International Journal of Pharmaceutics 422 (2012) 229–237

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Gemcitabine and tamoxifen-loaded liposomes as multidrug carriers for the treatment of breast cancer diseases

D. Cosco[a], D. Paolino[a,b], F. Cilurzo[a], F. Casale[a], M. Fresta[a,b,*]

[a] Department of Health Sciences, University "Magna Græcia" of Catanzaro, Campus Universitario "S. Venuta" - Building of BioSciences, Viale Europa, I-88100 Germaneto (CZ), Italy
[b] U.O.C. Farmacia Ospedaliera, Fondazione per la Ricerca e la Cura dei Tumori "Tommaso Campanella", Campus Universitario "S. Venuta", Viale Europa, I-88100 Germaneto (CZ), Italy

ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form 29 October 2011
Accepted 29 October 2011
Available online 9 November 2011

Keywords:
Liposomes
Multidrug carrier
Gemcitabine
Tamoxifen
Breast cancer cells

ABSTRACT

The effects of a lipid composition on the physico-chemical and technological properties of a multidrug carrier (MDC) containing both gemcitabine (GEM) and tamoxifen (TMX), as well as its *in vitro* antitumoral activity on different breast cancer cell lines, were investigated. In particular, the following three different liposomal formulations were prepared: DPPC/Chol/DSPE-mPEG2000 (6:3:1 molar ratio, formulation A), DPPC/Chol/DOTAP (6:3:1 molar ratio, formulation B) and DPPC/Chol/DPPG (6:3:1 molar ratio, formulation C). The colloidal systems were obtained by the TLE technique and the extrusion process allowed us to obtain vesicles having mean sizes of 150–200 nm, while the surface charges varied between 50 mV and −30 mV. Formulation A showed the best encapsulation efficiency between the two compounds and the presence of TMX influenced the release profile of GEM (hydrophilic compound) as a consequence of its effect on the fluidity of the bilayer. An MDC of formulation A was used to effectuate the *in vitro* cytotoxicity experiments (MTT-test) on MCF-7 and T47D cells. The liposomal MDC provided the best results with respect to the single drug tested in the free form or entrapped in the same liposomal formulation. The CLSM experiments showed a great degree of cell interaction of liposomal MDC after just 6 h.

1. Introduction

During the last decade, experimentation on the treatment of cancer diseases has been pursuing an innovative and comforting strategy through the association of two or more antitumoral compounds in order to reduce the effective dosages and their side effects (Theodossiou et al., 1998; Colomer, 2005). Unfortunately, the most important drawback of this approach is characterized by the absence of tissue selectivity and, sometimes, by the modification of the pharmacokinetic profiles of the drugs (Airoldi et al., 2008).

An innovative strategy to combine the synergic action of two drugs, thus favoring their selective localization may be their co-encapsulation in a drug delivery system. As in the case of Doxil®, liposomal colloidal devices allow the modulation of the biopharmaceutical properties of drugs having different physico-chemical characteristics, thus improving their pharmacological effects and increasing their blood circulation half-life (Papahadjopoulos et al., 1991; Robert et al., 2004; Gabizon et al., 2006). Our research team, for example, investigated the possibility of entrapping a nucleoside analogue, gemcitabine (GEM), inside a unilamellar PEGylated liposomal system in order to protect it from metabolic inactivation and increase the intracellular drug localization, obtaining a considerable improvement of its antitumoral effect both in *in vitro* and *in vivo* tests with respect to the free form and the commercial product GEMZAR® (Calvagno et al., 2007; Celia et al., 2008; Cosco et al., 2009a; Paolino et al., 2010). Moreover, we prepared a PEGylated liposomal formulation containing an innovative lipophilic tyrosine-kinase inhibitor allowing its *in vivo* administration in the absence of organic solvents or surfactant mixtures (Celano et al., 2008).

Our investigations have given us a good starting point for the design and the preparation of a liposomal multidrug carrier (MDC) containing both a water soluble drug (in aqueous environments) and a lipophilic compound (entrapped in the bilayers). So we recently characterized a PEGylated liposomal system containing both gemcitabine and paclitaxel which showed a greater degree of *in vitro* antitumoral activity against MCF-7 cells than single compound in the free or encapsulated forms or their association (Calvagno et al., 2006; Cosco et al., 2011). A similar approach was adopted by Tardi et al. (2007) who co-encapsulated irinotecan and floxuridine in a liposomal formulation, demonstrating that the simultaneous presence of the two hydrophilic compounds did not destabilize the colloidal structure. The rationale of this approach comes from the choice of drugs characterized by different

* Corresponding author at: Department of Health Sciences, University "Magna Græcia" of Catanzaro, Campus Universitario "S. Venuta" - Building of BioSciences, Viale Europa, I-88100 Germaneto (CZ), Italy. Tel.: +39 0961 369 4118; fax: +39 0961 369 4237.
E-mail address: fresta@unicz.it (M. Fresta).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.10.056

mechanisms of action and their partially non-overlapping toxicities (Colomer, 2005).

Taking into account the obtained results, in this manuscript we investigated the influence of the liposomal lipid composition on the physico-chemical and technological properties of a novel MDC carrier containing GEM and tamoxifen (TMX) with the aim of achieving a new nano-medicine against breast cancer disease. In fact, TMX, a non-steroidal selective estrogen receptor modulator, very effective against breast tumors, could represent an ideal synergic agent to increase the antineoplastic effect of GEM (Zheng et al., 2007). For example, Tomao et al. (2002) carried out a phase II study, testing the association GEM/TMX on patients affected by advanced pancreatic cancer disease, thus showing an innovative therapeutic approach for this pathology as a consequence of an effective antitumoral clinical response and a suitable toxicological profile.

The liposomal MDC containing these two drugs was characterized and its *in vitro* antitumoral efficacy was investigated in comparison to the association of the free drugs on MCF-7 and T47D cell lines. The interaction rate of liposomal MDC with breast cancer cells was also monitored by confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Chemicals and biochemicals

The phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phospocholine monohydrate (DPPC), dimyristoyl phosphatidylglycerol (DMPG) and N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG 2000), were purchased from Genzyme (Suffolk, UK). Cellulose membrane Spectra/Por MWCO 10000, used for drug release tests, were obtained by Spectrum Laboratories Inc. (Eindhoven, The Netherlands).

Gemcitabine hydrochloride (HPLC purity > 99%) was a gift of Eli-Lilly Italia S.p.a. (Sesto Fiorentino, Italy). Human breast cancer cells (MCF-7 and T47D) were provided by Istituto Zooprofilattico of Modena and Reggio Emilia. Minimum Essential Medium (MEM) with glutamine, trypsin/EDTA (1×) solution, foetal bovine serum and penicillin–streptomicin solution were obtained by Gibco (Invitrogen Corporation, Paisley, UK). Dioleoyl trimethylammonium propane (DOTAP), cholesterol (chol), phosphate saline tablets (for the preparation of phosphate buffer solution pH ~ 7.4), 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide salt (used for MTT-tests), dimethylsulfosside, amphotericin B solution (250 μg/ml) and tamoxifen powder were purchased from Sigma Chemicals Co. (St. Louis, Missouri, USA). Lissamine rhodamine B 1,2 dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine DHPE) was an Invitrogen (Eugene, Oregon, USA) product. All other materials and solvents used in this investigation were of analytical grade (Carlo Erba, Milan, Italy).

2.2. Liposome preparation

Three types of liposomes were prepared using the thin layer evaporation technique. Liposomes were made up of DPPC/Chol/DSPE-MPEG2000 (6:3:1 molar ratio, formulation A), DPPC/Chol/DOTAP (6:3:1 molar ratio, formulation B) and DPPC/Chol/DMPG (6:3:1 molar ratio, formulation C) (Table 1). The lipids (20 mg) were dissolved in a mixture of chloroform/methanol (3:1, v/v) in a graduated Pyrex® tube. The solvent was removed by means of a rotavapor Büchi R-210 (Flawil, Switzerland) and kept in overnight storage at room temperature in a Büchi T51 glass drying oven connected to a vacuum pump to obtain the formation of a lipid film on the inner walls of the tube. The lipid film was hydrated with 1 ml of a 250 mM sulphate ammonium solution to generate a pH-gradient (Celano et al., 2004; Calvagno et al., 2007). Multilamellar liposomes were obtained by submitting the lipid/aqueous phase mixtures to three alternate cycles (3 min each) of warming to 58 °C (water bath with thermostat) and vortexing at 700 rpm. The excess of ammonium sulphate was removed after centrifugation with a Beckman Coulter Allegra 64R at 20,000 × *g* for 60 min at 4 °C. The supernatant was removed while the pellet was re-suspended in 1 ml of an aqueous solution of GEM 1 mM and was kept at 58 °C for 3 h in order to stabilize the formulation. To reduce the liposomal mean sizes, the vesicles were extruded at 60 °C by a Lipex Extruder™ (Vancouver, Canada) equipped with polycarbonate membrane filters (Costar, Corning Incorporated, NY, USA). At first, the liposomes were extruded through two polycarbonate filters of 400 nm pore size (10 cycles) and then through two polycarbonate filters of 200 nm pore size (10 cycles). The working pressure was 450 kPa and 880 kPa respectively. This procedure allowed the formation of GEM-loaded unilamellar liposomes (L-GEM).

Table 1
Lipid composition of various liposome formulations.[a]

Formulation	DPPC	Chol	DSPE-mPEG2000	DOTAP	DMPG
A	6	3	1	–	–
B	6	3	–	1	–
C	6	3	–	–	1

[a] The values represent the molar ratio of the different lipids.

To prepare TMX-loaded liposomes (L-TMX), the lipophilic drug (0.45 mg) was added to the solvent mixture used to solubilise the phospholipids and the same procedure as reported above was followed.

The liposomal MDC containing both gemcitabine and tamoxifen was prepared by adding the lipophilic drug to the solvent mixture and the hydrophilic compound during the hydration step (Fig. 1). Also in this case, the lipid film was pre-hydrated with a sulphate ammonium solution to generate a pH-gradient with the aim of increasing the amount of encapsulated GEM in the colloidal system. The formulation was successively submitted to the extrusion process in order to obtain 200 nm-vesicles.

Rhodamine-labelled liposomes were prepared by co-dissolving rhodamine-DHPE (0.1% molar) together with the lipids.

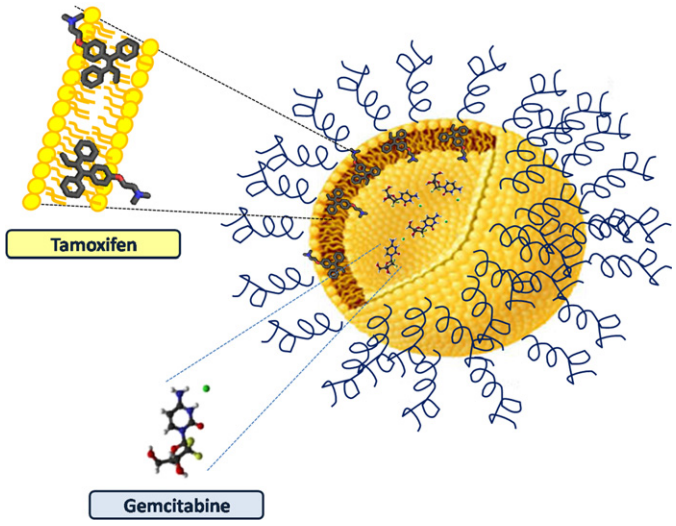

Fig. 1. Schematic representation of the GEM/TMX localization inside the MD carrier.

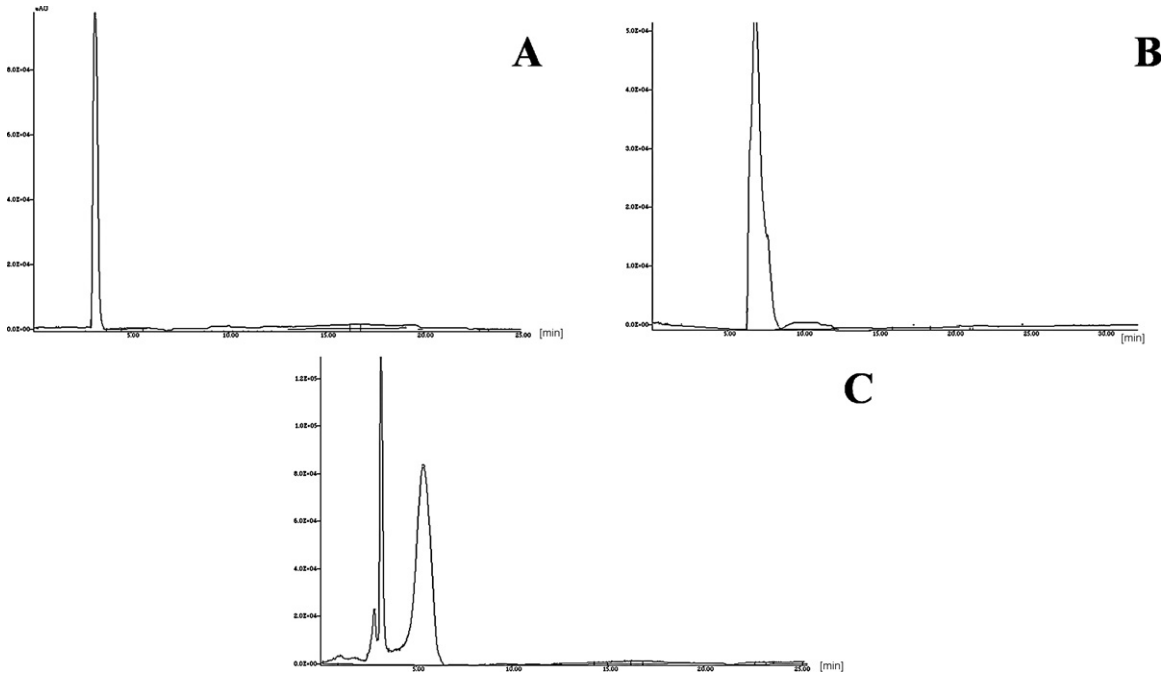

Fig. 2. HPLC chromatograms of TMX (A), GEM (B), and a mixture GEM/TMX (C). No interference peak was observed.

2.3. Physicochemical characterization

Mean size, size distribution and Z-potential were determined with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom), a dynamic light scattering spectrophotometer, by applying the third-order cumulant fitting correlation function. A 4.5 mW laser diode operating at 670 nm was used as a light source for size analysis and the back scattered photons were detected at 173°. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The medium refractive index (1.330), medium viscosity (1.0 mPa s), and dielectric constant (80.4) were set before the experiments. Quartz cuvettes were used for the analysis.

The Z-potential of the experimental colloidal suspensions was also measured with a Zetasizer Nano ZS. A Smoluchowsky constant F (Ka) of 1.5 was used to calculate this parameter as a function of the electrophoretic mobility of vesicles. The various measurements were carried out in triplicate on three different batches (10 determinations for each batch). Results were expressed as the mean ± standard deviation.

2.4. Evaluation of drug loading capacity

Vesicular formulations were centrifuged at 100,000 × g at 4 °C for 1 h using a Beckman Optima™ ultracentrifuge equipped with a TL S55 fixed angle rotor (Bechman Coulter Inc., Fullerton, CA, USA). The amount of GEM and TMX entrapped in the colloidal pellet was evaluated by HPLC after vesicle disruption. HPLC analysis showed that no interference was determined by the various components of liposomes (Fig. 2). The entrapment efficiency was evaluated as the percentage of the added drug that became liposome-associated, according to the following equation:

$$\mathrm{EE} = \frac{D_T - D_U}{D_T} \times 100 \quad (1)$$

where D_T is the total amount of compound added to the formulation during the preparation procedure, and D_U is the amount of unentrapped drug obtained after the purification procedure.

A Jasco PU-1580 intelligent HPLC pump (Tokyo, Japan) with a 20 μl loop injection valve was used. The chromatographic system was equipped with a Jasco MD 1510 diode array detector (Tokyo, Japan) which was set at the maximum absorption of GEM and TMX.

The separation was performed on an Eclipse XDB-C18 (Agilent) reversed-phase column (4.6 mm × 150 mm). The mobile phase used was water/acetonitrile (40:60, v/v) for 20 min and it was delivered at a flow rate of 0.4 ml/min through the column.

Data was processed using Borwin chromatography software (Version 1.5) from Jasco. Pure solutions of TMX and GEM compounds were prepared with acetonitrile and acetonitrile/water (50:50, v/v), respectively, at a drug concentration of 1 mg/ml. GEM and TMX quantifications were carried out using external standard curves in a linear concentration interval ranging from 0.1 to 10 μg/ml (Fig. 2), where x is the drug concentration (μg/ml) and AUC the area under the curve (mAu × min).

2.5. Drug release from liposomes

Drug 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.) sealed at both ends with clips. A pH 7.4 phosphate buffer solution (containing 4%, w/v of Tween 80)/ethanol (70:30, v/v), 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 the two antitumoral drugs. Before dialysis, the tubing was kept overnight in the buffer solution to allow the membrane to become thoroughly soaked. The liposomal formulations (1 ml) were placed in the dialysis bag, which was then transferred into a beaker containing 200 ml of the release medium thus complying with 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 by HPLC. The percentage of released drug was calculated using the following equation:

$$\mathrm{Release}\ (\%) = \frac{\mathrm{drug_{rel}}}{\mathrm{drug_{load}}} \times 100 \quad (2)$$

where $drug_{rel}$ is the amount of drug released at the time t and $drug_{load}$ is the amount of drug entrapped within liposomes. The release studies were carried out in triplicate.

2.6. Cell cultures

MCF-7 and T47D cells were maintained in culture as described previously (Sponziello et al., 2010). Briefly, they were incubated in plastic culture dishes (100 mm × 20 mm) (Guaire® TS Autoflow Codue Water-Jacketed incubator at 37 °C (5% CO_2) using MEM medium with glutamine, penicillin (100 UI/ml), streptomycin (100 μg/ml), amphotericin B (250 μg/ml) and FBS (10%, v/v). Fresh medium was substituted every 48 h. When ~80% confluence was reached, the cells were treated with trypsin (2 ml) to separate them from the dishes and then cells were collected into a centrifuge tube containing 4 ml of the culture medium. The dishes were then washed with 2 ml of PBS to remove the remaining cells and then the PBS was transferred into the 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 re-suspended in an appropriate culture medium volume and seeded in culture dishes before *in vitro* investigations.

2.7. Evaluation of cytotoxic activity

The cytotoxic effects of the association of GEM and TMX both as free drugs and entrapped in liposomes were evaluated by MTT-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 plates. The culture medium was then removed, replaced with the different formulations and incubated for 24, 48 or 72 h. Every plate had 8 wells containing untreated cells as the control and 8 wells with cells treated with empty PEGylated liposomes as the blank. After each incubation period, 10 μl of tetrazolium salt solubilised in PBS solution (5 mg/ml) was added to every well and the plates were incubated again for 3 h. The medium was removed and the formazan salts (which had precipitated to 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:

$$\text{cell viability}\,(\%) = \frac{\text{Abs}_{(T)}}{\text{Abs}_{(C)}} \times 100 \quad (3)$$

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 cell viability, which was reported as the mean of six different experiments ±standard deviation.

2.8. Cell/carrier interaction by CLSM

The interaction between the cancer cells and liposomes was evaluated by CLSM studies. Cells were placed in 6-well culture plates (4×10^4 cells/ml) with culture medium. A sterile glass slide had previously been positioned in each well. Plates were incubated for 24 h and then cells were treated with rhodamine labelled liposomes for 6 h. After incubation, each well was washed with PBS (3×) to remove the excess of liposomal suspension and cells were fixed on the sterile glass slides by using 1 ml of an ethanolic solution (70%, v/v). Each well was treated with 1 ml of Hoechst solution (1/1000), incubated for 30 min and then washed three times with PBS (2 ml).

Plates were stored at 4 °C until 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 Leika TCS SP2 MP laser scanning confocal microscopy operating at λ_{exc} = 560 nm and λ_{em} = 580 nm for rhodamine probe and at λ_{exc} = 405 nm and λ_{em} = 460 nm for Hoechst probe.

A scan resolution of up to 4096 × 4096 pixels with an Ar/Kr laser beam of 75 mW, equipped with a TRITC analyzer filter, was used for experimental investigations. Sample micrographs were recorded by a macro developer software package having multidimensional series acquisition and direct-access digital control knobs. An immersion oil lens 63× was used.

2.9. 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 average ± standard deviation.

3. Results and discussion

3.1. Multidrug carrier preparation and characterization

In order to produce a colloidal vesicular device able to deliver two drugs having different physico-chemical properties, an in-depth investigation of the most suitable formulations is necessary. Therefore, the first step was to examine the variation of mean sizes, the polydispersity indexes and zeta-potentials of the three liposomal formulations both in the presence and the absence of GEM and TMX in single form or in association.

As reported in Table 2, all the investigated liposomal formulations, were characterized by mean size values of about 200 nm and a polydispersity index of ≥0.1, thus having features suitably functional for systemic administration (Nagayasu et al., 1999). The presence of the two drugs, whether in single form or in association, did not vary the mean sizes of the liposomal formulations B and C. In the case of liposomal formulation A, the GEM favored a slight increase of colloidal mean size with respect to the empty liposomes while both the presence of the TMX alone and the association of the two drugs determined a decrease in this size value. This could probably be due to the TMX which, in itself, allowed a significant reduction of the carrier sizing. In fact, the lipophilic agent can act as a bilayer stabilizer thus behaving in a manner similar to that of cholesterol (Kayyali et al., 1994). To confirm this hypothesis, a TMX-loaded formulation (DPPC-DSPE-mPEG2000 7:1 molar ratio) was prepared in the absence of cholesterol and showed a mean size similar to that of TMX-loaded liposomal formulation A, thus evidencing the cholesterol-like stabilizing effect. But it also showed a decreasing of TMX entrapment efficiency while the addition of GEM to this formulation caused the destabilization of the colloidal structure (data not shown). This finding is in agreement with Bhatia's investigation which demonstrated a lesser degree of encapsulation of the TMX in a liposomal formulation without cholesterol as compared to a liposomal system containing sterol (Bhatia et al., 2004).

The two drugs did not unsettle the vesicles of formulations B and C, allowing the realization of different liposomal systems characterized by different surface charges (Table 2). This evidences a factor which is very important because the modulation of the Z-potential can influence blood circulation times, the opsonization process and hence reticuloendothelial system uptake, as well as interaction with biological compartments (Yan et al., 2005).

Table 2
Physico-chemical parameters of the different liposomal formulations.[a]

Sample (formulation)	Mean sizes (nm)[a]	Polidispersity index[a]	Zeta-potential (mV)[a]	LC%
A-empty	218.3 ± 3.7	0.117 ± 0.023	−8.4 ± 1.1	–
A-TMX	161.7 ± 1.095	0.040 ± 0.020	−41.1 ± 1.36	94.2 ± 2.7
A-GEM	224.1 ± 5.6	0.068 ± 0.033	−10.1 ± 3.2	92.2 ± 1.8
A-MDC	155.8 ± 0.782	0.063 ± 0.015	−35.7 ± 2.42	92.6 ± 2.0 (GEM) 89.7 ± 1.6 (TMX)
B-empty	178.4 ± 1.375	0.049 ± 0.019	45.0 ± 2.27	–
B-TMX	179.0 ± 1.320	0.101 ± 0.018	54.3 ± 2.19	84.3 ± 0.9
B-GEM	161.5 ± 2.011	0.119 ± 0.012	48.2 ± 1.90	71.8 ± 3.4
B-MDC	154.5 ± 1.065	0.047 ± 0.015	52.2 ± 2.16	66.7 ± 2.2 (GEM) 80.9 ± 3.6 (TMX)
C-empty	179.4 ± 2.098	0.080 ± 0.018	−37.4 ± 0.899	–
C-TMX	182.4 ± 1.577	0.105 ± 0.013	−29.8 ± 0.581	90.2 ± 2.5
C-GEM	176.1 ± 1.948	0.051 ± 0.019	−31.0 ± 2.69	91.7 ± 3.4
C-MDC	172.5 ± 0.886	0.073 ± 0.009	−30.4 ± 2.08	88.8 ± 3.4 (GEM) 86.4 ± 2.2 (TMX)

[a] Multilamellar vesicles extruded through 400 nm and 200 nm pore size filters. Each value represents the average of three different experiments ±standard deviation.

The TMX elicited a great reduction in the zeta-potential of liposomal formulation A in the case of encapsulation both in the single form and in association with GEM, while the nucleoside analogue alone furnished values similar to those of the empty formulation. The TMX probably modified the fluidity of the bilayer, possibly through an increased exposition of the phosphate groups of the polar heads, as a consequence of its localization between the alchylic chains of lipids (Table 2). There was a bit of variation of the surface charges of formulations B and C following the encapsulation of the two antitumoral drugs. Liposomal formulation B showed a zeta-potential of about 50 mV while formulation C showed a value of ∼−30 mV. Considering the aforementioned characterization, all the formulations could be used to deliver the active compounds; in fact different cationic liposomes having high positive zeta-potentials are normally used in experimental investigations, such as in the gene delivery field (Lonez et al., 2008), while, on the other hand, the surface charge plays an important role in systemic macrophage recognition of the colloidal systems after opsonization and so it is necessary to use a biocompatible hydrophilic polymer, like PEG, that can assure long circulation properties (Cosco et al., 2009b; Pasut and Veronese, 2009).

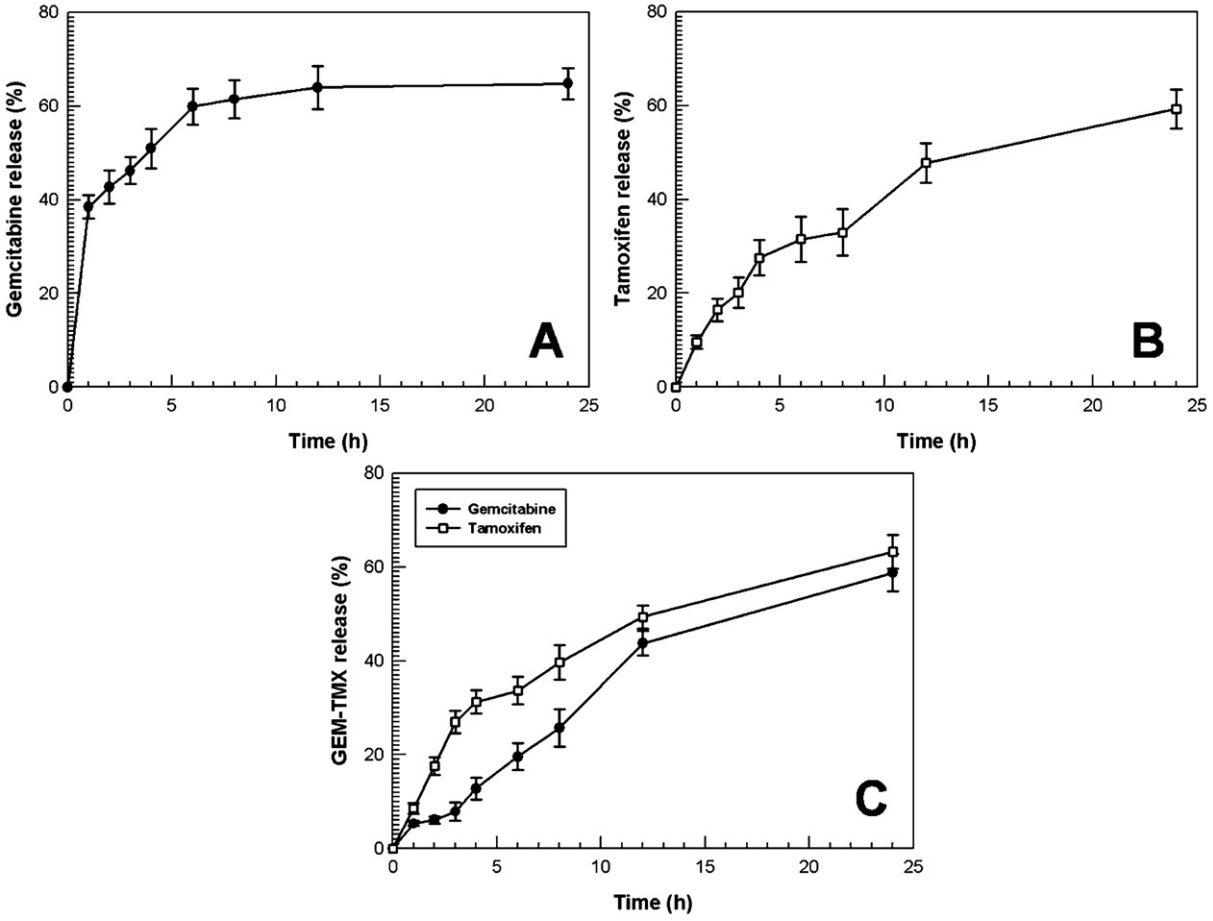

Fig. 3. Release profile of gemcitabine (GEM) and paclitaxel (TMX) from PEGylated liposomes after encapsulation in single form (panel A and B, respectively) and in association (panel C, MD). Experiments were carried out at room temperature. Values represent the average of three different experiments ±standard deviation.

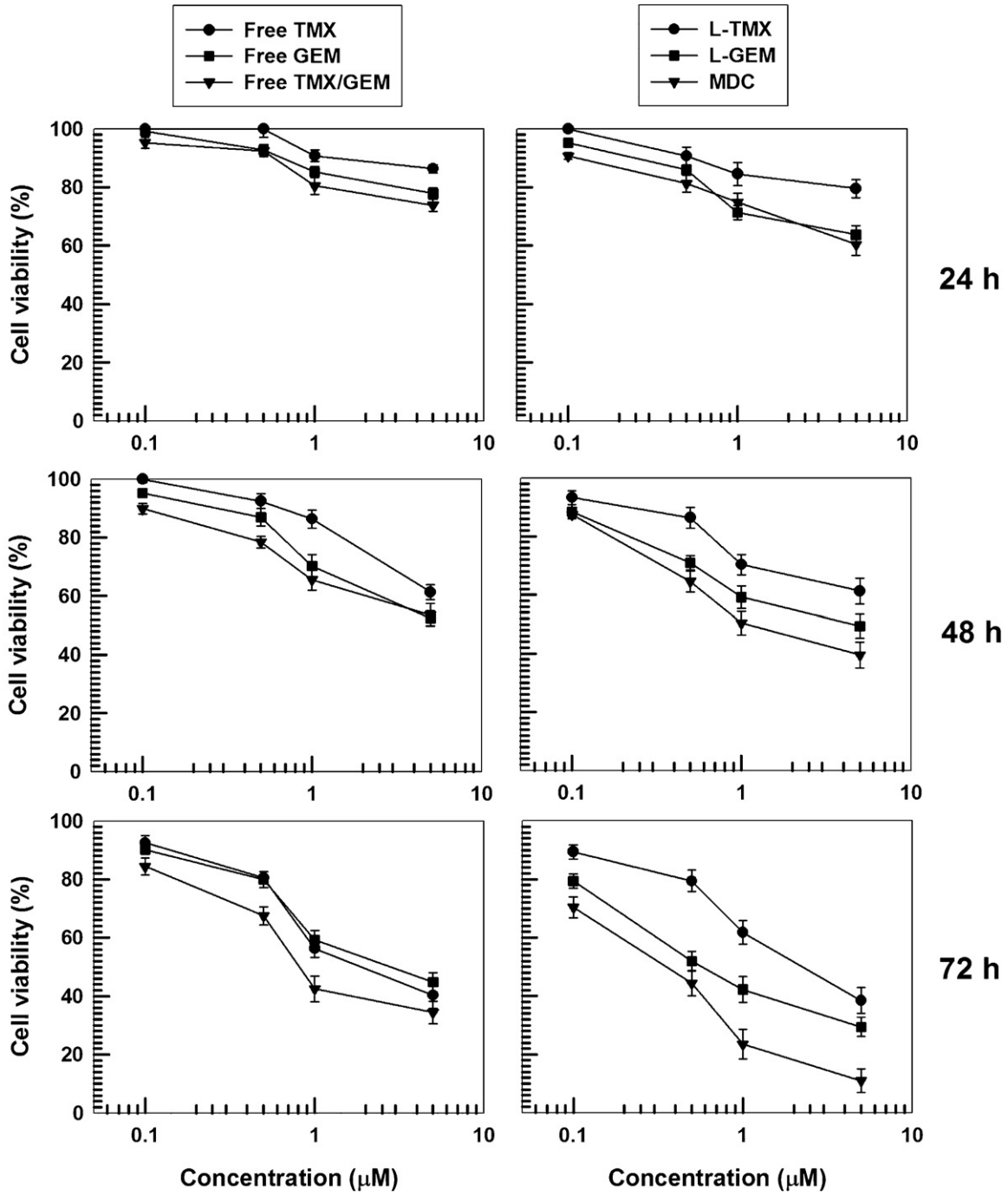

Fig. 4. *In vitro* cytotoxicity of GEM and TMX in free or PEGylated liposomal formulation on MCF-7 breast cancer cells as a function of drug concentration and exposition time. Data are expressed as percentage of cellular viability as evaluated by MTT-test. Results are the mean of six different experiments ±standard deviation. Error bars, if not shown, are within symbols.

The ability of the different liposome formulations to entrap the two antitumoral drugs was investigated (Table 2). Considering the possible applications of the best liposomal MDC formulation, the amount of the two antitumoral drugs in each system was modulated in order to obtain a similar final drug concentration (about 1 mM). GEM was effectively entrapped in all formulations as a consequence of the application of the pH gradient (Celano et al., 2004; Calvagno et al., 2007). In particular, liposomal formulations A and C showed a degree of GEM entrapment of about 90% (deriving also from the interaction between the antitumoral drug and the hydroxyl groups of PEG and glycerol, respectively) while formulation B showed a loading capacity value of about 70% (Table 2), probably as a consequence of the electrostatic repulsion between the amino groups of the drug and the ammonium residues of DOTAP.

It was very interesting to note the high entrapment efficiency of the TMX in the different formulations that varied between 85% and 94%, supporting Bhatia's evidence in which a loading capacity value of about 60% after the addition of 66 μg of TMX per mg of lipids to a mixture of DPPC/chol was obtained, with the aim of realizing topical liposomes (Bhatia et al., 2004). Moreover, the lipophilic antitumoral drug allowed for the effective co-encapsulation of GEM in the same formulation; in fact, all liposomal MDC formulations contained a great amount of both

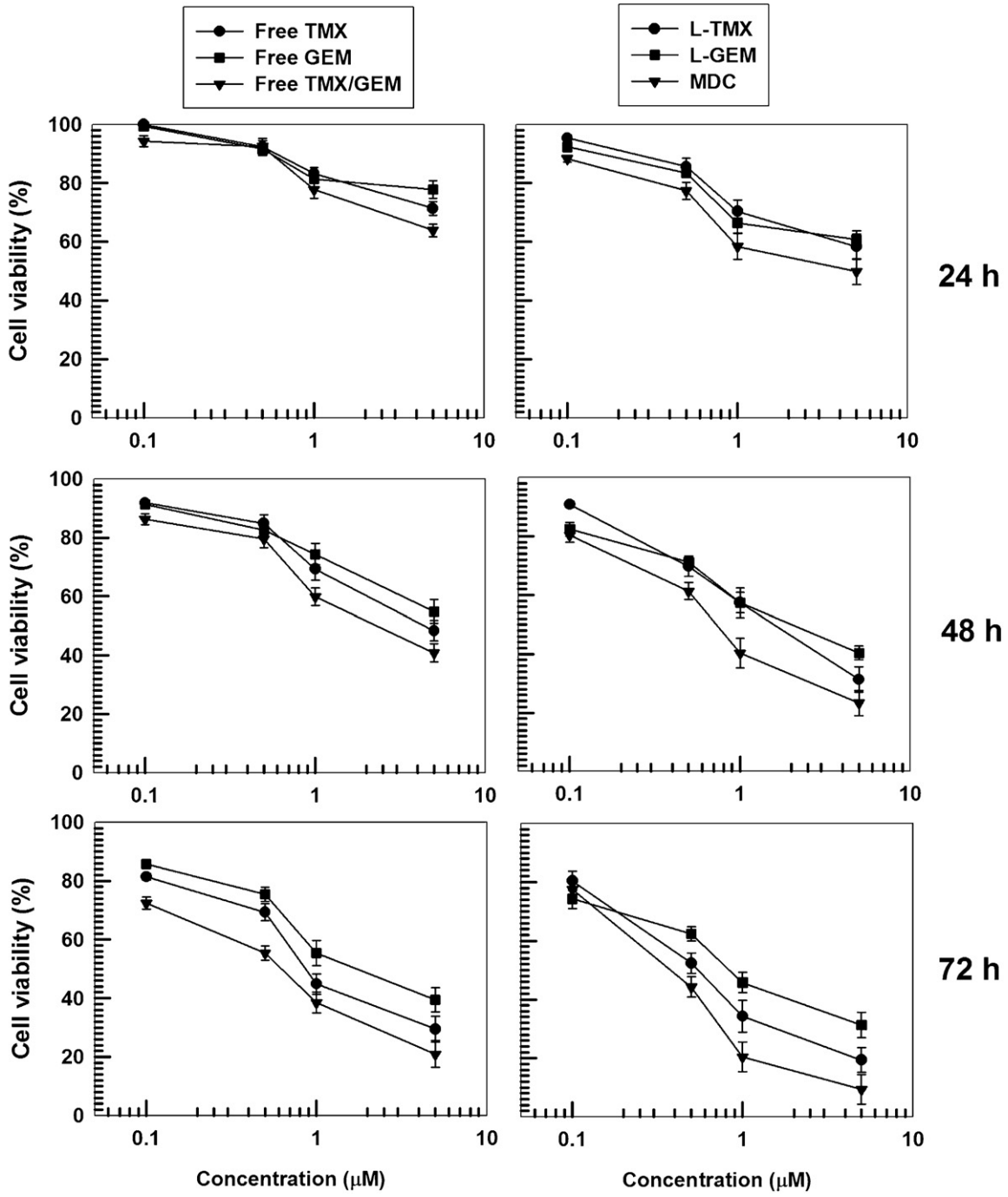

Fig. 5. *In vitro* cytotoxicity of GEM and TMX in free or PEGylated liposomal formulation on T47D breast cancer cells as a function of drug concentration and exposition time. Data are expressed as percentage of cellular viability as evaluated by MTT-test. Results are the mean of six different experiments ±standard deviation. Error bars, if not shown, are within symbols.

drugs. MDC liposomal formulations A and C showed similar encapsulation data compared to the single drug-loaded liposomal formulations, while formulation B showed a significant reduction in the encapsulation of GEM, probably because the TMX was not able to counteract the GEM leakage from the liposomal system due to the electrostatic repulsion hypothesized above.

Therefore, in light of the physico-chemical and technological investigation, the choice of the most suitable formulation for both *in vitro* and *in vivo* application was the MDC formulation A, on account of its ideal mean size, size distribution, zeta-potential and drug loading efficiency. Although formulation C showed similar values to those of formulation A, it did not assure long circulation properties after systemic administration due to the absence of PEG on its surface.

The successive step was to evaluate the release profile of the two drugs from formulation A after their encapsulation in single form and in association (Fig. 3). We previously described (Calvagno et al., 2007) a biphasic GEM leakage from formulation A, characterized by an initial burst effect, which depended on the GEM released by the PEG mushroom distributed on the vesicle bilayers which, being hydrophilic, can absorb the nucleoside analogue during the encapsulation procedure (Fig. 3). On the contrary, the TMX, encapsulated as a single form in the same formulation, showed an almost constant leakage (about 50% after 24 h). It was interesting to observe

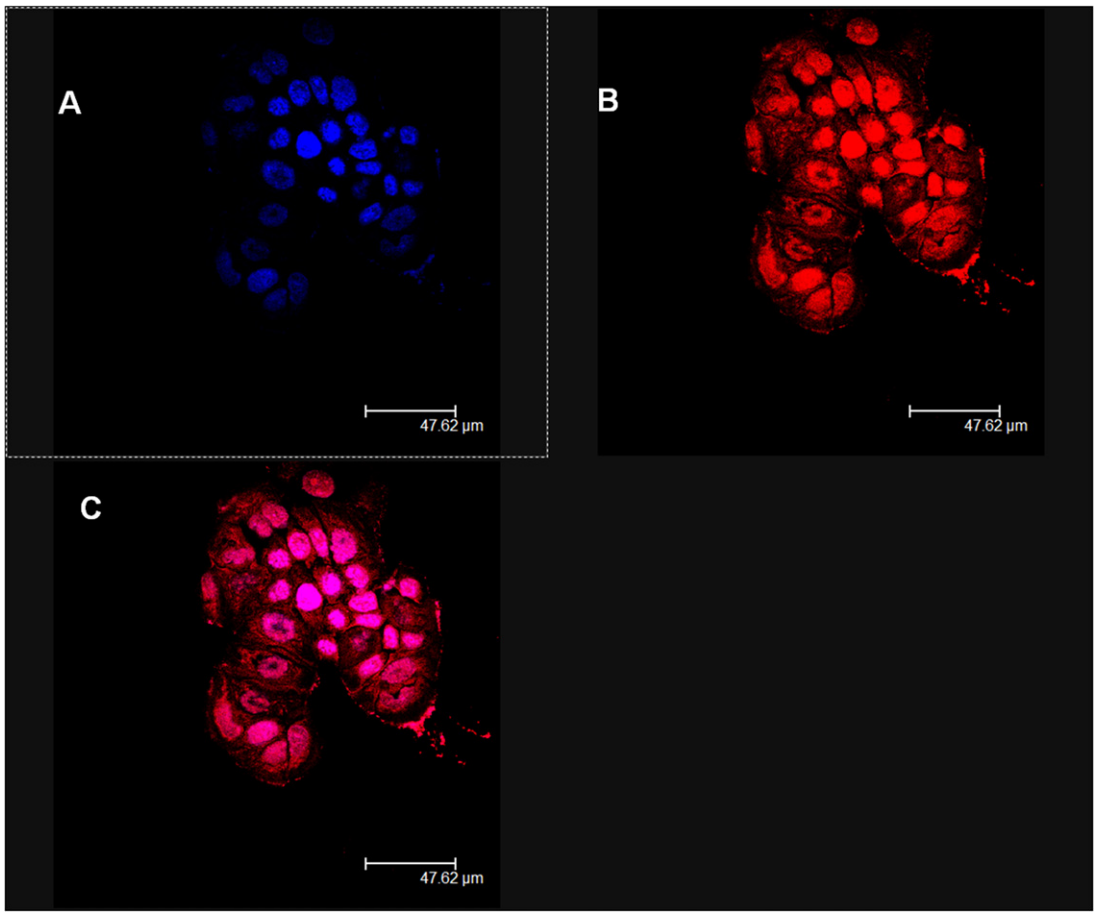

Fig. 6. CLSM micrographs of T47D cells treated with rhodamine-labelled pegylated unilamellar liposomes after 6 h incubation. Panel A: Hoechst filter; panel B: TRITC filter; panel C: overlay.

that the TMX allowed the modulation of the GEM leakage, probably thanks to its harsh effect on the liposomal bilayer (Fig. 3). Although the GEM-liposomal formulation showed a significantly better anticancer efficacy compared to the free drug in xenograft models, this phenomenon could be very important for a possible *in vivo* systemic administration of the MDC formulation that could mostly concentrate the nucleoside in the tumoral area, thus favoring the synergistic action of the TMX (Paolino et al., 2010).

3.2. Evaluation of cytotoxicity

The association of GEM and TMX can represent a valid and effective therapy in the treatment of advanced breast cancer (Silvestris et al., 2008; Brauch and Jordan, 2009). For this reason, the anticancer activity of the liposomal MDC formulation A was evaluated *in vitro* on MCF-7 and T47D cells, a widely used model of human breast cancer, responsive to both free GEM and TMX (Zheng et al., 2007). Cytotoxic effects were evaluated as a function of both the incubation time (24, 48 or 72 h) and the drug concentration (from 0.1 to 5 μM) to define the time-exposition and the dose response effects, respectively. Free TMX was solubilised in ethanol before testing and the obtained values were normalized with respect to those obtained after the treatment of the cells with an identical volume of solvent used to dissolve the antitumoral lipophilic compound.

After 24 h incubation, MCF-7 cells showed a significant reduction in cell viability only at the highest investigated free drug dosages (1 and 5 μM), while the single drug-loaded liposomes elicited more effective inhibition of cell vitality than the respective free drugs at a concentration of 0.5 μM (Fig. 4). The associations of the two antitumoral drugs both in free and liposomal forms furnished the best results in terms of cytotoxicity. After 48 and 72 h incubation, this behavior became more evident; namely liposomal formulations induced a higher reduction in cell vitality as compared to the free drug forms while the liposomal MDC allowed the best antitumoral action. After 72 h, the MDC favored a reduction of cell vitality of 95% at a drug concentration of 5 μM as compared to the association of the two free drugs, which determined a reduction in viability of 70%. It was interesting to observe that it was possible to decrease the effective dosage in the case of the liposomal MDC, because at a drug concentration of 0.1 μM it was possible to assess a cell mortality of ~60% as compared to the association of the two free drugs which determined a cellular mortality of 30% (Fig. 4). Therefore the following decreasing cytotoxic effect profile was observed: liposomal MDC > GEM–TMX free association ≥ L-GEM > L-TMX > free GEM and TMX.

The same test carried out on T47D cells showed similar results even though the liposomal MDC furnished better cytotoxic results as compared to the other formulations after just 24 h. In fact, as can be seen in Fig. 5, the liposomal MDC elicited a reduction in cell vitality of more than 50% at a drug concentration of 5 μM, while the other samples furnished a value of only 40%. This difference became more evident upon increasing the incubation times. In fact, liposomal MDC allowed almost total cell mortality at a drug concentration of just 1 μM after 72 h. Moreover, it was interesting to note that the cytotoxic profile for the T47D cells was slightly different with respect to MCF-7, and that is that the liposomal MDC > L-TMX ≥ GEM-TMX association > free TMX > L-GEM > free GEM.

A possible explanation of the improved antitumoral effect of the liposomal MDC on MCF-7 and T47D cells when compared to the other formulations could be due to the synergistic actions of the two compounds allocated in the same colloidal carrier which could favor their cellular accumulation while avoiding their destabilization phenomena. In fact, two anticancer compounds, acting with different cellular mechanisms, could overcome the cancer resistance phenomena allowing a decrease in the effective dosage. This phenomenon is favored by the internalization of the drug prompted by the colloidal device which, in the case of GEM, avoids and bypasses the cell nucleoside transporter, allowing a great accumulation of the drug in the cells. In particular, after just 6 h incubation, rhodamine-liposomes incubated with T47D cells showed a significant red coloration of the entire cell environment, a consequence of the carrier/cell interaction (Fig. 6). In fact, it is well known that a liposomal device can favor the intracellular uptake of the drug compound through different mechanisms, such as membrane fusion, endocytosis or lipid switch (Vono et al., 2010).

4. Conclusion

The association of two or more antitumoral compounds represents the modern treatment of different cancer diseases. The co-encapsulation of two bioactives having different physicochemical and pharmacological properties inside colloidal devices could open a new frontier in the drug delivery field, thus taking advantage of the synergistic action of the compounds in a specific area. Evidence of the increased antitumoral effect of GEM and TMX loaded into a PEGylated liposomal carrier as compared to the free single forms could open interesting perspectives in the cure of breast cancer. Moreover, the use of a PEGylated liposomal formulation, characterized by a colloidal mean diameter of 150 nm, a very narrow size distribution and a zeta-potential of −35 mV could assure an improvement in the biopharmaceutical properties of the encapsulated drugs.

These results represent a valid starting point for the use of this formulation for pre-clinical *in vivo* tests. In particular, as demonstrated in a previous work, the encapsulation of GEM in PEGylated liposomes allows to reduce the effective dosage of 10-fold in solid tumors with respect to the commercial formulation GEMZAR® as a consequence of the long circulating properties of the vesicles that facilitate their accumulation in the neoplastic areas (EPR effect) (Paolino et al., 2010). Considering the effect of TMX on the modulation of GEM leakage from liposomes, it is plausible that the lipophilic compound could further increase the efficacious dosage of the formulation for two reasons: (i) the existence of synergy of action with the nucleoside analogue and (ii) a lesser GEM leakage when liposomes are in the blood stream. All these considerations need to be investigated in order to verify a possible administration of MDC in humans.

Acknowledgements

The authors are very grateful to Lynn Whitted for her revision of the language of this manuscript.

References

Airoldi, M., Cattel, L., Milla, P., Pedani, F., Garzaro, M., Dosio, F., 2008. Paclitaxel and pegylated liposomal doxorubicin in recurrent head and neck cancer: clinical and unexpected pharmacokinetic interactions. Anticancer Res. 28, 2519–2527.

Bhatia, A., Kumar, R., Katare, O.P., 2004. Tamoxifen in topical liposomes: development, characterization and in-vitro evaluation. J. Pharm. Pharm. Sci. 7, 252–259.

Brauch, H., Jordan, V.C., 2009. Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the personalized approach? Eur. J. Cancer 45, 2274–2283.

Calvagno, M.G., Cosco, D., Celia, C., Paolino, D., Russo, D., Fresta, M., 2006. Liposomal multidrug carriers encapsulating gemcitabine and paclitaxel for the treatment of multi-drug resistant tumors. In: 33rd Annual Meeting & Exposition of the Controlled Release Society, Vienna, Austria, 22–26 July 2006, p. 678.

Calvagno, M.G., Celia, C., Paolino, D., Cosco, D., Iannone, M., Castelli, F., Doldo, P., Fresta, M., 2007. Effects of lipid composition and preparation conditions on physical-chemical properties, technological parameters and in vitro biological activity of gemcitabine-loaded liposomes. Curr. Drug Deliv. 4, 89–101.

Celano, M., Calvagno, M.G., Bulotta, S., Paolino, D., Arturi, F., Rotiroti, D., Filetti, S., Fresta, M., Russo, D., 2004. Cytotoxic effects of gemcitabine-loaded liposomes in human anaplastic thyroid carcinoma cells. BMC Cancer 4, 63–70.

Celano, M., Schenone, S., Cosco, D., Navarra, M., Puxeddu, E., Racanicchi, L., Brullo, C., Varano, E., Alcaro, S., Ferretti, E., Botta, G., Filetti, S., Fresta, M., Botta, M., Russo, D., 2008. Cytotoxic effects of a novel pyrazolopyrimidine derivative entrapped in liposomes in anaplastic thyroid cancer cells in vitro and in xenograft tumors in vivo. Endocr. Relat. Cancer 15, 499–510.

Celia, C., Calvagno, M.G., Paolino, D., Bulotta, S., Ventura, C.A., Russo, D., Fresta, M., 2008. Improved in vitro anti-tumoral activity, intracellular uptake and apoptotic induction of gemcitabine-loaded pegylated unilamellar liposomes. J. Nanosci. Nanotechnol. 8, 2102–2113.

Colomer, R., 2005. What is the best schedule for administration of gemcitabine–taxane? Cancer Treat. Rev. 31, S23–S28.

Cosco, D., Bulotta, A., Ventura, M., Celia, C., Calimeri, T., Perri, G., Paolino, D., Costa, N., Neri, P., Tagliaferri, P., Tassone, P., Fresta, M., 2009a. In vivo activity of gemcitabine-loaded PEGylated small unilamellar liposomes against pancreatic cancer. Cancer Chemother. Pharmacol. 64, 1009–1020.

Cosco, D., Paolino, D., Muzzalupo, R., Celia, C., Citraro, R., Caponio, D., Picci, N., Fresta, M., 2009b. Novel PEG-coated niosomes based on bola-surfactant as drug carriers for 5-fluorouracil. Biomed. Microdevices 11, 1115–1125.

Cosco, D., Paolino, D., Maiuolo, J., Russo, D., Fresta, M., 2011. Liposomes as multicompartimental carriers for the multidrug delivery in anticancer chemotherapy. Drug Deliv. Transl. Res. 1, 66–75.

Gabizon, A.A., Shmeeda, H., Zalipsky, S., 2006. Pros and cons of the liposome platform in cancer drug targeting. J. Liposome Res. 16, 175–183.

Kayyali, R., Marriott, C., Wiseman, H., 1994. Tamoxifen decreases drug efflux from liposomes: relevance to its ability to reverse multidrug resistance in cancer cells? FEBS Lett. 344, 221–224.

Lonez, C., Vandenbranden, M., Ruysschaert, J.M., 2008. Cationic liposomal lipids: from gene carriers to cell signaling. Prog. Lipid Res. 47, 340–347.

Nagayasu, A., Uchiyama, K., Kiwada, H., 1999. The size of liposomes: a factor which affects their targeting efficiency to tumors and therapeutic activity of liposomal antitumor drugs. Adv. Drug Deliv. Rev. 40, 75–87.

Paolino, D., Cosco, D., Racanicchi, L., Trapasso, E., Celia, C., Iannone, M., Puxeddu, E., Costante, G., Filetti, S., Russo, D., Fresta, M., 2010. Gemcitabine-loaded PEGylated unilamellar liposomes vs GEMZAR®: biodistribution, pharmacokinetic features and in vivo antitumor activity. J. Control Release 144, 144–150.

Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C., Martin, F.J., 1991. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc. Natl. Acad. Sci. U. S. A. 88, 11460–11464.

Pasut, G., Veronese, F.M., 2009. PEGylation for improving the effectiveness of therapeutic biomolecules. Drugs Today (Barc.) 45, 687–695.

Robert, N.J., Vogel, C.L., Henderson, I.C., Sparano, J.A., Moore, M.R., Silverman, P., Overmoyer, B.A., Shapiro, C.L., Park, J.W., Colbern, G.T., Winer, E.P., Gabizon, A.A., 2004. The role of the liposomal anthracyclines and other systemic therapies in the management of advanced breast cancer. Semin. Oncol. 31, 106–146.

Silvestris, N., Cinieri, S., La Torre, I., Pezzella, G., Numico, G., Orlando, L., Lorusso, V., 2008. Role of gemcitabine in metastatic breast cancer patients: a short review. Breast 17, 220–226.

Sponziello, M., Scipioni, A., Durante, C., Verrienti, A., Maranghi, M., Giacomelli, L., Ferretti, E., Celano, M., Filetti, S., Russo, D., 2010. Regulation of sodium/iodide symporter and lactoperoxidase expression in four human breast cancer cell lines. J. Endocrinol. Invest. 33, 2–6.

Tardi, P.G., Gallagher, R.C., Johnstone, S., Harasym, N., Webb, M., Bally, M.B., Mayer, L.D., 2007. Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo. Biochim. Biophys. Acta 1768, 678–687.

Theodossiou, C., Cook, J.A., Fisher, J., Teague, D., Liebmann, J.E., Russo, A., Mitchell, J.B., 1998. Interaction of gemcitabine with paclitaxel and cisplatin in human tumor cell lines. Int. J. Oncol. 12, 825–832.

Tomao, S., Romiti, A., Massidda, B., Ionta, M.T., Farris, A., Zullo, A., Brescia, A., Santuari, L., Frati, L., 2002. A phase II study of gemcitabine and tamoxifen in advanced pancreatic cancer. Anticancer Res. 22, 2361–2364.

Vono, M., Cosco, D., Celia, C., Paolino, D., Celano, M., Russo, D., Fresta, M., 2010. In vitro evaluation of the activity of gemcitabine-loaded pegylated unilamellar liposomes against papillary thyroid cancer cells. Open Drug Deliv. J. 4, 55–62.

Yan, X., Scherphof, G.L., Kamps, J.A., 2005. Liposome opsonization. J. Liposome Res. 15, 109–139.

Zheng, A., Kallio, A., Härkönen, P., 2007. Tamoxifen-induced rapid death of MCF-7 breast cancer cells is mediated via extracellularly signal-regulated kinase signaling and can be abrogated by estrogen. Endocrinology 148, 2764–2777.