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Innovative bola-surfactant niosomes as topical delivery systems of 5-fluorouracil for the treatment of skin cancer

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Abstract

An innovative niosomal system made up of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (Bola), Span 80® and cholesterol (2:5:2 molar ratio) was proposed as a topical delivery system for 5-fluorouracil (5-FU), largely used in the treatment of different forms of skin cancers. Bola-niosomes showed a mean size of ∼400 nm, which were reduced to ∼200 nm by a sonication procedure with a polydispersion index value of 0.1. Bola-niosomes showed a loading capacity of ∼40% with respect to the amount of 5-FU added during the preparation. 5-FU-loaded bola-niosomes were tested on SKMEL-28 (human melanoma) and HaCaT (non-melanoma skin cancer with a specific mutations in the p53 tumor suppressor gene) to assess the cytotoxic activity with respect to the free drug. 5-FU-loaded bola-niosomes showed an improvement of the cytotoxic effect with respect to the free drug. Confocal laser scanning microscopy studies were carried out to evaluate both the extent and the time-dependent bola-niosome–cell interaction. The percutaneous permeation of 5-FU-loaded niosomes was evaluated by using human stratum corneum and epidermis membranes. Bola-niosomes provided an increase of the drug penetration of 8- and 4-folds with respect to a drug aqueous solution and to a mixture of empty bola-niosomes with a drug aqueous solution.

Keywords: Niosomes; Bola-surfactant; 5-Fluorouracil; Percutaneous permeation; Cell cytotoxicity

1. Introduction

Niosomes are formed by the self-assembly of non-ionic amphiphiles in aqueous media thus providing closed bilayer structures. Niosomes are analogous to liposomes and, like them, can be multilamellar or unilamellar vesicles that are able to encapsulate both hydrophilic and lypophilic drugs (Bouwstra et al., 1997). Niosome properties make them a versatile carrier that is suitable for different systemic and topical applications, e.g. the administration of anti-inflammatory drugs (Shahiwala and Misra, 2002), non invasive vaccines (Vyas et al., 2005), and anti-cancer and anti-infective agents (Balasubramaniam et al., 2002).

Different types of surfactants are proposed as starting material to prepare niosomes, i.e. the SPAN® series and the Brij® series, and their physico-chemical properties can modulate the stability and the features of vesicular systems because they are able to influence the fluidity of bilayers (Jain and Vyas, 1995; Paolino et al., 2006). Usually, cholesterol is added to the formulations with the aim of reducing the temperature of the vesicular gel to liquid crystal phase transition and to decrease the overall HLB value of the surfactant mixture used for the preparation (Uchegbu and Vyas, 1998), thus allowing the formation of niosomes. In particular, it has been found that a molar ratio of 1:1 between cholesterol and non-ionic surfactants is an optimal ratio for the formation of physically stable niosomal vesicles (Nasseri, 2005).

New surfactants have been synthesized with the aim of preparing innovative niosomal systems (Muzzalupo et al., 1996).

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Bola-form amphiphiles are composed of two identical aza-crown ether units, as polar heads, linked to a long alkyl chain and represent a new class of non-ionic surfactants, which are able to assemble in colloidal structures if associated with cholesterol (Muzzalupo et al., 2005).

In a recent paper (Paolino et al., 2007), innovative niosomes made up of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (Bola C16), Span 80® and cholesterol (2:5:2 molar ratio) were designed, prepared and characterized in terms of both physicochemical properties and topical application potentialities as percutaneous drug delivery systems. *In vitro* experiments using human stratum corneum and epidermis showed that bola-niosomes were effective topical carriers in improving the percutaneous passage of drugs. Furthermore, *in vitro* and *in vivo* evidence showed that bola-niosomes are quite safe.

In this paper bola-niosomes are proposed as topical carriers for 5-fluorouracil (5-FU), a hydrophilic anticancer drug also used in the treatment of various forms of skin cancers (van Ruth et al., 2006; Gross et al., 2007), to improve the percutaneous drug permeation. In fact, this active compound showed a suitable antitumoral effect in the topical treatment of lesions related to squamous cell carcinoma such as actinic keratosis, Bowen's disease, and keratoacanthoma (Morse et al., 2003). Moreover, therapy for basal cell carcinoma and squamous cell carcinoma does not end with treatment of the initial lesion because almost 50% of patients with one non-melanoma skin cancer develop another one within the next 5 years (Nguyen and Ho, 2002).

Therefore, an improved percutaneous permeation of 5-FU is a fundamental requisite to achieve an effective topical therapeutic approach. Unfortunately, 5-FU *per se* shows a poor percutaneous permeation thus reducing its anticancer effectiveness following topical administration (Gupta et al., 2005; Singh et al., 2005).

The aim of this paper is also to improve the cytotoxic drug effect towards different skin cancer cell lines by using bola-niosomes. In particular, the SKMEL-28 cells (human melanoma skin cancer) and HaCaT (non-melanoma skin cancer) cells, which present an early stage of skin carcinogenesis by exhibiting UV-B type-specific mutations in the p53 tumor-suppressor gene (Lehman et al., 1993), were used for the *in vitro* cytotoxicity evaluation. In this attempt, confocal laser scanning microscopy (CLSM) experiments were carried out to evaluate the interactions between bola-niosomes and the investigated cancer cells.

2. Materials and methods

2.1. Chemicals

Cholesterol (Chol), α,ω-hexadecanedioic acid, 1-aza-18-crown-6 thionyl chloride, toluene, lithium aluminum hydride, tetra-hydro-furane (THF), *N*-(fluorescein-5-tiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (fluorescein-DHPE), 5-FU, phosphate saline tablets, 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide salt (used for MTT-tests), dimethysulfoxide and amphotericin B solution (250 μg/ml) were purchased from Sigma Chemicals Co. (St. Louis, USA). Sorbitan mono-oleate 80 (Span 80) was purchased from ACEF S.p.a. (Piacenza, Italy). SKMEL-28 cells (human melanoma) and HaCaT cells (human non-melanoma) were provided by Istituto Zooprofilattico of Modena and Reggio Emilia. Medium RPMI 1640 with glutamax, minimum essential medium (MEM) with glutamine, trypsin/EDTA (1×) solution, foetal bovine serum and penicillin–streptomicin solution were obtained by Gibco (Invitrogen Corporation, UK). All other materials and solvents used in this investigation were of analytical grade (Carlo Erba, Milan, Italy).

2.2. Synthesis of aza crown ether surfactant

The so-called bola-surfactant (α,ω-hexadecyl-bis-(1-aza-18-crown-6)), for its characteristic chemical structure (Fig. 1), was synthesized by condensation between *N*-aza-18-crown-6 and α,ω-hexadecanedioic acid according to the experimental procedure previously reported (Muzzalupo et al., 1996).

2.3. Bola-niosome preparation

Bola-niosomes having a multilamellar structure were prepared following the thin layer evaporation technique. A mixture (∼44 mg) of bola-surfactant, Span 80® and cholesterol (2:5:2 molar ratio) was dissolved in 2 ml of chloroform in a graduated pyrex tube. The organic solvent was removed by means of a rotavapor Büchi 461 under a slow nitrogen flux to obtain the formation of a thin film on the inner wall of the tube. This film was hydrated with 2 ml of a saline solution of 5-FU (10 mM) by submitting it to ten alternate cycles of warming at 67 °C (thermostated water bath) for 2 min and vortexing at 700 rpm for 1 min. The colloidal suspension of bola-niosomes was kept at 60 °C for 2 h to anneal the bilayer structure thus improving the

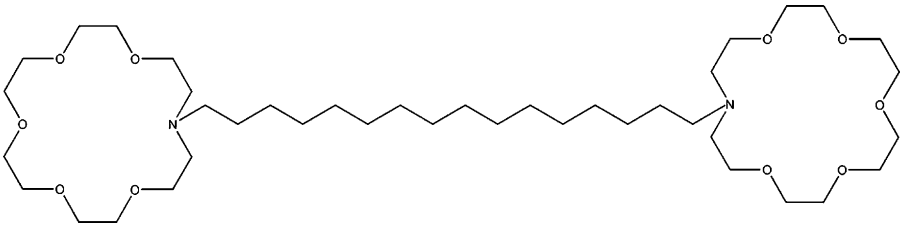

Fig. 1. Chemical structure of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (Bola C16).

stability of the system. Niosomal suspension was then submitted to a sonication procedure of 10 cycles of 1 min followed by a pause of 0.5 min by using a Sonopuls GM70 (Bandelin) with the instrument set at 30% of its maximum power to reduce the mean size of the vesicles. Unloaded niosomes were prepared following the same procedure but were hydrated with a simple saline solution without 5-FU. Fluorescent labelled bola-niosomes were prepared by adding the fluorescent probe (fluorescein-DHPE) to the lipid phase at a concentration of 0.1% molar with respect to other components. The probe was co-dissolved with other components during the film preparation.

2.4. Physicochemical characterization

Mean size and polydispersity index of bola-niosomes were evaluated by dynamic light-scattering experiments. The dimensional analysis was carried out by photocorrelation spectroscopy (PCS) (Zetamaster, Malvern Instruments Ltd., Spring Lane South, Worcs, England) using a 4.5 mW laser operating at 670 nm. Experiments were carried out at a scattering angle of 90°. A third-order cumulant fitting correlation function was performed by a Malvern PCS sub-micron particle analyzer. Samples were suitably diluted with a filtered (Sartorius membrane filters 0.22 μm) saline to avoid multiscattering phenomena and placed in a quartz cuvette. Experiments were carried out at room temperature.

2.5. Bola-niosome purification and loading capacity

Bola-niosomes were purified from unentrapped 5-FU by gel permeation chromatography. The instrument was an Äkta Prime Plus (Amersham Biosciences, Uppsala, Sweden), equipped with an UV detector at fixed wavelengths (254 and 280 nm). Gel permeation chromatography was carried out using a XK16/20 column (Amersham Biosciences) packed with Sephadex G-25 and a saline solution as the mobile phase. The equilibration volume of the column was 80 ml and the flow rate was 0.5 ml/min. The amount of 5-FU entrapped within the niosomal carrier was determined by HPLC (Fresta et al., 1993) following the destruction of the vesicular carrier using a mixture of $CH_3OH/CHCl_3$ (1:1, v/v). Unentrapped 5-FU was determined spectrophotometrically by means of a PerkinElmer Lambda 20 UV–vis spectrophotometer at a λ_{max} of 266 nm using a PerkinElmer UV WinLab™ 2.8 acquisition software (Perkin-Elmer GmbH Uberlingen, Germany). The following 5-FU calibration curve was used:

$$y = 18.409x - 0.1008$$

where y is the absorbance at 266 nm and x is the drug concentration (μM), the r^2 value was 0.9999.

Centrifugation was also used for niosome purification, when *in vitro* experiments had to be carried out, due to the dilution of niosomal suspension occurring during the gel permeation chromatography. In this case, bola-niosome suspension was centrifuged at 28,000 × g for 1 h at 4 °C by a Beckman Coulter Allegra 64R centrifuge. The supernatant was analyzed spectrophotometrically. The bola-niosome loading capacity of 5-FU was expressed as the percentage of entrapped drug with respect to the amount used for the preparation. Both purification methods provided similar results.

2.6. Drug release from niosomes

The 5-FU release was evaluated following the dialysis method by using cellulose acetate dialysis tubing (Spectra/Por with molecular cut-off 12,000–14,000 by Spectrum Laboratories Inc., Netherlands) sealed at both ends with clips (Saarinen-Savolainen et al., 1997). A pH 7.4 phosphate buffer solution, which was constantly stirred and warmed (GR 150 thermostat, Grant Instruments Ltd., Cambridge, UK) to 37 ± 0.1 °C throughout the release experiments, was used as the release fluid for 5-FU. Before dialysis, the tubing was kept overnight in the buffer solution to allow the complete wetting of the membrane. Bola-niosomes (1 ml) were placed in the dialysis bag, which was then transferred into a beaker containing 200 ml of the release buffer thus following sink conditions for 24 h experiments. At predetermined time intervals, a sample of release fluid (1 ml) was withdrawn and replaced with the same volume of fresh fluid. Samples were then analyzed spectrophotometrically at 5-FU λ_{max} 266 nm. No interference was observed from the components of bola-niosomes. The percentage of drug release was calculated using the following equation:

$$\text{Release}\,(\%) = \frac{5-\text{FU}_{\text{rel}}}{5-\text{FU}_{\text{load}}} \times 100$$

where 5-FU_{rel} is the amount of drug released at the time t and 5-FU_{load} is the amount of drug entrapped within bola-niosomes. The release studies were carried out in triplicate.

2.7. Permeation through human stratum corneum and epidermis

Samples of healthy adult human skin (mean age 29 ± 4 years) were obtained from abdominal reduction surgery of female subjects. Membranes of the stratum corneum and viable epidermis (SCE) were isolated as previously reported (Kligman and Christophers, 1963). Subcutaneous fat was surgically removed by means of a scalpel and the skin was immersed in distilled water at 60 ± 1 °C for 2 min. Then SCE was peeled off and immediately used for the various permeation experiments. The barrier integrity of SCE was checked by determining the tritiated water permeability coefficient ($K_p = 1.5 \pm 0.3 \times 10^{-3}$ cm h^{-1}), that resulted consistent with values previously reported (Saija et al., 2000). Skin permeation studies were carried out by using Franz-type diffusion cells. SCE membrane was mounted horizontally with the stratum corneum side up between the donor and the receptor compartments. The receptor was filled with saline solution that was constantly stirred. Two hundred microlitres of a 5-FU-loaded bola-niosomes or an aqueous solution of the drug, at the same concentration, were placed in donor compartments. The experiments were carried out in non-occlusive conditions for 24 h at a thermostated temperature of 35 ± 1 °C. A minimum of six diffusion cells were contemporarily used for each formulation and 1 ml of each receptor fluid was withdrawn every 1 h

up to 24 h of experiments by using an FC 204 fraction collector (Gilson Italia S.r.l., Cinisello Balsamo (MI), Italy) connected to a Minipuls 3 peristaltic pump (Gilson Italia S.r.l., Cinisello Balsamo (MI), Italy). The volume withdrawn was replaced by the same volume of fresh receptor phase. The samples were analyzed by HPLC (Fresta et al., 1993). The results are expressed as the mean value ± the standard deviation.

2.8. Cell cultures

SKMEL-28 cells were incubated (Guaire® TS Autoflow Codue Water-Jacketed-Incubator) in plastic culture dishes (100 mm × 20 mm) at 37 °C (5% CO_2) by using RPMI1640 medium with glutamate, penicillin (100 UI/ml), streptomycin (100 μg/ml) and FBS (10%, v/v). The culture medium used for the HaCaT cells was DMEM supplemented with glutamate, D-glucose, pyruvate, 10% FBS (v/v), penicillin (100 UI/ml), and streptomycin (100 μg/ml). The medium was replaced with fresh every 48 h. HaCaT cells, presenting mutations in the p53 tumor suppressor gene, were cultured at 40 °C for 7 days to obtain an *in vitro* cell line model of non-melanoma skin cancer (Lehman et al., 1993; Boukamp et al., 1999). When ∼80% the confluence was reached, cells were treated with trypsin (2 ml) to separate them from the dishes and collected into a centrifuge tube containing 4 ml of the culture medium. The dishes were further washed with 2 ml of PBS to remove the remaining cells and this was transferred into a centrifuge tube. The tube was centrifuged at 1000 rpm at room temperature for 10 min with a Heraeus Sepatech Megafuge 1.0. The pellet was resuspended in an appropriate culture medium volume and seeded in culture dishes before *in vitro* investigations.

2.9. Evaluation of cytotoxic activity

Cytotoxic effects of free or bola-niosome entrapped 5-FU were evaluated by MTT dye test (cell viability). The cultured cells were plated in 96-well culture dishes (5×10^3 cells/0.1 ml) and incubated for 24 h at 37 °C to promote their adhesion to the plate. The culture medium was then removed, replaced with the different formulations and incubated for 24, 48 or 72 h. Every plate had 8 wells with untreated cells as the control and 8 wells with cells treated with empty niosomes as the blank. After each incubation period, 10 μl of tetrazolium salt solubilized in PBS solution (5 mg/ml) were added to every well and the plates were incubated again for 3 h. The medium was removed and the formazan salts (precipitated on the well bottom after oxidation) were dissolved with 200 μl of a mixture of DMSO/ethanol (1:1, v/v), by shaking the plates for 20 min at 230 rpm (IKA® KS 130 Control, IKA® WERKE GMBH & Co., Staufen, Germany). The solubilised formazan was quantified with a microplate spectrophotometer (Multiskan MS 6.0, Labsystems) at a wavelength of 540 nm with reference at a wavelength of 690 nm. The percentage of cell viability was calculated according to the following equation:

$$\text{cell viability}\,(\%) = \frac{\text{Abs}_\text{T}}{\text{Abs}_\text{C}} \times 100$$

where Abs_T is the absorbance of treated cells and Abs_C is the absorbance of control (untreated) cells. The formazan concentration is directly proportional to the cell viability, that was reported as the mean of six different experiments ± standard deviation.

2.10. Confocal laser scanning microscopy (CLSM)

The interaction between the cancer skin cells and bola-niosomes was evaluated by CLSM studies. Cells were placed in 6-well culture plates (4×10^5 cells/ml) with culture medium. In each well a sterile glass slide was previously positioned. Plates were incubated for 24 h and then cells were treated with bola-niosomes labelled with fluorescein-DHPE for different incubation times, from 3 h up to 24 h. After incubation, each well was washed with PBS (three times) to remove the excess of bola-niosomes and cells were fixed on the sterile glass slides by using 1 ml of an ethanolic solution (70%, v/v). Each slide glass was washed again with PBS three times and PBS (2 ml) was added to each well. Plates were stored at 4 °C up to the confocal microscopy analysis. Before analysis, slide glasses were positioned on cover glass by using a glycerol solution (70%, v/v) to remove enclosed air and they were fixed by a transparent glue. The analysis was carried out using a laser scanning confocal microscopy (CLSM) Leika TCS SP2 MP at $\lambda_{exc} = 496$ nm and $\lambda_{em} = 519$ nm. A scan resolution up to 4096 × 4096 pixels with an Ar/Kr laser beam of 75 mW, equipped with a fluorescein analyzer filter, was used for experimental investigations. Samples were recorded by a macro developer software package having multi-dimensional series acquisition and direct-access digital control knobs. An immersion oil lens 100× was used.

2.11. Statistical analysis

One-way ANOVA was used for statistical analysis of the various experiments. A posteriori Bonferroni *t*-test was carried out to check the ANOVA test. A p value <0.05 was considered statistically significant. Values are reported as the mean ± standard deviation.

3. Results and discussion

3.1. Physical chemical characterization of niosomes

As shown in previous investigations (Muzzalupo et al., 2005; Paolino et al., 2007), bola-surfactant must be combined with cholesterol to obtain bola-niosome suspensions, because it is not able to form a stabile bilayer if used alone. The presence of a lipophilic non-ionic surfactant is useful to improve the stability of the system. In this investigation bola-surfactant, Span 80 and cholesterol were combined (2:5:2 molar ratio) to obtain bola-niosomes. These vesicles show partially aggregated, irregular structures when prepared in distilled water, while regular and spherical vesicles without the formation of any aggregate are achieved in the presence of saline solution. This finding is due to the cationic complexation in aza-crown-ether, whose cavity

Table 1
Physicochemical parameters of bola-niosomes[a]

Sample	Mean size (nm)	Polydispersion index
Empty niosomes	498 ± 5.3	0.312 ± 0.044
5-FU-loaded niosomes	479 ± 3.2	0.301 ± 0.031
Sonicated empty niosomes	251 ± 1.9	0.111 ± 0.029
5-FU-loaded sonicated niosomes	229 ± 2.4	0.102 ± 0.012

[a] Each value represents the average of three different experiments ± standard deviation.

size is selective with respect to sodium ions (Muzzalupo et al., 2005). For this reason the drug was dissolved in a sterile saline solution during the hydration phase to favour the formation of spherical niosomes and to prevent the formation of colloidal aggregates.

PCS analysis showed that the mean size of empty bola-niosomes was ∼500 nm while that of 5-FU-loaded bola-niosomes was slightly lower (Table 1). This finding is probably due to an interaction between the polar heads of bola-surfactant and the carbonyl groups of the anticancer drug. A polydispersity index value of 0.3 showed the presence of a wide size distribution of bola-niosomes. Considering that a reduced mean sized of a vesicular carrier can be an important parameter to improve the topical biopharmaceutical properties (Fresta and Puglisi, 1996; Boinpally et al., 2003), bola-niosomes were submitted to sonication thus achieving a colloidal vesicular suspension with a mean size of ∼200 nm (Table 1). Also in this case, the mean size of the 5-FU-loaded bola-niosomes is lower with respect to that of the empty sonicated bola-niosomes, thus confirming a possible interaction between the drug and the bola-niosome structure. The sonication also elicited a reduction of the polydispersity index up to a value of 0.1 (Table 1), thus showing the formation of a vesicular colloidal system characterized by a narrow size distribution.

Another important parameter for a possible application of a colloidal drug delivery system is the loading capacity of the drug within the carriers (Manconi et al., 2006). In particular, bola-niosomes showed a loading capacity towards 5-FU of ∼45% (Table 2, Fig. 2). This value was higher than that expected by a drug encapsulation in the aqueous compartment of a vesicular niosome delivery system. This finding was probably due to a significant interaction of 5-FU with the niosome membrane. It is interesting to observe that the two different purification methods used to separate the unentrapped 5-FU provided no significant difference in the values of loading capacity of bola-niosomes.

Table 2
Loading capacity of 5-FU in Bola-niosomes as assayed by two different methods[a,b]

Sample	Centrifugation method (%)	GPC method (%)
5-FU-loaded niosomes	45.3 ± 2.2	44.1 ± 1.6
5-FU-loaded sonicated niosomes	40.7 ± 3.1	39.5 ± 2.9

[a] Each value represents the average of three different experiments ± standard deviation.
[b] Loading capacity of colloidal vesicles is expressed as the percentage of the drug that became niosome associated.

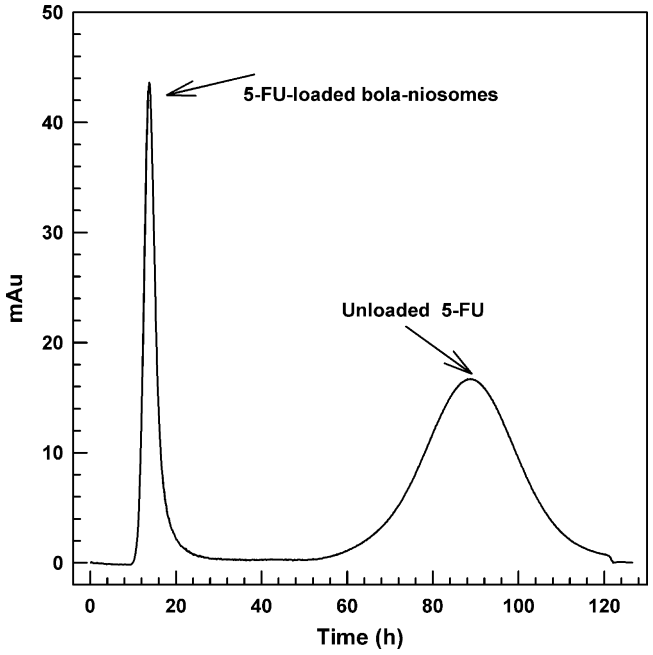

Fig. 2. Typical gel permeation chromatogram of an un-purified suspension of 5-FU-loaded bola-niosomes. The gel permeation chromatographic analysis was carried out at room temperature using Sephadex G-25 as the stationary phase and isotonic saline solution as the eluent with a flow rate of 0.5 ml/min.

The sonication of 5-FU-loaded bola-niosomes elicited a slight reduction of the carrier loading capacity.

The release of 5-FU from bola-niosomes is reported in Fig. 3. The release profile of 5-FU was characterized by the absence of a burst effect, thus showing the absence of unentrapped drug or the presence of drug weakly bound to bola-niosomes. The release profile was characterized by a first phase (0–10 h) of

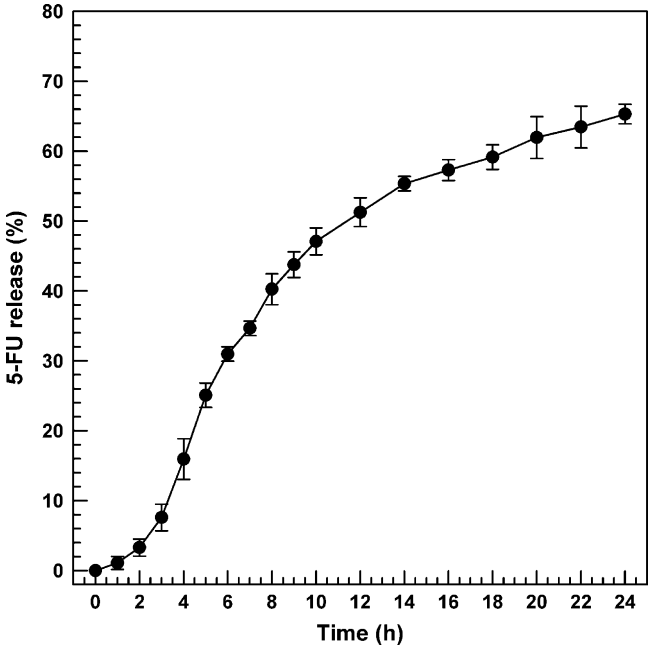

Fig. 3. Release profile of 5-FU from bola-niosomes. Experiments were carried out at room temperature. Values represent the average of three different experiments ± standard deviation.

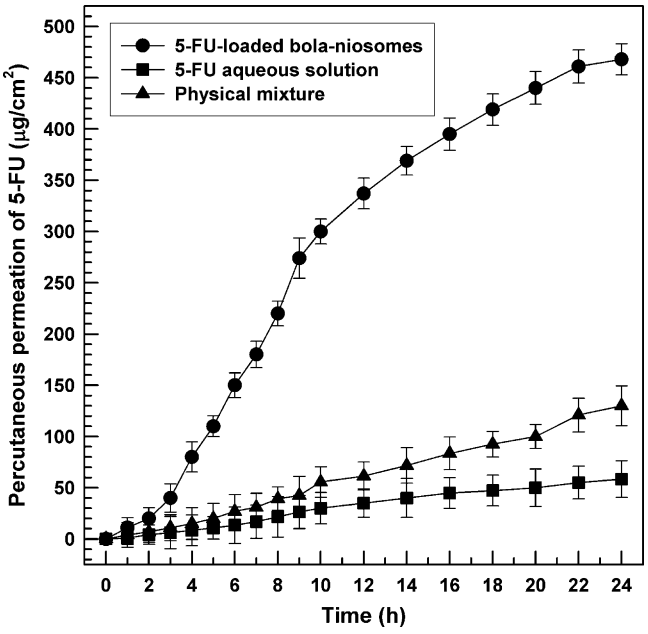

Fig. 4. *In vitro* percutaneous permeation through human SCE membranes of 5-FU-loaded bola-niosomes, 5-FU aqueous solution and a physical mixture between unloaded bola-niosomes and a 5-FU aqueous solution. Each value is the average of six different experiments ± standard deviation.

faster 5-FU release rate followed by a second phase of slower release. This situation is typical of compounds that are not simply entrapped within the aqueous compartments of a vesicular carrier, but have also the ability to interact with bilayer structures of vesicular carriers thus increasing the carrier loading capacity and providing drug release profiles that results from the drug permeation through bilayers and the retention phenomenon arising from the drug-bilayer compounds interaction (Kulkarni et al., 1995; Hao et al., 2002; Pardakhty et al., 2007). Also the presence of chol, as a constituent of bola-niosomes, can have an active role in the release profile of 5-FU due to its stabilizing function on the bilayer structures (Uchegbu and Vyas, 1998). Bola-niosomes provided a 5-FU release of ∼60% with respect to the entrapped amount after 24 h.

3.2. In vitro human skin percutaneous permeation

To investigate the *in vitro* percutaneous permeation of 5-FU through the stratum corneum, an essential aspect to be evaluated for the potential application of a drug delivery system as a topical device (Fang et al., 2001; Fiume, 2001; Paolino et al., 2005), a dynamic Franz diffusion cell apparatus was used. Although it is likely within the clinical setting that 5-FU would be permeating through skin exhibiting cancerous changes, SCE membranes coming from healthy subjects were used because it is not possible to obtain cancerous skin specimens. In any case healthy SCE membranes can provide relevant scientific data on the 5-FU (both as a free form and niosomally encapsulated) percutaneous permeation, that represent the limiting factor in the clinical topical treatment (Gupta et al., 2005; Singh et al., 2005).

In particular, 5-FU-loaded bola-niosomes showed (Fig. 4) an improvement of the drug percutaneous permeation through human SCE membranes of ∼8-folds with respect to an aqueous solution of the antitumoral drug (468.3 ± 15.2 μg/(cm^2 h^{-1}) vs. 58.5 ± 17.9 μg/(cm^2 h^{-1}), respectively). This phenomenon is probably due to the enhanced percutaneous carrier capacity of bola-niosomes (Paolino et al., 2007) coming from the flexibility and deformability of the structure of these colloidal systems, which enables them to pass through human skin (Menger and Keiper, 2000; El Maghraby et al., 2004), similarly to other deformable and flexible vesicular carriers, e.g. ethosomes® (Touitou et al., 2000; Paolino et al., 2005).

To confirm this hypothesis the permeation of a physical mixture of a 5-FU solution with unloaded Bola-niosomes through human SCE membranes was also investigated (Fig. 4). In this case, percutaneous permeation data after 24 h showed a permeation of 130.1 ± 19.3 μg/(cm^2 h^{-1}), that was 2-folds higher than that observed for just the aqueous solution of the drug, but was about 4-folds lower than that observed for 5-FU-loaded bola-niosomes. The increase of percutaneous permeation of 5-FU following the co-administration of unloaded bola-niosomes was probably due to the presence of niosomal surfactants which could act as penetration enhancers destabilizing the packing order of stratum corneum lipids. These findings showed that the improvement of the drug percutaneous permeation was mainly related to the encapsulation of 5-FU and hence to the topical carrier capacity of bola-niosomes.

3.3. Evaluation of anticancer activity

The finding achieved from physicochemical and technical characterization of bola-niosomes showed that this carrier can be suitable as topical drug delivery system of 5-FU being able to improve its percutaneous permeation. Another issue to be addressed is the evaluation of the anticancer activity of 5-FU-loaded bola-niosomes with respect to the free drug to exploit the potential advantages of this carrier in terms of therapeutic effectiveness. Since bola-niosomes were proposed as topical carriers for the treatment of cancer skin diseases, two different human cell lines of skin cancer, i.e. SKMEL-28 (human melanoma) and mutated HaCaT cells (human non-melanoma), were used in the experiments for the *in vitro* evaluation of anticancer activity.

Melanoma is a type of cancer which cannot be treated with topic formulations but only with some palliative cures (Ryan et al., 1988). On the other hand 5-fluorouracil is normally employed to treat several skin pathologies related to melanoma (actinic keratosis, precursor lesions) and, for this reason, the cytotoxic activity of free or niosome-encapsulated 5-FU was also evaluated on melanoma cells. Therefore, SKMEL-28 cell line was used as a model to mime the basal skin carcinoma, a subtype of non-melanoma skin cancer, that is treated normally with topical 5-FU solutions (Stockfleth and Sterry, 2002), while the second cell line was used as a model of squamous cell carcinoma (SCC), i.e. actinic keratosis, Bowen's disease, and keratoacanthoma, which represents the most aggressive form of non-melanoma skin cancer.

The *in vitro* anticancer activity was evaluated in terms of cytotoxicity by using the cell viability MTT test. Cytotoxic effect was evaluated as a function of both the incubation time (24, 48

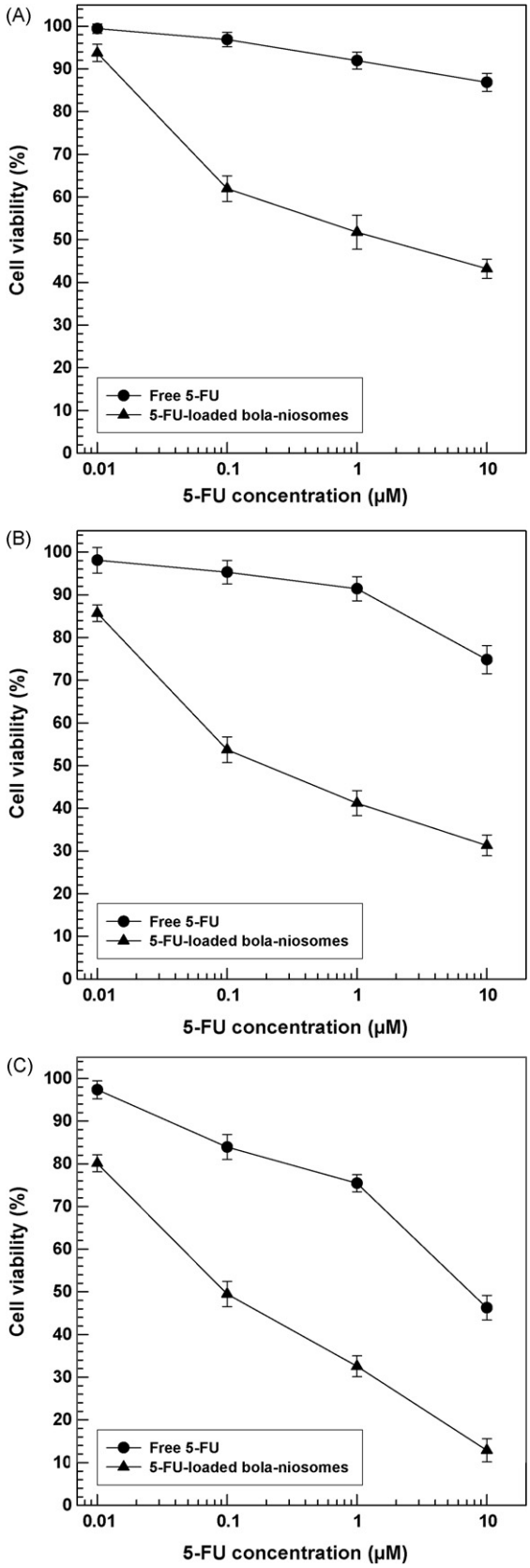

Fig. 5. *In vitro* cytotoxicity on SKMEL-28 cells of 5-FU and 5-FU-loaded bola-niosomes as a function of drug concentration and exposition times, i.e. 24 h (panel A), 48 h (panel B) and 72 h (panel C). Data is reported as percentage of cellular viability as evaluated by MTT test. Error bars, if not shown, are within symbols. Results are the mean of six different experiments ± standard deviation.

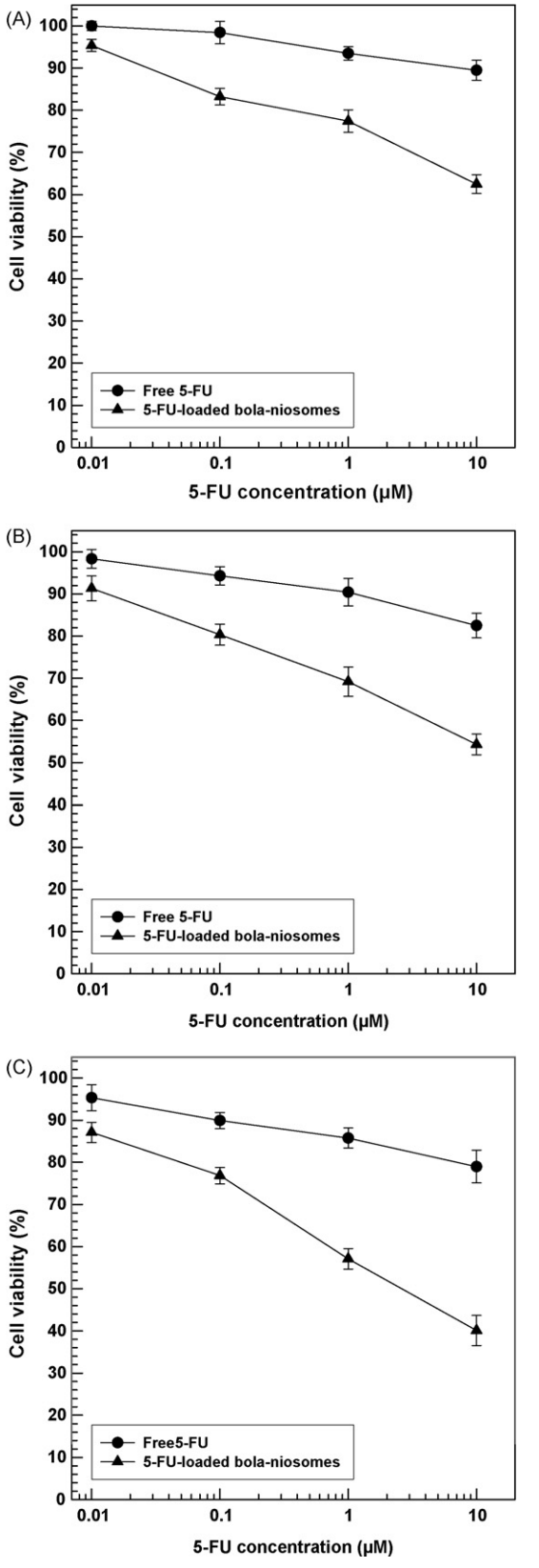

Fig. 6. *In vitro* cytotoxicity on mutated HaCaT cells of free 5-FU and 5-FU-loaded bola-niosomes as a function of drug concentration and exposition times, i.e. 24 h (panel A), 48 h (panel B) and 72 h (panel C). Data is reported as percentage of cellular viability as evaluated by MTT test. Error bars, if not shown, are within symbols. Results are the mean of six different experiments ± standard deviation.

or 72 h) and the drug concentration (from 0.01 to 10 μM) to define both the time-exposition and the dose–response effects, respectively. A previous work showed that empty bola-niosomes had a certain cytotoxicity at concentrations >10 μM (Paolino et al., 2007). In this investigation this concentration was never exceeded and hence empty bola-niosomes showed no cytotoxic effect at the drug concentrations used (data non-reported).

Initially, 5-FU-loaded bola-niosomes was tested on SKMEL-28 cells. Just after 24 h incubation it was possible to observe an improvement of the cytotoxic effect on SKMEL-28 cells elicited by 5-FU-loaded bola-niosomes with respect to the free drug at each investigated concentration. In particular, free 5-FU showed no appreciable cytotoxic effect up to a concentration of 1 μM. Only at a concentration of 10 μM a slight anticancer activity was observed, while the drug encapsulated within bola-niosomes elicited a significant inhibition of SKMEL-28 cell vitality at all the investigated concentrations (Fig. 5 panel A). An inhibition of ∼50% was achieved at 10 μM drug concentration.

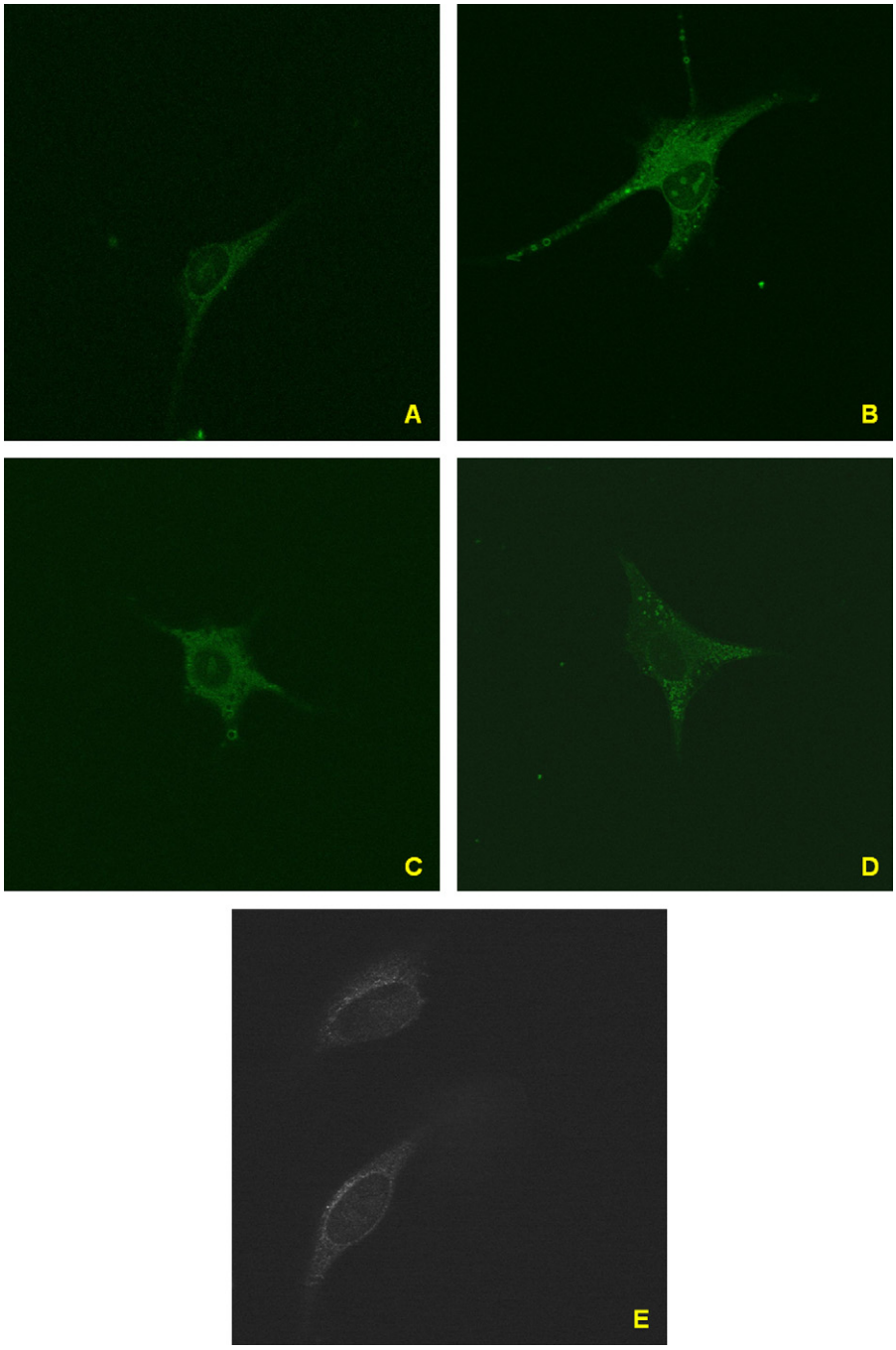

Fig. 7. Confocal laser scanning micrographs showing the interaction between bola-niosomes and SKMEL-28 cells and the intracellular localization of fluorescein-DHPE labelled niosomes as a function of the incubation time: panel A, 3 h; panel B, 6 h; panel C, 12 h; panel D, 24 h. Photomicrograph of untreated SKMEL-28 cells (control) achieved in transmission mode (panel E). No significant cellular fluorescence was observed coming from the autofluorescent phenomena caused by the cellular proteins.

After 48 h incubation, the difference of cytotoxicity between the two formulations is more pronounced. It was very interesting to observe that 5-FU-loaded bola-niosomes at the concentration of 0.1 μM determined a reduction of cell viability of ∼50%, while the free drug showed a reduction of vitality of ∼25% but at a concentration 100-fold higher (10 μM) (Fig. 5 panel B).

The free 5-FU showed a significant cytotoxic effect at a lower concentration than 10 μM only at an incubation time of 72 h (Fig. 5 panel C). Also in this case 5-FU-loaded bola-niosomes showed a better anticancer efficacy than the free drug. In particular, the maximum effect of the vesicular formulation was observed at the concentration of 10 μM with a reduction of cell viability of ∼80%, while the free 5-FU elicited a reduction of ∼50% (Fig. 5 panel C). This drug concentration is not too high for a dermal administration of bola-niosomes to treat basal carcinomas (Jorizzo et al., 2004; McGillis and Fein, 2004), especially considering the selective administration due to the topical administration.

The 5-FU-loaded bola-niosomes and the free drug were tested, successively, on mutated HaCaT cells (presenting mutations on p53 gene following incubation at 40 °C for 7 days) to assay the cytotoxicity of the active compound on a model of squamous cell carcinoma. Also in this case the cytotoxicity of 5-FU-loaded bola-niosomes is greater than that determined by the free drug, although the difference of the cell viability inhibition induced by the two formulations is less pronounced with respect to that observed in the case of SKMEL-28 cells. After 24 h of incubation, free 5-FU showed a significant cytotoxicity only at a concentration of 10 μM (∼10%), while the encapsulated drug induced significant reductions of cell viability just starting at a concentration of 0.1 μM (∼15%) (Fig. 6 panel A). The same trend but to a greater extent for both the 5-FU-loaded bola-niosomes and the free drug was also observed after 48 h of incubation (Fig. 6 panel B). The difference between the two formulations in terms of extent of cell viability inhibition became more evident after 72 h of incubation (Fig. 6 panel C). In fact, the free 5-FU showed a ∼35% reduction of cell vitality at a concentration of 10 μM, while 5-FU-loaded bola-niosomes determined the same effect at a concentration 100-fold lower, i.e. 0.1 μM. The treatment of HaCaT cells with 5-FU-loaded bola-niosomes determined a reduction of cell viability up to ∼60% at a concentration of 10 μM (Fig. 6 panel C).

Taking into consideration the anticancer activity findings and the low skin permeation of the 5-FU solution with respect to the bola-niosome formulation, the great potential advantages of using bola-niosomes as topical carrier of 5-FU for the treatment of non-melanoma skin cancer diseases are clear.

3.4. CLSM studies on bola-niosome/cell interaction

An important feature of an innovative drug delivery system is to improve the amount of active compound in the target zones causing a better therapeutic effect of the drug. In this attempt it was interesting to evaluate the mechanisms of interaction between bola-niosomes and the human skin cancer cells used in this investigation. For this reason CLSM experiments on SKMEL-28 cells were carried out by using bola-niosomes labelled with fluorescein-DHPE. Fig. 7 shows how the fluoresceinated bola-niosomes interacted with the melanoma cells at different incubation times. In particular, a green fluorescence distribution was observed in the cells just after 3 h incubation. After 6 h incubation the fluorescence of the membrane and the cytoplasm began more intense and increased slightly up to 24 h incubation. It is noteworthy that in Fig. 7 panels B–D it is possible to observe some bola-niosomes inside the cells. This finding can explain the observed intracellular diffused green fluorescence. These data allow us to suppose that the main mechanism involved in the bola-niosome/cell interaction was the endocytosis of this carrier, which enables a rapid internalization in the cytoplasm. This phenomenon could explain the difference in the cytotoxicity between 5-FU-loaded bola-niosomes with respect to the free drug. In fact, the active compound, after a rapid access to the intracellular compartments and its release from the colloidal vesicular carrier, could interact more easily with its target, thus eliciting an increase of the cellular death.

4. Conclusions

Considering the safety and tolerability of the bola-niosomes, we used these innovative carriers to encapsulate the antitumoral compound 5-FU with the aim of improving its cytotoxic effect on different cancer cell lines. Our studies showed that the inclusion of the active compound inside the vesicles does not modify the physicochemical properties of the carriers. CLSM studies demonstrated that bola-niosomes were able to promote the intracellular delivery thus improving the anticancer activity of the entrapped 5-FU. In fact, the antitumoral compound, when entrapped in bola-niosomes elicited a reduction of cellular viability with respect to the free drug on both cancer cell lines used. These results in association with the technological features of bola-niosomes are very encouraging for a possible topical application of this carrier for the treatment of certain cancerous skin diseases.

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