by *Streptomyces distallicus*, is a potent antiviral agent active against Herpes simplex virus (HSV) and Varicella zoster virus (VZV) in cell cultures with a mechanism of action different from that of acyclovir and its analogues. It is a minor groove double strand-DNA binder, since the curvature of its molecular surface makes it complementary in geometrical terms to the DNA double helix in AT rich regions of DNA (Casazza et al., 1966; Chandra et al., 1971; Verini et al., 1976; Broyles et al., 2004). Recently, the activity of distamycin A against acyclovir resistant Herpes viruses has been demonstrated (Matteoli et al., 2008).

DA was developed as an ointment under the name Herperal® useful for treatment of cutaneous localizations in herpetic infections. Although clinical trials showed that Herperal® could be safe when administered for various weeks, its clinical efficacy was limited probably for its scarce penetration into tissues.

The application of liposomes as topical drug delivery systems able to improve tissue penetration has gained wide interest (Ebrahim et al., 2005; El Maghraby et al., 2008; Nounou et al., 2008; Korting and Schäfer-Korting, 2010) and liposomes with different compositions have been reported for ophthalmic therapy, aimed at increasing and/or prolonging the therapeutic effects either by faciliting trancorneal uptake/ penetration or by prolonging the precorneal residence time (Gregoriadis and Florence, 1993; Chetoni et al., 2004; Araújo et al., 2009; Gaudana et al., 2009; 2010). When applied topically as eye drops, liposomes can attach to the hydrophobic corneal epithelium, where they continuously release the encapsulated drug , improving pharmacokinetics and decreasing toxic side effects (Chetoni et al 2007; Habib et al., 2010; Agarwal et al, 2014; Yu, et al., 2015). Therefore, DA entrapped in liposomes could be useful for treatment of ocular viral infections that remain an important cause of external and corneal disease (Green et al., 1984). Humans are the only natural host to HSV and more than 80% of the population carries systemic antibodies to it (Al-Dujaili et al., 2011). Most herpes simplex keratitis in adult is caused by HSV type 1, but cases of simultaneous ocular infection with type 1 and type 2 in patients with AIDS have

been reported. Herpes simplex virus is mainly treated when confined to epithelium, even if recent studies indicate that herpetic stromal disease and iritis are effectively treated with a combination of a corticosteroid and an antiviral drug (Toma et al., 2008; Pepose et al., 2006). However, the extensive use of acyclovir and other nucleoside analogues exerts a continuous selective pressure on the HSV virus population and antiviral drug resistance is not infrequent. On the other hand, DA liposomes are endowed with potential therapeutic properties in the treatment of ocular infections, especially of acyclovir-resistant herpes simplex keratitis.

Thus, the purpose of this study was the preparation and evaluation of a liposomal formulation containing DA aimed at increasing the uptake by ocular tissues and therefore the efficacy of the drug. The liposomal formulation (DA-Lipo) was prepared using phosphatidylcholine and cholesterol and, after its characterization from technological point of view (DA encapsulation efficiency, diameter, *in vitro* DA release rate), the vesicular dispersion was used for biological characterization. The following tests were performed: *a)* antiviral activity against clinical isolates of HSV type 1 (HSV1) and type 2 (HSV2), *b)* cytotoxicity test on VERO cell line and on rabbits corneal epithelial cell line (RCE), *c)* transcorneal permeation of DA both *in vitro,* using reconstituted corneal epithelium and *in vivo* in the rabbit. The main pharmacokinetic parameters of DA in ocular tissues (concentrations in tear fluid, aqueous humor and cornea) after instillation in rabbit eyes of DA-Lipo and of a reference aqueous solution containing free DA hydrochloride (DA-Sol) were determined.

2. Materials and methods

2.1. Chemicals

Distamycin A hydrocloride (DA $C_{22}H_{28}ClN_9O_4$, MW= 517.969) was obtained from Chemper, Prato, Italy). DA contains three pyrrole rings, four amide bonds and one strongly basic amidine function and therefore has properties in common with basic peptides. Among the pKa values, calculated using the ChemSpider pKa program (http://www.chemspider.com), the more interesting is 12.55. Consequently the major microspecie at physiological pH (pH=7.4) is the ionized form in the amidine group with log P = -1.39. In physiological conditions its solubility was calculated to be of 0.5 mg/ml. Phosphatidylcholine (Phospholipon®80, PC) was kindly donated by AVG Srl (Milan, Italy). This is a soy bean lipid extract with 76% (w/w) content of phosphatidylcholine and 3% (w/w) of lysophosphatidylcholine. Cholesterol (Chol) and trypsin/EDTA solution (Sigma Chemical Co., St, Louis, MO. USA); cell proliferation reagent WST-1 (Roche Diagnostic, cat. N° 1644807, F. Hoffmann-La Roche Ltd, Diagnostics Division,

Basel, Switzerland) were used as received. Ultrapure water was prepared using Milli-Q® plus apparatus (Millipore, Milan, Italy). All other chemicals and solvent were of analytical grade.

2.2. Cell cultures and animals

The rabbit corneal epithelial cell line (RCE) was obtained from the European Cell Culture Collection (N° 95081046, ECACC, Salisbury, UK) and was used for cytotoxicity test. The growth medium had the following composition: Dulbecco's modified Eagle's medium (DMEM) with Ham's nutrient mixture F12 (1:1) added of L-glutamine (1% v/v), penicillin (100 IU/mL), streptomycin (0.1 mg/mL), amphotericin B (0.25 μg/mL), foetal bovine serum (15% v/v) (Gibco Invitrogen S.r.l., Milan, Italy); epidermal growth factor (10 ng/mL), insulin (5 mg/mL) (Sigma Chemical Co., St Louis, MO).

The African Green monkey kidney cell line (VERO) was supplied by the Zooprophylactic Institute of Lombardia and Emilia Romagna, "Bruno Ubertini" (Brescia, Italy) and used for antiviral and cytotoxicity tests. The growth medium had the following composition: minimum Eagle's medium, MEM, supplemented with foetal bovine serum (FCS, 5% v/v); penicillin (100 IU/mL), streptomycin (0.1 mg/mL) all from Gibco Invitrogen S.r.l., Milan, Italy. All cell lines were grown at 37 °C in a humidified atmosphere with 5% CO_2.

Male New Zealand albino rabbits weighing 2.8-3.5 kg were purchased from Pampaloni Rabbitry (Pisa, Italy). They were housed in standard cages in a light-controlled room (10 h dark/14 h light cycle) at 19 ± 1°C and 50 ± 5% relative humidity and were given a standard pellet diet and water *ad libitum*. During the experiments the rabbits were placed in restraining boxes to which they had been habituated in a room with dim lighting and they were allowed to move their heads and eyes freely (Stella et al., 2012).

2.3.Preparation of DA liposomal formulation (DA-Lipo)

A liposomal formulation containing DA was prepared by partially modified reverse phase evaporation technique (REV) (Szoka and Papahadjopoulos, 1978). A chloroform:ethanol (1:1) phospholipid solution (30.0 mL) and a pH 7.4 Sørsensen phosphate buffer solution (PBS, 10.0 mL) containing a 1:25 molar ratio between DA (5.0 mg) and PC (248.6 mg), were mixed in a round bottom flask. The organic solutions contained a 4:1 molar ratio of PC (248.6 mg):Chol (23.7 mg) mixture. In order to remove the organic solvent, the dispersion was evaporated at 37°C in a rotary evaporator at 130 rpm under reduced pressure (Rotavapor R II, Büchi Corporation, New Castle, DE, USA). The hydrated vesicles were reduced in size by sonication for 20 seconds (Microson® XL 2000 Ultrasonic Homogenizer, Misonix, N.Y., USA). The DA-containing liposomes were separated from free DA by ultracentrifugation (Sorval® Discovery 90 Ultracentrifuge, Rotor TFT-50.38,

Kendo Laboratory Products, Newton, CT, USA), at $8.0x10^4$ rpm for 20 min at 4°C, followed by dispersion of the liposomal pellet in an appropriate volume of isotonic PBS to obtain the DA-Lipo formulation.

2.4. Preparation of the reference isotonic DA aqueous solution (DA- Sol)

DA was dissolved in PBS solution to a final concentration of 1.38 mg/mL. The osmolality of the preparation was adjusted to a physiological value (308 mOsmol/kg) with sodium chloride (Roebbling Osmometer, Camlab, Cambridge UK).

2.5. Characterization of DA-Lipo formulation

The mean particle size of liposomes was measured by dynamic light scattering (Coulter® N4 Plus Particle Size Analyzer, Coulter Corporation, Miami, FL, USA) after a suitable dilution of the liposomal dispersion with ultrapure water. The size analyses were carried out at 90.0° and 62.6° scattering angles and at a temperature of 20°C by repeated scans for each scattering angle (run time 200 and 400 s for 90.0° and 62.6°, respectively). The zeta potential of liposome (ζ) was measured on a Marvel Zetasizer Nano ZS (Marvel Instruments Ltd., Worcestershire UK) on samples (20 µl) of diluted DA-Lipo formulation in ultrapure water. The value was the average of three measurements.

The amount of DA in liposomes was determined by HPLC after lysis of liposomes with absolute ethanol. The liposomal dispersion was mixed with ethanol in 1:10 v/v ratio; after centrifuging at 13,000 rpm for 5 minutes (IEC Micro CL 17, Thermo Electron Corporation, Waltham, MA, USA) the supernatant was diluted and analysed. Each analysis was performed in triplicate. The encapsulation efficiency (E%) was calculated using the formula: E% = ED/TD x 100 where ED represents the amount of encapsulated DA, and TD the total amount of drug initially added to solution.

In vitro release was determined by the dialysis bag method (Omotosho et al., 1989). To perform the study, a fixed amount (1.0 mL) of liposomal dispersion containing 1.38 mg of DA was taken into a dialysis bag (3.5 kDa MWCO; Spectra/Pore 3 Dialysis Membranes, Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA) and then introduced into 25.0 ml of isotonic PBS release medium. The whole system was incubated at 32 ± 0.5°C under stirring condition at a constant speed (20 rpm) and aliquots of 10.0 mL were withdrawn from the release medium at selected time intervals and replaced with fresh medium. The released amount of DA was detected by means of the HPLC methods. Control experiments were done by taking the same amount of reference isotonic DA aqueous solution (DA-Sol, 1.38 mg/mL). Drug release experiments were

repeated at least thrice to ensure reproducibility. The mechanism of drug release was analyzed by a relationship proposed by Ritger and Peppas (1987) and using Prism software (GraphPad Software Inc., San Diego, CA, USA).

2.6. Antiviral activity

Antiviral activity of DA-Sol and DA-Lipo formulations was evaluated using two different viral strains and VERO epithelial cell cultures. The HSV1 strain was isolated from a *herpes labialis* cutaneous-mucosal lesion and the HSV2 strain from a *herpes genitalis* genital-mucosal lesion (Matteoli et al., 2008). Both HSV strains were collected from immune compromised solid organ-transplanted patients. Sensitivity of HSV2 isolates to DA-Sol and DA-Lipo was tested by the plaque reduction assay (PRA_{50}) (Leary et al., 2002). Since a long period was necessary for HSV1 isolates to induce plaque formation, an immunofluorescence assay (IFA) was performed to investigate HSV1 drug susceptibility after 48 h of treatment (Mentel et al., 1996). In our experimental conditions, results obtained for HSV1 by IFA mirrored those found by plaque assay. All viral isolates were treated with DA concentrations ranging from 0.0772 to 772 μM. Briefly 500,000 VERO cells were seeded on circular sterile slides lodged on well plates and infected with 200 μL of the HSV1 dilution, that produced 200 fluorescent cells, or of the HSV2 dilution that produced 200 plaques after 48 h. After viral infection and 48 h of incubation with DA formulations, cells were treated with a fluorescein-conjugated antibody and analyzed with a fluorescence microscope (Matteoli et al., 2008). The cytopathic effect was determined on the basis of the number of positive fluorescent cells (Mentel et al., 1996). Drug concentration that inhibited viral cytopathic effect by 50% with respect to the control (IC_{50}) was calculated using Prism software.

2.7. Cytotoxicity assay on Vero cells and on rabbit corneal epithelial cell line (RCE)

Cytotoxicity tests were performed on VERO cells by means of the neutral red uptake assay (Martin and Clynes, 1993). Cells were plated at 5×10^6 cells/well in 24-well microtiter plates and were treated with DA concentrations ranging from 0.00346 to 346 μM obtained by dilution of DA-Lipo and DA-Sol formulations with MEM containing 5% FCS. After incubation at 37°C for 48 h, the formulations were removed from each well and replaced with an equal volume of the neutral red working solution (40 μg/mL). Cells were then incubated at 37°C for 3.5 h and after incubation the neutral red working solution was aspirated and the attached cells rinsed with phosphate-buffered saline before being fixed with a formalin/calcium solution (0.5% v/v formalin/1% v/v calcium chloride in ultrapure water). The fixative solution was removed and cells were then treated with an ethanol/acetic acid solution (50% v/v ethanol and 1% v/v acetic acid) to release the remaining

neutral red from within the lysosomal or cytoplasmic compartments. The absorbance of this final solution was assayed at 570 nm using a microplate reader (Asys UVM 340, Biochrom, Cambridge, UK). The results were obtained from six replicate experiments.

Cytotoxicity of the DA formulations was also carried out on rabbit corneal epithelial cells (RCE) using WST-1 cell proliferation reagent (Burgalassi et al., 2001). The RCE cells were plated at 5 x 10^3 cells/well, in 96-well microtiter plates and treated with DA-Lipo and DA-Sol preparations suitably diluted in growth medium to obtain DA concentrations ranging from 5.8 10^{-5} to 58 μM. Briefly, after 1 hour of exposure the medium was removed, the cells were washed twice with DMEM/F12 and 100 μL fresh growth medium and 10μL reagent WST-1 were added in each well. The cells were incubated for 2 h at 37°C, then the microplate was thoroughly shaken for 1 min and the absorbance was measured at 450 nm using the microplate reader. Cytotoxicity data were obtained from 4 to 6 separated experiments. Drug 50% cytotoxic concentration (CC_{50}) was calculated by Prism software using a non-linear regression analysis.

2.8. Permeation of DA through reconstituted rabbits corneal epithelial membranes (RRCE)

RCE were seeded onto the Snapwell® membranes at a concentration of 8 x 10^4 cells/cm^2 (Snapwell®, surface area 1.0 cm^2 and pore size 0.4 μm; Costar Italia, Milan, Italy) and were grown in standard conditions at 37°C in a humidified atmosphere containing 5% CO_2, with growth medium both in the apical and basolateral compartment, for 15 days until the cells had become confluent and were stratified (Burgalassi et al., 2004). The Snapwell® devices were mounted on diffusion chambers consisting of six individual chambers, thermostated at 37°C (Harvard Apparatus Inc., Holliston, MA, USA). The donor phase (apical phase, 0.65 mL) contained 0.013 mg of DA as DA-Sol and DA-Lipo diluted in cell grown medium. The receiving phase (basolateral phase, 1.50 mL) consisted of cell growth medium. The permeation studies lasted 4 hours and at appropriate time intervals during the experiment, 0.50 mL were collected from the receiving compartment and were replaced with an equal volume of fresh medium. The amount of DA permeated through (permeation) and retained into (penetration) RRCE was assayed by HPLC after deproteinization by mixing each sample with an equal volume of methanol containing 6% v/v perchloric acid followed by centrifugation for 10 minutes at 13,000 rpm. DA retained into reconstituted tissue at the end of the permeation experiments was evaluated after extraction of drug. The treatment consisted of adding methanol to the pre-weighted samples, sonication in an ultrasound bath for 20 minutes and centrifugation for 10 minutes at 13,000 rpm.

2.9. Ocular pharmacokinetics

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, following a protocol approved by the Ethical-Scientific Committee of the University of Pisa and under veterinary supervision. Different groups of rabbits were used for: 1) DA retention time in the tear fluid (36 rabbits); 2) DA intraocular penetration (36 rabbits); 3) DA corneal uptake (12 rabbits). A single dose (50 μL) of both appropriately diluted DA-Lipo dispersion or DA-Sol was instilled in the lower conjunctival sac of one eye of each rabbit. The amount of administered DA was 0.05 mg in all cases. For precorneal DA retention study at appropriate time intervals (1, 3, 5, 10, 20 and 30 min) after administration of each formulation, 1.0 μL of tear fluid was collected using disposable glass capillaries (Drummond "microcaps" Fisher Scientific, St. Louis, MO, USA) and after appropriate dilution (1:100 ratio) with HPLC mobile phase, the samples were submitted to HPLC analysis. The DA levels in the aqueous humor were monitored at 15, 30 and 60 min after instillation, withdrawing about 50-60 μL of aqueous humor from the anterior chamber of anaesthetized animals (Stella et al., 2012). All samples were frozen immediately and stored at -18C. For analysis, the thawed samples were mixed with an equal volume of HPLC mobile phase and centrifuged for 5 min at 13,000 rpm; 20 μL of each sample were submitted to HPLC analysis.

The analysis of DA concentration in the corneal tissue was carried out 30 min after instillation of the formulations. The animals were sacrificed by i.v. administration of an overdose of sodium pentobarbital solution (Pentothal sodium®, Gellini Farmaceutici, Milan, Italy) in the ear marginal vein, the eyes were rapidly enucleated, trimmed of all adventitial tissue and rinsed with normal saline solution and finally the corneas were isolated, frozen and stored at -18°C. For analysis, the thawed corneas were weighted and transferred in a glass tubes where 1.0 mL of ethanol was added. The samples were homogenized (10 minutes), sonicated in an ultrasound bath for 20 min, and centrifuged for 5 min at 13,000 rpm. A measured amount of alcoholic samples was concentrated under vacuum up to complete desiccation and then 30 μL of ethanol were added to the dry samples before HPLC analysis.

2.10. HPLC analysis of DA

HPLC analysis of DA was performed on a Shimadzu LC-10AS apparatus equipped with LC-6AS pump, SPS-10AV detector, C-R4A integrating system, 20 μL Rheodyne injector sample loop (Shimadzu Italia s.r.l., Milan, Italy) using a reversed-phase C_{18} column (Bondclone 30mm × 3.9 mm i.d., Phenomenex, Torrance, CA, USA). The mobile phase, delivered at a flow rate of 0.7 mL/min, was a 72:28 ratio mixture of phosphate buffer solution and ethanol. The buffer solution consisted of potassium dihydrogen phosphate (0.68% w/v) added with an appropriate amount of

H_3PO_3 up to pH = 3.0. The detection wavelength was 303 nm and the retention time of DA under these conditions was 8.1 min. The amount of drug in the samples was determined by comparison with appropriate external standard curves. The calibration curves were obtained applying the least square linear regression analysis by using Prism software.

2.11. Pharmacokinetics and statistical analysis

The area under the curve values of DA concentration in tear fluid were calculated from the first observation (1 min after instillation, t_{1min}) to the end of observation (30 min) from appropriate graphs, applying the linear trapezoidal rule. The apparent first-order elimination rate constants of DA from tear fluid (K_{tf}) and the corresponding half-lives ($t_{1/2}$) were calculated from the log-linear phase of drug concentration *vs* time profiles (Stella et al., 2012).

Statistical differences 1) between DA recovered from RRCE membranes after permeation study and 2) DA concentration in the tear fluid of rabbit eyes after topical administration of the formulations 3) DA concentration in the cornea were evaluated applying Student's two-tailed unpaired t-test (Prism software). The evaluation included calculation of means and standard error (S.E). Differences are considered statistically significant at $p < 0.05$.

3. Results

3.1. *Preparation and characterization of DA liposomal formulation (DA-Lipo)*

The liposomal dispersion, prepared by means of the REV method, was heterogeneous in size immediately after preparation, with significant differences between six different batches, as shown by the standard deviation of the mean diameter (707.41 ± 121.20 nm). After sonication, the mean diameter decreased to 358.41± 48.01 nm and the polidispersity changed from 0.579 to 0.311, indicative of a unimodal liposomal dispersion. The encapsulation efficiency was 34.53% (E%) and the volume of isotonic PBS added at the end of the preparation allowed to obtain a final concentration of DA encapsulated of 1.38 ± 0.26 mg/mL. The zeta potential (ζ) of DA-Lipo was negative (-15.80 ± 6.5 mV) probably due to the presence of phosphatidic acid in PC. Furthermore, DA-Lipo preparation is expected to be sufficiently stable without sedimentation or increase (< 10% increase of means diameter) in vesicle size within 3 months at 4°C. This results might be partially due to the zeta potential value that provide sufficient electrostatically repulsion or enough steric barriers to favour the repulsion between vesicles.

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The analysis of the drug release showed a fast diffusion of DA from the aqueous reference solution (DA-Sol) and a more controlled release rate from DA-Lipo vesicles (Figure 1). The release kinetics was evaluated by fitting the data into Zero order, Higuchi, and Ritger-Peppas models using Prisma software. The DA release kinetic data of the formulations were depicted in Table 1. Based on the results, the release of DA from liposome best-fitted the semi-empirical power law relationship proposed by Ritger and Peppas (1987), $Mt/M\infty = Kt^n$, where $Mt/M\infty$ is the fraction of drug released at each time point (t), k a kinetic constant relative to the properties of the matrix and n is the release exponent, indicative of the mechanism of drug release. As the release mechanism from drug delivery systems is complex, this model is used when the exact mechanism is unknown or when multiple release mechanisms are involved. The value of k and n can be calculated from the intercept and slope of the straight line considering only the portion of the release curve where $Mt/M\infty < 60\%$. It is well known that for spherical carrier n should be equal to 0.5 for a pure Fickian release, 1 for zero-order kinetics and $0.5 < n < 1$ for anomalous (non-Fickian) release. We obtained good linear fits with corresponding R values of 0.997 and 0.991 for liposome and solution, respectively. In the present system the release of DA was found to be Fickian in nature for DA-Sol (n = 0.592 h^{-1}; k=1.070), while followed a zero order kinetic (n = 1.085 h^{-1}; k=22.63) for DA-Lipo (Ritger and Peppas, 1987). After 5.0 h, more than 50% of DA content was released in the receiving phase from DA-Sol, while the amount of DA released from the DA-Lipo was less than 5.0%. After 24 hours about 25% of DA content was released by DA-Lipo while after 50 hours the released amount di DA was 35%. These results are not influenced by the dialysis membrane chosen with appropriate cut-off or by the system since the volume of receiving phase was 40-fold larger than that of the donor and the DA concentration in the acceptor compartment was maintained lower than 10% throughout the experiment. The membrane with pores of 3.5 kDa retains substances with MW 1355 and in particular globular molecules (e.g., proteins). More linear molecules, which may have a small diameter in two of three dimensions, may be able to pass through the pores more freely. Moreover, the long DA release time, also by DA-Sol,could depend by the experimental protocol (stirring, surface area of the membrane in relationship to the volume of the sample).

3.2. Antiviral activity and cytotoxicity assay

The IC_{50} values determined after 48 h treatment are showed in Table 2. The results showed that DA is appreciably active against both HSV1 and HSV2 isolated: in particular, IC_{50} values for DA-Sol obtained by IFA reduction assay for HSV1 isolates and by PRA_{50} assay for HSV2 isolates were 0.079 µg/mL and 3.78 µg/mL, respectively. Similar IC_{50} values were determined for treatment with DA-Lipo, namely 0.086 µg/mL and 2.71 µg/mL after treatment of HSV1 and

HSV2 isolate respectively. The data demonstrate that liposomal DA is just as potent as DA-Sol and therefore that the liposomal formulation maintains the whole activity of the plain drug.

In the same cell strain (VERO), both formulations produced analogous results with average CC_{50} values corresponding to 29.8 μg/mL for DA-Sol and of 20.1 μg/mL for DA-Lipo by using the neutral red cytotoxicity assay. These values are more than two orders of magnitude higher than those of the antiviral IC_{50} for HSV1 and less than one order of magnitude in the case of HSV2.

A different result was obtained with cytotoxicity test performed on RCE cell line: cell viability was maintained above 80% for DA-Lipo and above 60% for DA-Sol regardless of the concentration (up to 30.1 μg/mL). Therefore, DA-Lipo was definitively less cytotoxic than DA-Sol.

3.3. Permeation of DA through reconstituted rabbits corneal epithelial membranes (RRCE)

The time-course profile of cumulative amount of DA transported through RRCE were similar and lag time comparable for both the formulations (Figure 2). The good linearity at steady-state as resulting by the coefficients calculated by linear regression analysis proved the integrity of the corneal epithelial barrier during all the experiments (R^2 = 0.978 and 0.972 for DA-Lipo and DA-Sol respectively). The steady-state flux values (J) calculated from the linear ascents of the permeation graph were 72.5 10^{-6} and 64.71 10^{-6} μg/cm^{-2} s^{-1} and the corresponding lag time were 74.3 and 81.4 min for DA-Lipo and DA-Sol, respectively. The permeation coefficients, P, calculated by the equation P = J/Co where Co was the initial drug concentration in the donor compartment, were 1.31 for DA-Lipo and 1.16 cm/min for DA-Sol, revealing a fairly low DA permeability that was not influenced by the type of formulation. However, DA-Lipo appears to provide an appreciable increase DA retention in the cornea, although statistically significant differences were not observed: the amount of DA recovered into reconstituted corneal tissue was similar for both DA formulations and corresponded to 3.40 and 4.58% of the applied dose for DA-Sol and DA-Lipo, respectively.

3.4. Ocular pharmacokinetic

As expected, after instillation into rabbit eye, the formulations were well tolerated and in no case symptoms of ocular inflammation and irritation were evidenced. Figure 3 shows the precorneal DA concentration-time profile after instillation into the conjunctival sac of rabbits of the same dose of drug as DA-Sol and DA-Lipo. The relevant pharmacokinetic parameters calculated from DA concentration *vs* time curves, DA concentrations in tear fluid 1 min after administration (C_{1min}), the apparent first order elimination rate constants of DA from tear fluid (k_{tf}) and the half-life for elimination of drug from the precorneal area ($t_{1/2}$) and the area under curve values (AUC), are

reported in Table 3. The inspection of Figure 3 evidences the typical high DA concentration in the tear fluid of rabbits immediately after instillation of both formulations (C_{1min} = 0.364 ± 0.018 and 0.321 ± 0.038 mg/mL for DA-Lipo and DA-Sol, respectively) and its rapid decrease 10 minutes after instillation up to DA concentration of 2.59 and 0.87 μg/mL for DA-Lipo and DA-Sol respectively after 30 minutes. At this time point the amount of DA was higher than IC_{50} values for HSV1 for both formulations, while for HSV2 the two values (IC_{50} and DA concentration at 30 min) were similar only for DA-Lipo (see Table 2). In addiction, the tear fluid DA concentration values were significantly higher at 3, 5 and 10 minutes ($p< 0.05$) for DA-Lipo formulations than for the reference. In both cases the profiles (up to 30 minutes) were well depicted with a mono-exponential equation with apparent first-order elimination rate constants of 38.16 10^{-2} ± 0.022 min^{-1} and 25.23 10^{-2} ± 0.016 min^{-1} and half-life values of 1.82 and 2.75 min for DA- Sol and DA-Lipo, respectively (R^2 = 0.9965-0.9955). These values were indicative of a less rapid elimination of DA after instillation of the liposomal formulation. This behaviour was confirmed by DA half-life in tear fluid, whose values were 1.82 and 2.75 min for DA-Sol and DA-Lipo, respectively. The AUC values were clearly indicative of the greater DA bioavailability produced in tear fluid by DA-Lipo formulation (about 1.73-fold) with respect to DA-Sol even if statistically significant differences were not observed.

The HPLC analysis on the DA content in the aqueous humor samples carried out at 15, 30, 60 min after instillation of both formulations allowed to assure the lack of trace of drug in this ocular fluid (LOQ and LOD values of analytical method 21.2 ng/mL and 9.23 ng/mL, respectively).

Conversely, a significant amount of DA corresponding to 1.579 ± 0.087 and 2.028 ± 0.063 ng/mg were detected in the corneal tissues 30 min after administration of DA-Sol and DA-Lipo formulations, respectively ($p<0.05$). These data reveals the greater affinity of the liposomal formulation for ocular structures.

Discussion

Liposomes provide a promising tool to realize an advantageous ophthalmic drug delivery system since they have the convenience of the eye drops, restrain drug activity at site of action, and often present a reduced toxicity if compared with free drug (Rawas-Qalaji and Williams, 2012). The knowledge that liposomes may cause an increased drug uptake into the cornea and conjunctiva and that these effects could be attributed to the physical adsorption of lipid vesicles onto the epithelial membrane surface (Lee et ., 1984; Stratford et al., 1993; Gaudana et al., 2009) prompted us to develop a liposomal vesicles, targeting the corneal structure with the antiviral drug Dystamicin A. Even though the contact time of the liposome with the cornea seem to be related to the presence of a

positive charge on the vesicles (Chetoni et al., 2004; Gaudana et al., 2009), we overlooked this aspect, focusing our attention on the preparation of large bio-compatible liposomes able to encapsulate a high amount of distamycin A by using REV technique as reported by other authors (Cortesi et al., 2004; Cortesi et al., 2010a). The formulation studied in this paper was chosen among other previously screened formulations (data no reported) having different molar ratio between lipid and drug and prepared with different amount of solvent), because of the high DA encapsulation yield and for the homogeneous and suitable for ocular administration dimensions (about 200 nm).

Liposomes are subjected to clearance mechanisms and such as occur for other foreign bodies coming in contact with the ocular surface, they tend to be drained from the precorneal area by physiological mechanisms. While, small colloidal particles may be retained into the cornea and/or the scleral tissues favouring the intraocular absorption of the drugs, large colloidal particles may be more likely entrapped under the eyelids or in the inner canthus, remaining in contact with the eye structures for extended periods of time. This last mechanism, together with the high biocompatibility of DA-Lipo, might explain the increase of precorneal residence time of DA and the nearby two-fold increase bioavailability of DA in the tear fluid for DA-Lipo formulation. Moreover, even if the *in vitro* release test are not perfectly correlated to *in vivo* behaviour, the low cytotoxicity of the liposomal formulation might be partially related to the slow release rate of DA from liposomes. In fact the *in vitro* release test showed that less than 20% of DA content was released in the dissolution medium after 1 hour. The possibility to maintain pharmacologically active DA concentrations in the corneal tissue and avoid its transcorneal permeation, was also investigated. The *in vitro* studies performed on reconstructed rabbits corneal showed a permeability of DA similar to those measured for other drugs (Burgalassi et al., 2004; Reichl, 2008) However, even if permeation studies with this corneal model often enable the determination of variations in ocular drug bioavailability from different formulations does not take into account the complete structure of the cornea, the composition of tear fluid and aqueous humor, the mechanical stress of eyelids and the dynamics of tear fluid. The in vivo investigations highlighted the high bioavailability of DA in tear fluid for the liposomal formulation that at the same time allowed an appreciable uptake of drug into the cornea up to concentration values able to produce the inhibition of viral replication (IC_{50}) and without any evidence of transcorneal permeation. Indeed, the DA concentrations in the cornea were 1.579 and 2.028 μg/g 30 min after instillation of reference solution and of liposomal vesicles respectively, confirming the effective antiviral activity of DA-Lipo formulation. The IC_{50} values are reported in Table 2 as μg/mL but considering that 1 mL of growth medium may be assumed to weight 1 g, the IC_{50} could be expressed as μg/g as reported by

other authors (Monti et al., 2013). The promising results obtained in term of ocular bioavailability and IC_{50} using DA-Lipo formulation has been also emphasized by the cytotoxicity tests performed on both cell lines and in particular on VERO cell line. The CC_{50} values were higher of the antiviral IC_{50}s for HSV1 and HSV2, thereby demonstrating that the antiviral activity of DA was not influenced by their cytotoxicity.

These investigations confirm the potential usefulness of liposome as drug delivery systems for the treatment of ocular viral infections. The liposomal formulation, as well as limiting the cytotoxicity of DA, was capable of enhancing its ocular bioavailability, favouring its retention into the cornea. Other authors (Cortesi, 2010b) demonstrated the potentialities of liposome containing distamycin for its *in vitro* antiproliferative activity and hypothesized that the presence of phospholipids influenced drug uptake by cells. In conclusion, beside to be in accord with literature data these results improved new knowledge on the correlation between experimental *in vitro* data (drug release rate, cytotoxicity, antiviral activity) and the ocular pharmacokinetic of the drug following topical application. Further studies aimed at a fuller assessment of the biological activity of liposomal formulation could be interesting to perform.

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Figure captions

Figure 1 - Fractional DA release, Mt/M∞ *vs* time for DA-Lipo (♦) and DA-Sol (◇) formulations. Means ± S.E. of 3 runs.

Figure 2 – Permeation profile of DA across RCE for DA-Lipo (♦) and DA-Sol (◇). Means ± S.E.; n = 6.

Figure 3 - Tear fluid concentration-time profiles of DA upon topical administration of the formulations DA-Lipo (♦) and DA-Sol (◇). Means ± S.E.; n = 6. * Significantly different from DA-Sol ($p<0.05$, t-test).

Table 1 –VERO cells: IC_{50} values of DA-Lipo and DA-Sol formulations against HSV1 and HSV2 , with 95% confidence interval in parentheses

Formulation	**HSV1** µg/mL	**HSV2** µg/mL
DA-Lipo	0.086 (0.059-0.127)	2.71 (0.724-10.1)
DA-Sol	0.079 (0.046-0.138)	3.78 (0.302-47.2)

Table 2 - Pharmacokinetics parameters in tear fluid after administration of the formulations under study, means ± S.E.; n = 6.

Formulations	C_{1min} (mg/mL ± S.E.)	K_{tf} ($10^2 min^{-1}$ ± S.E)	$t_{½}$ (min)	AUC (min mg mL^{-1} ± S.E.)
DA-Sol	0.321 ± 0.038	38.16 ± 0.022	1.82	1.04 ± 0.72
DA-Lipo	0.364 ± 0.018	25.23 ± 0.016	2.75	1.80 ± 0.58

Table 3 - DA release kinetic data for the formulations

	DA-Sol			DA-Lipo		
	R^2	K	n	R^2	K	n
Zero-order model	0.891	8.817		0.978	0.662	
Ritger-Peppas model	0.991	1.070	0.592	0.997	22.630	1.085
Higuchi model	0.950	26.790		0.941	4.334	

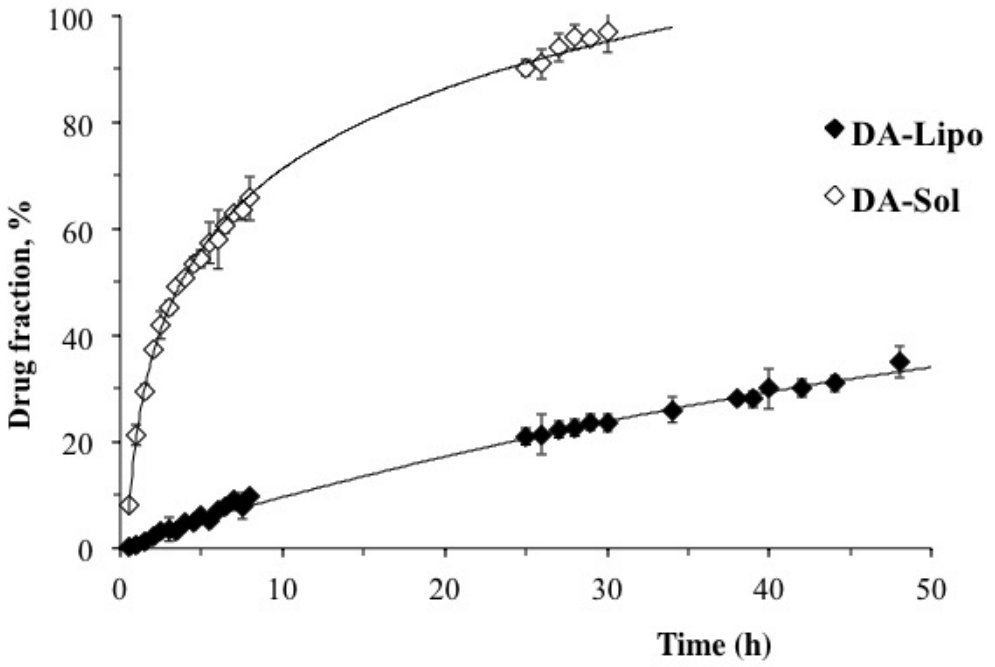

Fig. 1

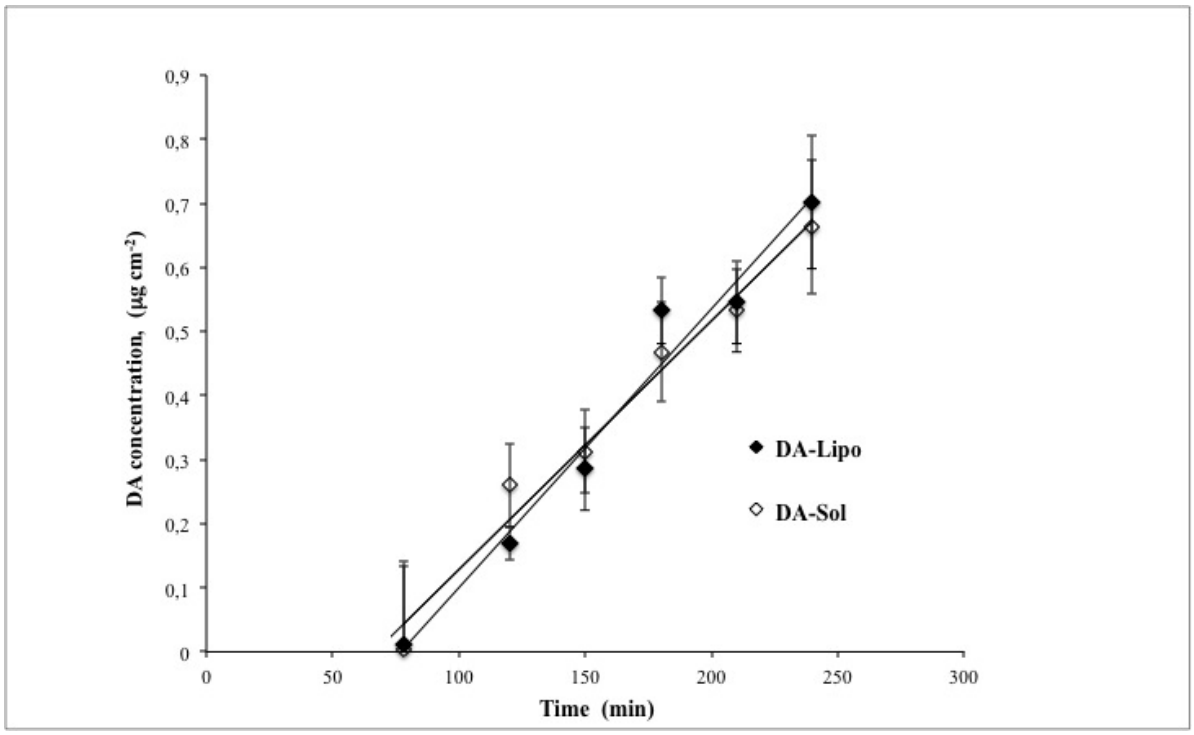

Fig. 2

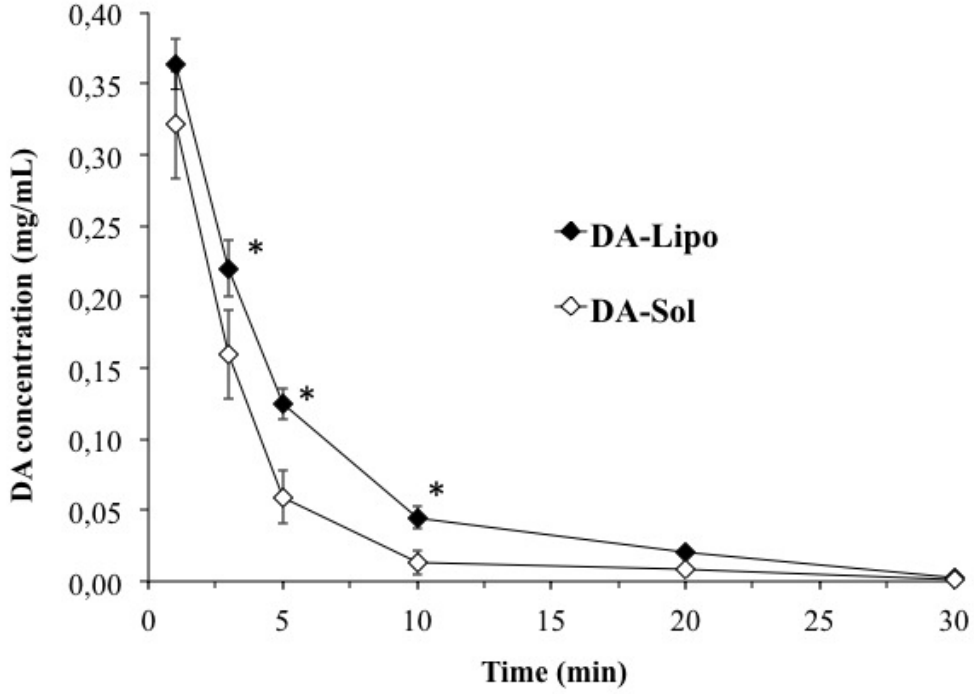

Fig. 3