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New chimeric advanced Drug Delivery nano Systems (chi-aDDnSs) as doxorubicin carriers

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ABSTRACT

Since the late 1960s, the field of drug delivery has focused on the creation of new formulations with improved properties, taking much attention to drug release from the carrier. Liposomes and dendrimers represent two of the most studied drug carriers. A Modulatory Liposomal Controlled Release System (MLCRS) combining liposomal and dendrimeric technology has been recently published as well as Liposomal *locked-in* Dendrimers (LLDs) technology which was considered to be a class of MLCRSs. Chimeric advanced Drug Delivery nano Systems (chi-aDDnSs) can be defined as mixed nanosystems due to the combination of the bionanomaterials used and can offer advantages as drug carriers. This work deals with the production of two new chi-aDDnSs incorporating the newly synthesized dendrimer PG1. One of the two formulations bears the exact lipidic composition as the commercial liposomal drug "Myocet". Doxorubicin (Dox) was incorporated into conventional (free of dendrimer) liposomal formulations and into the corresponding chi-aDDnSs, and the physicochemical characteristics, the *in vitro* drug release and the *in vitro* cytotoxicity against human cancer cell lines were assessed. The results revealed a different modulation release effect of doxorubicin from the chi-aDDnS, compared to the Myocet replica. Pharmacological cytotoxicity concerning all the chi-aDDnSs was very close to that of the conventional liposomal systems.

1. Introduction

Doxorubicin (Dox) is an anthracycline antibiotic used for cancer therapy. Because of its high effectiveness it is used against a wide range of cancers, carcinomas and soft tissue sarcomas. A problem associated with the use of Dox as a drug against cancer, is its side-effects that make its application sometimes unbearable for the patient. Dox also exhibits dose-dependent and sometimes lethal cardiomyopathy, thus limiting its applicability (Abraham et al., 2005). In order to overcome this problem, the scientific community has put a lot of effort towards formulating Dox in carriers that have been shown to limit the drug's release in blood and, thus, increase its therapeutic index (TI). Such carriers include micelles, dendrimers, solid-lipid nanoparticles and liposomes (Ke et al., 2008; Ma et al., 2009; Papagiannaros et al., 2006; Sun et al., 2009).

Liposomes are by far the most well studied of all Dox formulations, with a vast number of publications appearing in the literature. Furthermore, liposomes, are the only Dox carriers that have found their way to the market. At present, two commercial products are, in clinical application: Doxil (or Caelyx) that is composed of hydrogenated Soy phosphatidyl choline (HSPC):cholesterol (CHOL):polyethyleneglycol (PEG) attached to phosphatidylethanolamine (50:40:5 molar ratio) and Myocet which is composed of egg phosphatidyl choline (EPC):CHOL (55:45 molar ratio). Doxil/Caelyx exhibits a very prolonged circulation time due to the presence of the PEG outer layer which leads to significant changes in the biodistribution of the drug and high accumulation in the skin. This is an advantage for the treatment of skin cancers, such as sarcoma Kaposi, but also leads to a new side-effect, the "hand and foot syndrome". Myocet, on the other hand, releases Dox significantly faster and its circulation time has been shown to be three times higher than the free drug. Myocet has also been shown clinically to reduce the cardiomyopathy and gastrointestinal toxicity of Dox.

Both formulations act through the mechanism of passive targeting, taking advantage of the enhanced permeation and retention (EPR) effect that concerns vesicles with size smaller than 200 nm. Such vesicles are easily capable of crossing the leaky tumor vasculature and releasing their content in the target area in a controlled

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way. The result of this modulation on the pharmacokinetic and pharmacological profile of the drug is a better tolerated and more effective therapy (Maeda, 2001; Maeda et al., 2000, 2009).

Dendrimer are another class of nanocarrier that have the potential to be used in effective Dox formulations (Lai et al., 2007; Papagiannaros et al., 2005). They are a relatively new class of polymers and the total control over the synthesis offers the advantage of providing a product with a single molecular weight (Gillies and Frechet, 2002, 2005; Tomalia, 2005; Tomalia and Fréchet, 2002; Tomalia et al., 1990). A recent significant application of dendrimers, is their combination with liposomes to produce Liposomal *locked-in* Dendrimers (LLDs) (Gardikis et al., 2010b, c; Tekade et al., 2009). LLDs could more precisely be termed 'chimeric' to reflect the combination of two different nanobiomaterials in one single nanocarrier. Such nanocarriers have been classified as chimeric advanced Drug Delivery nano Systems (chi-aDDnSs) due to the combination of two independent technologies for producing an overall system with unique properties (Demetzos, 2010a,b) which could have advantages over conventional liposomal carriers. Important benefits of such formulations are (a) the increase of the drug load taken up by the system, this being important for reasons related to manufacturing cost and also to carrier-related side-effects (such as the hand and foot syndrome) and (b) the modification of the release of the drug from the chi-aDDnS compared to that of the liposomal formulation leading to higher TI (Gardikis et al., 2010b; Khopade et al., 2002; Tekade et al., 2009). As the dendrimer acts as a modulator for the release rate of the encapsulated Dox, chi-aDDnSs belong to the Modulatory Controlled Release nano Systems (MCRnSs) (Demetzos, 2010a,b; Papagiannaros et al., 2005; Tekade et al., 2009). Allthough the exact mechanism of release modulation is still unclear, there are several studies that shed light on the physicochemical interaction of the substances of such combined nanosystems composed of different nanobiomaterials (i.e. liposomes and dendrimers) (Gardikis et al., 2006, 2010b, c; Klajnert and Epand, 2005).

In the present study, two conventional liposomal systems (i.e. DOPC/CHOL and EPC/CHOL) one of which (EPC/CHOL) had the same lipid composition as the commercial product "Myocet", were incorporated into the newly synthesized PG1 dendrimer (Gardikis et al., 2010b) in order to produce two new chi-aDDnSs. These chi-aDDnSs were subsequently loaded with Dox and evaluated in terms of their physicochemical characteristics, drug loading efficiency, *in vitro* drug release of Dox and *in vitro* cytotoxicity, vs the respective conventional liposomal carriers.

2. Materials and methods

2.1. Materials

PG1 (empirical formula $C_{25}H_{44}O_{20}$; formula weight: 664.2) is a hydroxyl-terminated dendrimer and was synthesized following a divergent strategy. The synthesis and the structure of the dendrimer is presented in the literature (Gardikis et al., 2010b).

Egg-phosphatidyl-choline (EPC) and dioleoyl-phosphatidyl-choline (DOPC) were purchased from Avanti Polar Lipids. Cholesterol (CHOL) was purchased from Sigma–Aldrich. All solvents were of analytical grade.

2.2. Methods

2.2.1. Pure liposome and advanced Drug Delivery Systems preparation

The liposomes prepared in this study consisted of (a) DOPC and CHOL in a 65:35 molar ratio and (b) EPC and CHOL in a 55:45 molar ratio (Myocet replica). These specific molar ratios were chosen because they gave the best results in terms of vesicular characteristics after reconstitution of their lyophilized forms: relatively narrow size distribution (Polydispersity Index, or PI <0.4 and mean diameter within the 50–250 nm range). In the case of chi-aDDnSs, PG1 dendrimeric solutions in methanol were mixed with the lipid solutions. The initial dendrimer/lipid molar ratio was 0.1. $(NH_4)_2SO_4$ 150 mM pH 5.5 with 150 mM sucrose as a cryoprotectant was added and the mixture was vortexed until the induction of a homogenous emulsion. MLV preparation was carried out using the reverse phase evaporation method (REV) (Szoka and Papahadjopoulos, 1978). Sonication was applied to afford SUVs with reduced PI and the extraliposomal pH was changed to 7.5 through gel permeation chromatography using a Sephadex G75 column with PBS 10 mM pH 7.5/150 mM sucrose as a mobile phase. This chromatographic technique, in parallel, removes extraliposomal (excess) of dendrimer molecules from the chi-aDDnS suspension (Gardikis et al., 2010b).

Doxorubicin was loaded to either conventional liposomes or to chi-aDDnSs by incubation either at room temperature (DOPC/CHOL system) or at 37 °C (EPC/CHOL system) for 1 h. Unentrapped Dox was removed by gel permeation chromatography using a Sephadex G75 column.

2.2.2. Freeze drying of liposomal suspensions

Free or Dox loaded conventional liposomes and chi-aDDnSs were frozen at −80 °C overnight and were subjected to lyophilization in order to overcome stability issues concerning liposomal suspensions (Wang et al., 2009). The lyophilization was achieved using a freeze drier (TELSTAR Cryodos-50, Spain) under the following conditions: condenser temperature from −50 °C, vacuum 8.2×10^{-2} mb. Reconstitution was made by adding the appropriate amount of HPLC-grade water.

2.2.3. Characterization of free and Dox loaded formulations

The hydrodynamic diameter of empty and Dox-loaded formulations was measured by light scattering. 100 μL of the liposomal suspension was 30-fold diluted immediately after preparation or after reconstitution and *z*-average mean and ζ-potential of the empty and Dox loaded formulations were measured. Samples were scattered (633 nm) at 90° angle, and measurements were made at 25 °C in a photon correlation spectrometer (Zetasizer 3000HS, Malvern Instruments, Malvern, UK) and analysed by the CONTIN method (MALVERN software). Dendrimer and lipid quantification was done by HPTLC-FID (Iatroscan) (Hatziantoniou and Demetzos, 2006) with chloroform/methanol/water 60:20:3.2 (v:v) as a mobile phase. The incorporation of Dox into all formulations was determined by UV spectrometry (UV – 1700, UV–visible spectrophotometer, Shimadzu, Pharmaspec, Kyoto, Japan) at a wavelength of 480 nm after the addition of methanol to the liposomal suspension and with the aid of a Dox calibration curve in methanol. Pure methanol was used as blank.

2.2.4. In vitro release studies

Dox loaded conventional liposomal formulations or chi-aDDnSs were placed in 12000 MWCO 25 mm width dialysis sacks (Sigma–Aldrich). Dialysis sacks were inserted in RPMI 5% medium in shaking water bath (Selecta) set at 37 °C. The Dox concentration was at least 10 times lower than the saturation point for this specific solution throughout the experiment leading to the conclusion that the experimental conditions for the *in vitro* release study are very close to perfect sink conditions. Aliquots of samples (1 mL) were taken from the external solution at specific time points and that volume was replaced with RPMI incubated at 37 °C. Dox concentrations were measured with UV spectrometry after the addition of 2 mL HPLC-grade water. RPMI incubated at 37 °C, diluted 3-fold with HPLC-grade water, was used as reference sample. The cumulative percentage of drug release was calculated and plotted versus

Table 1
Mean diameter and ζ-potential of conventional liposomes and chi-aDDnSs before lyophilization and after freeze-drying.

System	Mean diameter (nm)	SD	PI	S.D.	ζ-Potential (mV)	SD	Lyophilization
DOPC/CHOL	75.3	0.9	0.241	0.033	−15.4	4.5	Before
	78.5	1.6	0.228	0.022	−20.8	2.2	After
EPC/CHOL	92.15	6	0.166	0.030	−12.6	0.1	Before
	99.7	8.3	0.199	0.020	−13.7	0.4	After
DOPC/CHOL/PG1	75.3	1.1	0.212	0.004	−15.8	0.3	Before
	77.3	1.9	0.242	0.006	−16	0.7	After
EPC/CHOL/PG1	101.6	1.1	0.133	0.004	−2.8	0.3	Before
	110.4	1.1	0.185	0.003	−2.6	0.8	After
DOPC/CHOL/DOX	78.4	4.4	0.261	0.034	−16	4.7	Before
	98.5	1.8	0.23	0.044	−10.9	3.1	After
EPC/CHOL/DOX	98.9	4.1	0.187	0.033	−11.8	2.1	Before
	185.9	5.3	0.376	0.098	−7.9	7.5	After
DOPC/CHOL/PG1/DOX	104.4	2.3	0.199	0.011	−15.7	0.6	Before
	131.4	2.3	0.246	0.011	−16	0.5	After
EPC/CHOL/PG1/DOX	116.6	1.4	0.194	0.004	−7.5	0.3	Before
	220.3	2.9	0.341	0.02	−8.4	0.7	After

time using the equation:

$$\%\ \text{Released Dox} = \frac{\text{Dox}_{\text{released}}}{\text{Dox}_{\text{initial}}}$$

2.2.5. In vitro pharmacological studies

The *in vitro* activity of free Dox, the four Dox-loaded systems: DOPC/CHOL, DOPC/CHOL/PG1, EPC/CHOL, EPC/CHOL/PG1 as long as the activity of the empty carriers and PG1 dendrimer alone was performed against MCF7 and MB231 human breast cancer cell lines using the SRB (sulphorodamine B) (Gardikis et al., 2010a) assay. Cell viability was assessed at the beginning of each experiment by the trypan blue dye exclusion method, and was always greater than 95%.

Cells were seeded into 96-well cell culture plates in 100 μL of medium at a density of 7500 for MCF7 cells and 20,000 cells per well for MB231 cells. Subsequently the plates were incubated at standard conditions for 24 h to allow the cells to resume exponential growth prior to the addition of Dox. Then, in order to measure the starting cell population, cells in one plate were fixed *in situ* with TCA followed by SRB staining, as described elsewhere (Gardikis et al., 2010a). Dox and the formulations (empty or loaded with doxorubicin) were further added at five 10-fold dilutions (from 100 to 0.01 μM) to the cell culture plates and the incubation continued for an additional period of 48 h. The assay was terminated by addition of cold TCA followed by SRB staining and absorbance measurement at 530 nm, in an EL-311 BIOTEK microelisa reader (BioTek, Winooski, VT, USA), to determine the three parameters GI_{50}, TGI and LC_{50} (Gardikis et al., 2010a). Therapeutic Index (TI) was defined as the ratio of LC_{50}/GI_{50}. All experiments were done in triplicate and repeated at least twice.

2.2.6. Statistical analysis

Results are shown as mean value ± SD of three independent experiments. In order to analyse differences in variables before and after freeze-drying the paired Student's *t*-test was used. Comparison between different groups was done using one-way ANOVA followed by Tukey multiple comparison test when equal variances were assumed and Dunnett's C multiple comparison test when equal variances were not assumed. In order to assess the correlations between the pairs of the variables' parameters of the regression line were estimated together with the regression coefficient *r*. The no correlation hypothesis was rejected on the $P = 0.05$ significance level. *P* values <0.05 were considered statistically significant. Statistical analysis was done using "SPSS 14.0."

3. Results

3.1. Characterization of free and Dox loaded formulations

The physicochemical characteristics of free and Dox-loaded liposomes and chi-aDDnSs before freeze-drying or after reconstitution are presented in Table 1.

In all cases, before freeze drying, Small Unilamellar Vesicles (SUVs) with a slight negative charge were produced. The incorporation of PG1 into DOPC/CHOL liposomal vesicles did not significantly affect their mean diameter, while in the case of EPC/CHOL vesicles it induced the formation of slightly larger vesicles. On the contrary, PG1 incorporation seemed to ameliorate the Polydispersity Index (PI), calculated from cumulant analysis of the correlation functions, of the EPC/CHOL system and the repeatability of the ζ-potential measurements in both systems.

The incorporation of Dox into the liposomal vesicles did not provoke any changes to the vesicular characteristics. On the other hand, when Dox was incorporated into chi-aDDnSs, the liposomal mean diameter was found to be significantly higher. On the contrary, this pattern was not followed by the PI meaning that Dox-loaded chi-aDDnSs demonstrate similar size distribution to empty chi-aDDnSs or liposomes.

Reconstitution after freeze-drying of empty conventional liposomes or chi-aDDnSs was successful in all cases, as both the mean diameter and the ζ-potential remained unaffected. Only the EPC/CHOL/PG1 system exhibited a small increase in vesicular size after reconstitution. On the contrary, all Dox-loaded systems demonstrated a significant increase in mean diameter after reconstitution. EPC/CHOL liposomes and chi-aDDnSs, especially, were found to be almost double size and more polydisperse after the lyophilization process.

Dendrimer locking into liposomes was almost quantitative as measured by HPTLC-FID since more than 90% of the initial dendrimer was entrapped in the liposomal vesicle leading to a PG1:lipid ratio of 0.14 ± 0.01 and 0.13 ± 0.02 for DOPC/CHOL/PG1 and EPC/CHOL/PG1 chi-aDDnSs, respectively (see Table 2). Freeze-drying of chi-aDDnSs did not affect dendrimer entrapment. There was no significant dendrimer loss as measured by HPTLC-FID after gel permeation chromatography that followed the lyophilization process (data not shown).

Dox loading to either conventional liposomes or chi-aDDnSs using ammonium sulphate gradient was 95%, in consistence with what is reported in the literature (Fritze et al., 2006). The Dox/lipid molar ratios for EPC/CHOL and DOPC/CHOL liposomes and chi-aDDnSs is presented on Table 2. DOPC/CHOL liposomes and chi-aDDnSs encapsulated a significantly larger quantity of Dox

Table 2
PG1/lipid and Dox/lipid molar ratios (lipid refers to moles of phospholipid plus moles of cholesterol).

System	Molar ratio PG1/lipid	SD	Molar ratio Dox/lipid	SD
DOPC/CHOL/PG1	0.14	0.01		
EPC/CHOL/PG1	0.13	0.02		
DOPC/CHOL/Dox			0.46	0.14
EPC/CHOL/Dox			0.21	0.02
DOPC/CHOL/PG1/Dox	0.14	0.01	0.43	0.08
EPC/CHOL/PG1/Dox	0.13	0.02	0.26	0.02

compared to the respective systems consisting of EPC/CHOL. The presence of the dendrimer did not seem to affect Dox loading except the case of EPC/CHOL chi-aDDnS system that exhibited slightly higher Dox/lipid molar ratio than the respective conventional liposomal system. Freeze-drying did not affect Dox entrapment, as reported in the literature (vanWinden and Crommelin, 1997). More than 95% of Dox remained in the vesicle, as measured by UV–vis after gel permeation chromatography following reconstitution.

3.2. In vitro release studies

The cumulative *in vitro* release of Dox from conventional liposomes and chi-aDDnSs consisting of DOPC/CHOL or EPC/CHOL in RPMI 5% medium at 1, 4, 8, 24, 72 and 96 h is presented in Fig. 1.

During the first hour of the experiment the EPC/CHOL liposomal system released 16.6 ± 2.3% of entrapped Dox. From that point and on, the Dox release rate slowed down and after 96 h 40.6 ± 4.8% was released in the RPMI medium. In the case of EPC/CHOL/PG1 chi-aDDnSs, only 7.2 ± 4.6% of Dox is released during the first hour. After 8 h the cumulative release reaches a plateau and remains practically stable until the end of the experiment. At that point 18.2 ± 1.5% is released, 50% lower than the respective percentage of the EPC/CHOL liposomal system. On the contrary, the release from the DOPC/CHOL system was very low during the first hour of the experiment. Significant release takes place after 8 h, when 8.6% ± 2.8% of the initial Dox is released in the medium. At 96 h, the cumulative release is 24.1 ± 5.8%. The incorporation of PG1 in the DOPC/CHOL system did not affect the release profile of Dox significantly, differently to what was observed in the case of EPC/CHOL chi-aDDnSs and liposomes.

3.3. In vitro pharmacological experiments

All empty carriers (DOPC/CHOL, DOPC/CHOL/PG1, EPC/CHOL, EPC/CHOL/PG1) were tested for their toxicity against MB231 and MCF7 human breast cancer cell lines at various concentrations up to 100 μM and were found to be inactive. Free Dox was used as positive control. The growth rate of MB231 and MCF7 cell lines treated with various concentrations of the loaded formulations is shown in Fig. 2.

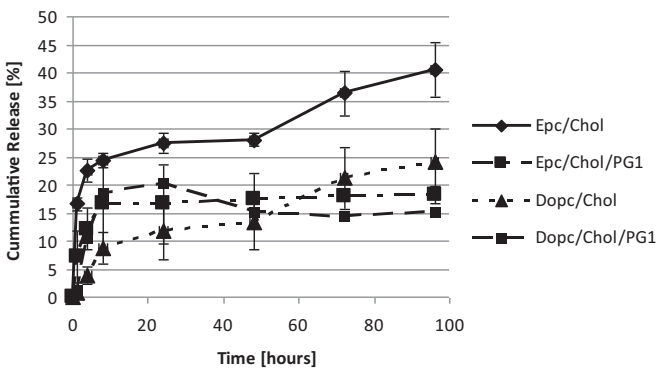

Fig. 1. Cumulative release of Dox from conventional liposomes and chi-aDDnSs consisting of DOPC/CHOL or EPC/CHOL in RPMI 5% medium over time

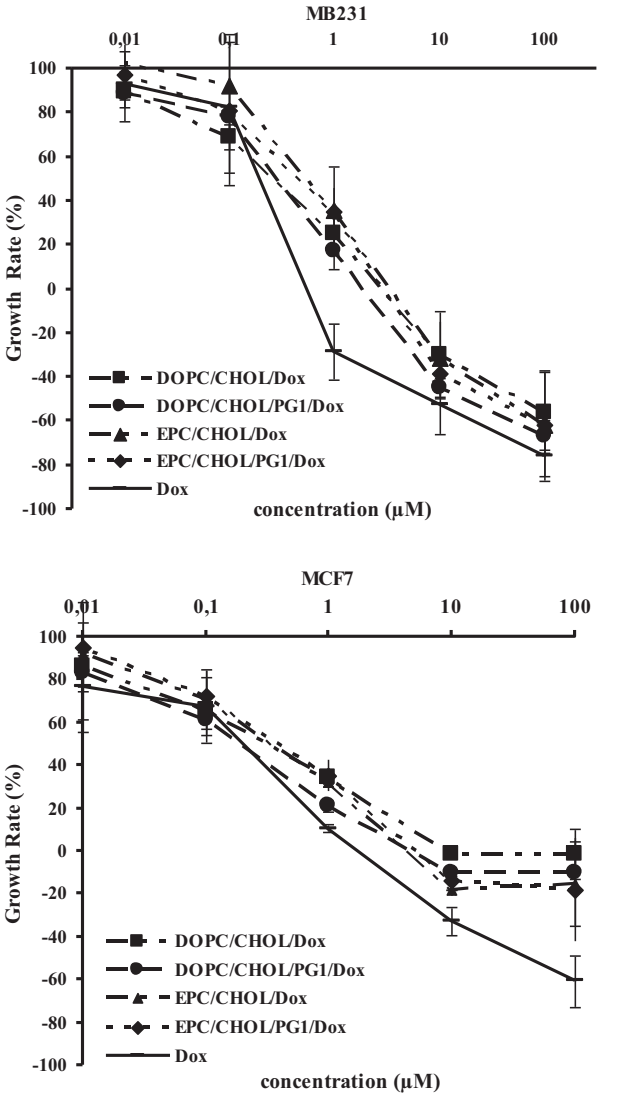

Fig. 2. Percent growth rate of MB231 and MCF7 human breast cell lines upon treatment with various concentrations of free Dox and the formulations: DOPC/CHOL/DOX, DOPC/CHOL/PG1/DOX, EPC/CHOL/DOX, EPC/CHOL/PG1/DOX for 48 h. Points represent the means of the two independent experiments carried out in triplicates ±SD.

Tables 3 and 4 illustrate the concentrations that induce 50% growth inhibition (GI_{50}), total growth inhibition (TGI) and death of the 50% (LC_{50}) of cancer cells of MB231 and MCF7 cell lines. In both cell lines, GI_{50} and TGI indices of liposomes or aDDnSs incorporating Dox were found to be quite similar to those of free Dox, indicating that all formulations retained the growth inhibiting activity of Dox. On the contrary, the LC_{50} value was significantly higher for

Table 3
GI_{50}, TGI and LC_{50} values of SRB assay for the systems: DOPC/CHOL/DOX, DOPC/CHOL/PG1/DOX, EPC/CHOL/DOX, EPC/CHOL/PG1/DOX as long as for free Dox in MB231 human breast cancer cell line. Values correspond to the mean of two independent experiments carried out in triplicates. CV < 10%.

	DOPC/CHOL	DOPC/CHOL/PG1	EPC/CHOL	EPC/CHOL/PG1	DOX
GI_{50}	0.49	0.52	0.77	0.71	0.46
TGI	5.12	3.51	5.79	5.28	1.37
LC_{50}	78.35	31.57	64.82	53.11	16.88
TI	159.90	61.1	84.18	74.80	36.70

Table 4
GI_{50}, TGI and LC_{50} values of SRB assay for the systems: DOPC/CHOL/DOX, DOPC/CHOL/PG1/DOX, EPC/CHOL/DOX, EPC/CHOL/PG1/DOX as long as for free Dox in MCF7 human breast cancer cell line. Values correspond to the mean of two independent experiments carried out in triplicates. CV < 12%.

	DOPC/CHOL	DOPC/CHOL/PG1	EPC/CHOL	EPC/CHOL/PG1	DOX
GI_{50}	0.55	0.35	0.58	0.64	0.46
TGI	9.72	7.01	6.73	7.47	1.37
LC_{50}	>100.00	>100.00	>100.00	>100.00	16.88
TI	181.82[a]	281.75[a]	172.41[a]	156.26[a]	36.70

[a] The LC_{50} could not be determined as it was higher than 100 μM, the highest concentration of Dox tested. Thus the TI was calculated by assuming LC_{50} = 100 μM.

all formulations compared to that of free Dox. Consequently, the Therapeutic Index (TI) was found to be significantly improved for all conventional liposomal or chi-aDDnS systems, compared to that of free Dox. Interestingly, the DOPC/CHOL/PG1 chi-aDDnS exhibited the best TI against MCF7 cell line as it demonstrated a very low growth inhibiting activity, as this is depicted by the GI_{50} parameter. This was even higher than that of the free anthracycline.

4. Discussion

Drug Delivery Systems (DDSs) seem to be an attractive field for research that can offer advantages for the production of drug nanocarriers with tailored characteristics, mainly concerning increased drug loads and controlled release of drugs. (Abraham et al., 2005; Papagiannaros et al., 2005; Tekade et al., 2009). A new synthesized poly-ester-ether PG1 dendrimer has already been incorporated into liposomes to afford a drug delivery nanosystem that demonstrates modulated rate of the release of Dox compared to that of conventional liposomal carriers (Gardikis et al., 2010b). This effect can be explained by modification of the stability of the liposome membrane and the cooperativity of the relevant gel-to-liquid crystal transition, which is enhanced in the presence of both dendrimer and the drug (Gardikis et al., 2010c). This process implies a practically complete displacement of the dendrimer and the drug into the aqueous core of the liposome, the membrane of which behaves as an homogeneous system with high cooperativity, like as simple (non liposomal) lipid bilayer (Gardikis et al., 2010c). A Modulatory Liposomal Controlled Release System (MLCRS) incorporating Dox has been recently published (Papagiannaros et al., 2005). Furthermore, the concept of Liposomal *locked-in* Dendrimers (LLDs) technology was also recently published and was considered to be a class of MLCRSs (Gardikis et al., 2010b,c; Tekade et al., 2009). Chimeric advanced Drug Delivery nano Systems (chi-aDDnSs) could be considered as a new class of advanced drug delivery nanosystems with a modulatory controlled release profile which combined liposomal and dendrimeric technologies and can offer advantages as drug carriers.

In this work, PG1 dendrimer was incorporated for the first time into liposomes composed of EPC or DOPC containing cholesterol.

Conventional liposomes composed of EPC/CHOL, exhibited significantly increased mean diameter compared to conventional liposomes consisting of DOPC/CHOL (92.1 nm and 75.3 nm, respectively). This result should be attributed to the heterogeneity of EPC that consists of phospholipids with shorter and longer, saturated and unsaturated, acyl chains that bear different degree of bending leading to larger and more polydisperse vesicles. On the contrary DOPC vesicles consist of a single phospholipid that induces the formation of smaller and more homogeneous liposomes. Furthermore, the ratio of cholesterol used for the liposomes consisting of EPC/CHOL (55/45) is higher compared to DOPC/CHOL (65/35). Cholesterol, due to increased concentration in EPC liposomes probably hinders stereochemically the bending of phospholipids upon SUV formation, leading to vesicles with increased mean diameter.

The incorporation of PG1 dendrimer into conventional liposomes composed of EPC/CHOL and DOPC/CHOL was considered to be successful in terms of the incorporation efficiency (>95% of the initial dendrimer quantity) as well as in terms of physicochemical characteristics before freeze-drying and after reconstitution. PG1, in both systems, seems to lead to the formation of less polydisperse vesicles with ζ-potential with lower heterogeneity between batches, as was reported in a recent work by our group (Gardikis et al., 2010b). This fact could be attributed to the stabilization of the liposomal membrane provoked by the incorporation of PG1 molecule. The fact that no change in vesicle size was observed between conventional liposomes and chi-aDDnSs should be attributed to the fact that the size of PG1 is very small (MW: 664, 2). Results in the literature were similar in the case of DOPC/DPPG/PG1 drug carrier compared to the respective liposomes (Gardikis et al., 2010b). The encapsulation of Dox into both conventional liposomal systems, similarly, did not alter the physicochemical characteristics of the vesicles. On the other hand, in the case of the chi-aDDnS a slight increase of the mean hydrodynamic diameter was observed. This "swelling" of the liposomes should be attributed to the co-existence of Dox and PG1, with probable Dox-PG1 complex formation in the interior cavity of the liposome, as this effect did not take place upon the presence of either just Dox or PG1. A possible explanation is the increased osmotic pressure of the aqueous interior of the vesicles due to the large quantity of the entrapped molecules (Gardikis et al., 2010b; Hupfeld et al., 2010).

Reconstitution of freeze-dried material in the case of both EPC/CHOL and DOPC/CHOL systems incorporating Dox gave rise to vesicles with increased hydrodynamic diameter. This occurs probably due to liposomal fusion caused by leakage of Dox during the lyophilization process. In the case of EPC/CHOL vesicles, the increase of the above parameters was almost 100%, which means that there is probably MLVs formation at some population of liposomes. It is known that the presence of cholesterol in the liposomal membrane reduces its tendency to fuse and aggregate. This fact is due to the rigidity that cholesterol provides, at some specific incorporation percentages, to the liquid crystalline membranes, as aggregation and fusion phenomena are favored in fluid and highly mobile bilayers. As a consequence, aggregation of liposomes containing cholesterol should be attributed to either modulation of the fluidity of the membrane caused by the incorporated molecules, or phenomena that can influence the external surface of the vesicles. The fact that there is no increase in the mean diameter or PI in any case, except the reconstituted Dox-loaded liposomes or chi-aDDnSs, could lead to the conclusion that the dendrimer, Dox, as well as their complex probably locate in the interior cavity of the liposome without interfering significantly with the physicochemical properties of the membrane. This hypothesis is supported by published calorimetric data from our group that demonstrate that, upon Dox loading into similar chi-aDDnSs, the PG1-Dox complex migrates to the interior of the vesicle leading to structural and energetic changes of the liposomal membrane (Gardikis et al., 2010c). At the stage of lyophilization, though, probably during the freezing procedure (Van Bommel and Crommelin, 1984) a leakage of Dox is eager to take place. Free Dox during the hydration stage upon reconstitution is capable of inducing fusion or aggregation of cholesterol containing liposomes, as reported in the literature (Martí et al., 1991). Probably the largest amount of Dox released during freeze-drying is re-encapsulated inside the vesicles during fusion or aggregation, as drug loss was less than 5% after reconstitution, as measured by UV–vis spectroscopy after gel permeation chromatography.

The encapsulation percentage of Dox was 95% similar to what has been reported in the literature (Abraham et al., 2005). Inter-

estingly, the conventional liposomes composed of DOPC/CHOL exhibited very high drug to lipid molar ratio, almost twice as high as to the respective ratio of the EPC/CHOL systems. This can probably be attributed to the longer acyl chain of DOPC (18C) that creates a stronger lipophilic barrier compared to EPC that is a mixture of lipids with shorter and longer chains.

Furthermore, it is possible that EPC cannot incorporate cholesterol as homogenously as DOPC, creating areas poor in cholesterol that are more permeable, leading to Dox leakage. This hypothesis is probably the base of the slower release of Dox from DOPC/CHOL liposomes in RPMI 5% medium, compared to EPC/CHOL liposomes.

Chi-aDDnS composed of DOPC/CHOL liposomes did not demonstrate any significant changes to the release rate of Dox compared to that of the conventional system. On the contrary, chi-aDDnSs composed of EPC/CHOL exhibited significant retardation of Dox release compared to the conventional liposomes composed of EPC/CHOL. This fact could be interpretated by the PG1/Dox molar ratio that was found to be 0.32 for DOPC/CHOL chi-aDDnSs compared to a ratio of 0.50 for EPC/CHOL chi-aDDnSs. It is possible that the increased relative concentration of dendrimer in the case of EPC is responsible for the retardation of the leakage of Dox. Taking into consideration that the PG1-incorporated quantity was the highest possible – with the specific production method applied – a possible solution for the lowering of the release rate of Dox from the chi- aDDnS DOPC/CHOL would be the incorporation of higher generation dendrimers. On the other hand, this could create very large vesicles, if it is taken into consideration that the reconstituted chi-aDDnS exhibited 100% size increase compared to the respective liposome. In a recent publication from our group it has been shown that the incorporation of second generation PG dendrimer (PG2) gave rise to significantly larger DOPC/DPPG vesicles compared to the respective PG1 chi-aDDnSs (Gardikis et al., 2010b). Large Unilamellar Vesicles (LUVs >200 nm) are prone to higher rate of uptake from the reticuloendothelial system that leads to lower drug bioavailability (Abraham et al., 2005).

Finally, the *in vitro* activity studies revealed that both liposomal and chi-aDDnSs preparations were able to retain the growth inhibiting activity of the free anthracycline while they diminished the drug toxicity of the drugs (i.e. exhibited much higher LC_{50}s) against the two cell lines tested. Thus, all systems under consideration seem to increase the TI of Dox. An interesting observation was that amongst all preparations tested, the DOPC/CHOL/PG1 chi-aDDnS presented the highest growth inhibiting activity and the best TI. This fact makes this system a basic candidate for further *in vivo* investigation that will confirm the increase of TI provoked by the formulation of Dox in this chi-aDDnS and evaluate it as an optimum drug carrier.

5. Conclusions

The combination of liposomes and dendrimers in a single drug carrier is gaining attention in the pharmaceutical nanotechnology field of research. The main challenges concerning drugs with low TI, namely fast or unsustained drug release and low drug encapsulation, seem to be able to be overcome by using the concept of chi-aDDnSs. Careful tailoring of the physicochemical properties of the carrier components may lead to products with higher bioavailability, effectiveness and fewer side-effects. In this study it was shown that lyophilized products with very interesting properties can be prepared using the chi-aDDnS technology. Interestingly, it was shown that EPC/CHOL as chi-aDDnS can encapsulate Dox more effectively and release it more slowly than the respective liposomes (Myocet replica). This means that a final lyophilized product ready for the shelf, which exhibits more interesting kinetic properties while retaining the TI of the commercial product can be prepared without any further production step. On the other hand, the incorporation of the dendrimer into DOPC/CHOL liposomes did not affect Dox encapsulation or release rate, a fact that could be attributed to the lower dendrimer/Dox ratio achieved for this system. Interestingly, this chi-aDDnS exhibited an increase of the TI in MCF7 cell line compared to the respective conventional liposomal formulation.

In vivo experiments are under design in order to confirm and get the proof-of-concept for the superiority of chi-aDDnSs for the delivery of highly toxic drugs such as Dox, compared to conventional liposomal drug carriers.

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