DOI 10.1002/mnfr.200700474 Mol. Nutr. Food Res. 2009, *53*, 592 – 599

Research Article

Does the lipid membrane composition of arsonoliposomes affect their anticancer activity? A cell culture study

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Sonicated arsonoliposomes were prepared using arsonolipid with palmitic acid acyl chain (C16), mixed with phosphatidylcholine (PC)-based or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)-based, and cholesterol (Chol) with C16/DSPC/Chol 8:12:10 molar ratio. PEG-lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine conjugated to polyethylenoglycol 2000) containing vesicles (PEGylated-arsonoliposomes; PC-based and DSPC-based) were also prepared. The cytotoxicity of these arsonoliposomes towards different cancer cells (human promyelocytic leukaemia NB4, Prostatic cancer PC3, human breast adenocarcinoma MDA-MB-468, human T-lymphocyte (MT-4) and also towards human umbilical vein endothelial cells (HUVECs) was evaluated by calculating the arsonoliposome-induced growth inhibition of the cells by the MTT assay. IC-50 values were interpolated from cell number/arsonoliposome concentration curves. The results reveal that all types of arsonoliposomes evaluated significantly inhibit the growth of most of the cancer cells studied (PC3, NB4, MT4) with the exception of the MDA-MB-468 breast cancer cells which were minimally affected by arsonoliposomes; in some cases even less than HUVEC. Nevertheless, for the same cell type the differences between the different types of arsonoliposomes were significant but not proportional to their stability, indicating that the formation of arsonoliposomes with very stable membranes is not a problem for their anticancer activity. Thereby it is concluded that arsonoliposome composition should be adjusted in accordance to their *in vivo* kinetics and the desired, for each specific application, biodistribution of As and/or encapsulated drug.

Keywords: Anticancer / Arsenic / Arsonolipid / Arsonoliposome / Lipid composition

Received: April 25, 2007; revised: June 16, 2007; accepted: June 18, 2007

1 Introduction

Liposomes containing a type of Arsonolipids (Ars) (Fig. 1), referred to as 'arsonoliposomes' have been prepared and characterized in our laboratories previously [1]. As summarized before [2], promising results were obtained with some of the arsonoliposome types prepared, for which a differential toxicity towards cancer and normal cells was demonstrated [3, 4] as well as *in vitro* antiparasitic activity [5]. However, it has been found that the membrane integrity of arsonoliposomes [6, 7] and their *in vivo* distribution (of arsenic) [8, 9] are influenced by the lipid composition of the arsonoliposome membrane, and this has been pinpointed as the reason – or at least one of the reasons – for the different antiparasitic activities demonstrated (*in vitro* and *in vivo*), by different arsonoliposome types (with different lipid compositions) [10].

The glutathione induced reduction of As(V) (of Ars) to As(III) which has higher toxicity, and the fact that glutathione concentration is much higher in tumour cells as compared to normal cells [11], is one of the mechanisms proposed to explain the demonstrated differential toxicity of

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Abbreviations: DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; **FCS,** fetal calf serum; **HUVEC,** human umbilical vein endothelial cell; **PC,** phosphatidylcholine

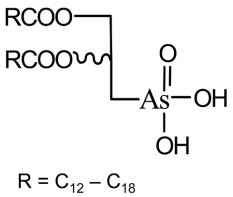

Figure 1. Chemical structure of the synthetic arsonolipid (Ars) used in this study for construction of arsonoliposomes.

arsonoliposomes towards cancer cells (compared to normal). Recently arsonoliposomes have been demonstrated to be 'sensitive' to glutathione [12], after they were incubated in presence of glutathione (10 mM) and their integrity was evaluated. Indeed, as the As(V) of the Ars molecules present on the outer bilayer of arsonoliposomes are reduced by glutathione and form an arsenic (III) diglutathiolate [13] (according to the reaction presented in Fig. 2), the vesicle membrane integrity was affected; resulting in the release of vesicle encapsulated fluorescent dye, which was easily measured.

Nevertheless, in those latter studies, the arsonoliposomes with more stable membranes, as 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)-based arsonoliposomes (composed of 27 mol% arsonolipid C16 and distearoyloglycero phosphatidylcholine (DSPC)) and DSPC-based PEGylated arsonoliposomes (composed of C16, DSPC and 8 mol% polyethyneleglycol (PEG)-conjugated phospholipid) were found to be less sensitive to glutathione, compared to the arsonoliposomes with more leaky membranes; the phosphatidylcholine (PC)-based ones (composed of 27 mol% arsonolipid C16 and PC). This phenomenon could be attributed either to a lower reactivity of Ars towards glutathione, when the latter lipids are packed in the more stable membranes, or perhaps to the fact that the vesicles which have more stable membranes do not release their contents after they react with glutathione – at least to the same degree with the vesicles that have less stable (or more leaky) membranes. If the first theory is valid, the anticancer activity of arsonoliposomes could also be affected by arsonoliposome lipid composition and the more stable DSPC-based (nonPEGylated and PEGylated) arsonoliposomes would posses lower anticancer activities compared to the less stable PC-based ones.

In order to investigate the latter possibility we evaluated herein the anticancer activity of PC-based and DSPC-based, nonPEGylated and PEGylated arsonoliposomes, by measuring the arsonoliposome-induced growth inhibition of four different cancer cell lines. Additionally, the same experiments were performed with one type of normal cells human umbilical vein endothelial cells (HUVECs), in order to test if all arsonoliposome types behave similarly to the PC-based arsonoliposomes which were found to exhibit very low toxicity towards HUVECs (as well as other normal cell types) and profoundly higher toxicity towards cancer cells [3, 4] (when co-incubated under identical conditions).

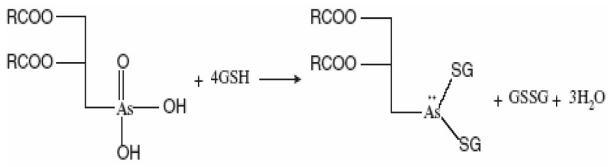

Figure 2. Reaction between Ars (on the outer bilayer of arsonoliposomes) and glutathione.

For the first time PC-based PEGylated arsonoliposomes were constructed, characterized and studied, in accordance to our previous suggestions [9, 10], that this specific arsonoliposome type might posses a better equilibrium between vesicle integrity and ability to react with cell membranes, compared to the more stable DSPC-based arsonoliposome types.

2 Materials and methods

2.1 Reagents

PC (from egg, grade 1), DSPC (synthetic, grade 1) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolanine conjugated to PEG (M_W 2000) (DSPE-PEG2000) (synthetic, grade 1) were purchased from Avanti Polar Lipids. Cholesterol (Chol) (pure) and Triton X-100 were obtained from Sigma–Aldrich ((O.M.), Athens, Greece). The water used was deionized and then distilled.

All other reagents and solvents used throughout the study were of analytical grade and were purchased from Sigma–Aldrich. All media used for cell growth and handling as well as fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany), and were of cell culture grade.

Human leukaemia NB4 cells were a kind gift of Professor Michael Lannote (University of Paris, France); Prostatic cancer PC3, human breast adenocarcinoma MDA-MB-468 and human T-lymphocyte (MT-4) cells were from ATCC, USA. HUVECs were isolated from human umbilical cords as previously described [14].

The *rac*-arsonolipid (C16) (Ars) (2,3-dipalmitoyloxypropylarsonic acid) with a palmitic side chain ($R = C_{15}H_{31}$) (Fig. 1) was synthesized and characterized, as described in detail before [15–18].

2.2 Arsonoliposome composition

Using Ars, PC or DSPC, Chol and in some cases DSPE-PEG, liposomes having the following lipid compositions were prepared: (i) PC-based arsonoliposomes (PC/Ars/Chol 12:8:10 by mole, with 27 mol% arsonolipid content and in some cases PC/Ars/Chol 17:3:10 by mol, with 10 mol% Ars content); nonPEGylated and PEGylated (in which 8 mol% DSPE-PEG_{2000} lipid was incorporated). And (ii) DSPC-based arsonoliposomes (DSPC/Ars/Chol

12:8:10 by mol, with 27 mol% Ars content and in some cases DSPC/Ars/Chol 17:3:10 by mol, with 10 mol% Ars content); nonPEGylated and PEGylated (in which 8 mol% DSPE-PEG_{2000} lipid was incorporated).

2.3 Preparation of arsonoliposomes

Arsonolipid (Ars)-containing liposomes were prepared as described previously [1, 2, 6, 7]. In brief, lipids (after removing the organic solvents with a nitrogen stream) were mixed with 5 mM phosphate buffer (pH 7.4) and 20 mM NaCl and magnetically stirred vigorously on a hot plate for 4 h at 70–80°C. After formation of liposomes, the samples were left to anneal for at least 1 h at the liposome preparation temperature.

In order to reduce liposome size, the large liposome suspension initially produced was sonicated, using a Vibra-cell probe sonicator (Sonics and Materials, UK) equipped with a tapered microtip, for at least two 5 min cycles. In all cases the initially turbid liposomal suspension was clear (transparent) after sonication. Following sonication, the liposome suspensions were left to stand for 1 h at 65°C (or higher than the transition temperature of the lipids used in each case), in order to anneal any structural defects. The titanium fragments and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at $10\,000 \times g$ for 10 min.

The lipid content of the samples was routinely determined using a colorimetric technique, which is widely applied for phospholipids, the Stewart assay, in which the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution is utilized [19]. This assay was found to also detect Ars (at the high concentrations of the initial dispersions). In brief, liposome samples (20 μL) are vortexed with 2 mL of a solution of ammonium ferrothiocyanate (0.1 M) and 2 mL of chloroform. The OD-485 nm of the chloroform phase is measured and the lipid concentration of samples is calculated by comparison with a calibration curve.

The exact arsenic content of all arsonoliposome preparations was determined by graphite furnace atomic absorption spectroscopy (GFAAS), as previously described [20, 21]. In brief, 20 μL of arsonoliposomes were digested with nitric acid at 80°C and after this the residue was dissolved in a mixture of 0.5 mL 0.4% v/v nitric acid and 0.5 mL of H_2O_2. These samples were kept at 4°C until GFAAS analysis.

Liposome samples were kept at 4°C until use in cell culture experiments.

2.4 Characterization of arsonoliposome (size, zeta potential and integrity)

The liposomes prepared were characterized by measuring their size by DLS (dynamic light spectroscopy) with a Malvern Zetasizer 5000 (Malvern, UK), as described before [1, 9, 10]. In brief, liposome dispersions were diluted with filtered PBS pH 7.40 and sized immediately.

The arsonoliposome electrophoretic mobility was also measured at 25°C (Zetasizer 5000 Malvern Instruments), after diluting the vesicle dispersion with filtered PBS pH 7.40. Zeta potentials of the dispersions were calculated (by application of the Helmholtz–Smolowkovski equation).

Measurement of arsonoliposome integrity: For some arsonoliposome types, vesicle integrity was evaluated by measuring the latency of vesicle encapsulated calcein [22]. Calcein was encapsulated in the vesicles (during their preparation as described above) at a quenched concentration (100 mM) and the vesicles were incubated in PBS buffer or in presence of serum proteins (80% v/v FCS) for periods up to 24 h at 37°C and under mild agitation (30 rpm).

For calcein latency calculation, samples from the incubates were drawn (20 μL) and diluted with 4 mL PBS, pH 7.40, and fluorescence intensity (FI) was measured (EM 470 nm, EX 520 nm) by a Shimatzu RF1000 Spectrofluorimeter, before and after addition of Triton X-100 at a final concentration of 1% v/v (that ensures liposome disruption and release of all encapsulated dye). Per cent latency (% latency) was calculated from the following equation:

$$\%\text{Latency} = \frac{(1.1F_{AT} - F_{BT})}{1.1F_{AT}} \times 100 \quad (1)$$

where, F_{BT} and F_{AT} are calcein FIs before and after the addition of Triton X-100, respectively.

2.5 Cell culture

HUVECs were cultured in M199 supplemented with 20 mM Hepes pH 7.4, 10% heat-inactivated FCS, 150 μg/mL endothelial cell growth supplement (Sigma) and 5 U/mL heparin and used at passages 1–5. PC3 and MDA-MB-468 cells were cultured in HAM's F-10 supplemented with 10% FCS and used at passages 19–21. NB4 and MT-4 cells were cultured in RPMI supplemented with 10% FCS. All media also contained 100 IU/mL penicillin and 100 μg/mL streptomycin and cultures were maintained at 37°C, 5% CO_2 and 100% humidity.

2.6 Cell Viability studies

For cell viability assays, cells were seeded at an initial concentration of 1×10^5 cells/mL in 24-well tissue culture plates, and incubated in medium with or without arsonoliposomes for periods of 24 h. Cell viability after incubations was assessed by: (i) tryptan blue exclusion using a hemocytometer, and (ii) by measuring the number of cells, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay [23]. For this, MTT stock (5 mg/mL in PBS) at a volume equal to one-tenth of the volume of

medium was added to all wells and plates were incubated at 37°C for 2 h. After incubation, the medium was removed, the cells were washed with PBS pH 7.4 and 100 μL of acidified isopropanol (0.33 mL HCl in 100 mL isopropanol) was added to all wells and agitated thoroughly to solubilize the dark blue formazan crystals. The solution was transferred to a 96-well plate and immediately read on a microplate reader (BioRad) at a wavelength of 490 nm.

The effect of arsonoliposomes on cell viability was assessed by comparing the number of live cells in the treated wells with those in the control wells in which plain buffer but no arsonoliposomes were added. Growth inhibition concentrations (50%, IC 50) were calculated from interpolations of the graphical data. In all cases studied, additional controls were performed, in which the effect of sonicated conventional phospholipid liposomes PC/Chol (2:1 mol/mol) on the cell viability was evaluated under identical experimental conditions (lipid concentration, time of exposure, *etc*.). In all cases PC/Chol liposomes did not demonstrate any cytotoxic effects. Similar results were obtained (no significant cytotoxicity was observed) when PEGylated PC/Chol liposomes (or DSPC/Chol, PEGylated or nonPEGylated) were incubated under the same experimental conditions, with all the cell types used herein.

3 Results

3.1 Arsonoliposome physicochemical properties

The arsonoliposome mean diameter ranged between 79.9 and 112.5 nm, depending on the arsonoliposome type (Table 1). PEGylation results in significant (at $p = 0.05$) increase in the mean diametre of arsonoliposomes as also reported before.

Additionally, as demonstrated previously [2], Ars give a negative surface charge to vesicles (Table 1), while this charge is abolished when PEG molecules are present on the arsonoliposome surface, proving that the surfaces of arsonoliposomes are indeed coated with PEG molecules.

Since PEGylated PC arsonoliposomes were prepared for the first time, the integrity of this type of arsonoliposomes was evaluated by measuring the release of vesicle-encapsulated calcein, during incubation of the vesicles in buffer or in presence of serum proteins (80% FCS), for 24 h, as studied before for PEGylated (and non-PEGylated) DSPC arsonoliposomes [7]. The integrity of non-PEGylated PC arsonoliposomes was also evaluated under identical conditions for comparison. As seen in Fig. 3, while both types (PEGylated and non-PEGylated PC arsonoliposomes) demonstrate high integrity during incubation in buffer (calcein latency is always higher than 85%) the nonPEGylated PC arsonoliposomes are very unstable during incubation in presence of serum proteins (calcein latency is 65%, after 5 min of incubation and only 15.4% after 24 h), as demonstrated also before [6]. However, PEGylation results in a

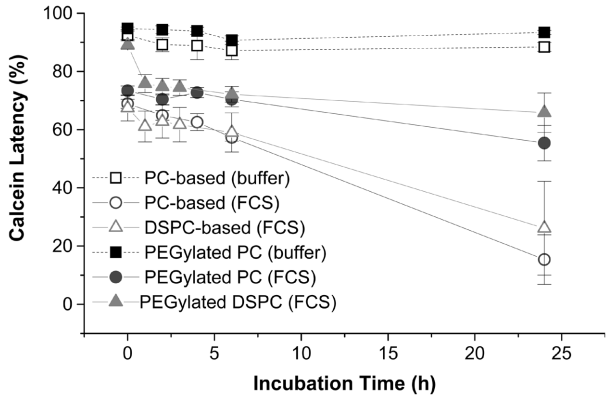

Figure 3. Retention of encapsulated calcein (Latency%) in calcein-entrapping PC-based (open symbols) and PEGylated PC (solid symbols) arsonoliposomes during incubation in buffer (black squares) or in presence of serum proteins (80% FCS – blue circles) at 37°C for 24 h. Each point is the mean of at least four different experiments and bars represent the SD of the mean. For direct comparison the retention of calcein-entrapping DSPC-based liposomes (studied previously under identical experimental conditions [7]), without PEG (open red triangles) and PEGylated DSPC (solid red triangles) were also incorporated in this graph.

significant stabilization of these arsonoliposomes, since in the presence of FCS the calcein latency is 70% after 5 min and 55% after 24 h of incubation (Fig. 3). A similar stabilizing effect of PEGylation (performed by adding 8 mol% of DSPE-PEG2000 in the lipid phase during arsonoliposome preparation) was demonstrated previously in the case of DSPC-based arsonoliposomes (with the specific lipid composition which is used also in this study) [7]. Nevertheless, when the stability (in FCS) of the PEGylated PC arsonoliposomes studied herein, is compared to that of the PEGylated DSPC asronoliposomes studied before (under the same conditions) [7], it becomes obvious that PEGylated DSPC arsonoliposomes (the values for which were added in Fig. 3 (as triangles) for direct comparison) are more stable (calcein latency is 90 and 65% after 5 min and 24 h incubation, respectively) compared to the PC ones. This observation is in good accordance with our previous suggestions [9, 10] that PEGylated PC arsonoliposomes should have intermediate stability, being more stable compared to the PC-based arsonoliposomes (nonPEGylated ones) and less stable compared to the PEGylated DSPC arsonoliposomes.

3.2 Arsonoliposome induced inhibition of cell growth

The effect of increasing concentrations of arsonoliposomes (expressed as Ars concentration) on the viability of the cells (after 24 h of co-incubation), is presented in Figs. 4 – 8. IC50 values for all cell types studied were calculated (for each type of arsonoliposome studied), and are listed in Table 2.

Table 1. Physicochemical properties of the arsonoliposomes used in this study

Arsonoliposome (lipid composition)	Vesicle mean diameter (nm)[a]	Zeta-potential (mV)[a]
PC-based arsonoliposomes (27%)[b] (PC/Ars/Chol (12:8:10))	82.55 (0.45)	−20.45 (0.22)
PEGylated PC (27%) (PC/Ars/Chol (12:8:10) +8mol% DSPE-PEG2000)	94.00 (0.80)	0.8 (2.1)
DSPC-based arsonoliposomes (27%) (DSPC/Ars/Chol (12:8:10))	79.9 (2.9)	−23.2 (1.4)
PEGylated DSPC (27%) (DSPC/Ars/Chol (12:8:10) +8mol% DSPE-PEG2000)	112.5 (1.7)	0.3 (1.2)

These measurements were performed in PBS buffer, pH 7.4, at 25°C as described in detail in Section 2. The corresponding values of PC-based arsonoliposomes are also stated, for direct comparison.

a) All values are mean values measured from at least three different batches of liposomes, and SD of each mean is reported.

b) The value in parenthesis is the mol% content of arsonoliposomes in Ars.

Table 2. Growth-inhibitory concentrations (50%, IC_{50}) of arsonoliposomes (expressed as the Ars content of arsonoliposomes in each case, Ars (mM)/10^5 cells) for the various cell types studied

	IC_{50}[a] (Ars (mM)/10^5 cells)				
Arsonoliposome composition	HUVEC	PC3	NB4	MDA-MB-468	MT4
PC-based (27%)[b]	0.36 (.014)	.0117 (.0024)	.0246 (.0047)	.96 (.18)	.0376 (.0072)
PC-based (10%)	ND	.172 (.010)	.22 (.013)	ND	ND
[c]PEGylated PC (27%)	.451 [.051]	.076 (.013).293 (.043)	.0135 (.0023)	.472 (.080)	.080 (.014)
PEGylated PC (10%)	ND	.293 (.043)	.219 (.032)	ND	ND
DSPC-based (27%)	ND > 1.17	.0262 (.0047)	.0157 (.0028)	.288 (.052)	.0192 (.0034)
DSPC-based (10%)	ND	.544 (.011)	ND > 0.26	ND	ND
PEGylated DSPC (27%)	.806 (.085)	.0132 (.0013)	.0824 (.0083)	.639 (.065)	.0522 (.0053)
PEGylated DSPC (10%)	ND	.1804 (.0034)	.126 (.026)	ND	ND

ND: not determined.

a) The values were calculated from interpolations of the graphical data presented in Figs. 4–8 using the Microcal Origin Program (Version 5). SD values for the IC_{50}'s (presented in brackets) were calculated as the mean of the SD's from the two closer measured viability values for each case.

b) The numbers in parentesis denote the mol% content of Ars, in each arsonoliposome type (27% means phosholipid/Ars/Chol 12/8/10, and 10% means phosholipid/Ars/Chol 17/3/10).

c) PEGylated PC arsonoliposomes contain also 8 mol% DSPE-PEG2000.

As demonstrated, HUVEC viability starts to be affected (significant decrease compared to control) when they are co-incubated with arsonoliposomes at concentrations higher than 10^{-4} M (Fig. 4). This is in good accordance with previous findings [3, 4]. Oppositely, all types of cancer cells used demonstrated significant arsonolipid-induced cell growth inhibition that was initiated at lower arsonoliposome concentrations; starting at 10^{-5} M for MT4 and MDA-MB-468, and even lower for NB4 and PC3 cells (3×10^{-6} M and 5×10^{-6} M, respectively) (Figs. 5–8).

Cancer cells were affected by arsonoliposomes in the order NB4 > PC3 > MT4 > MDA-MB 468, while the dependence between viability and concentration of (co-incubated) arsonoliposomes was different for the different types of cancer cells studied. Indeed, as the arsonoliposome concentration increases (starting from the concentration that initiates a significant decrease in cell viability) the viability of PC3 (Fig. 7A) and MT4 cells (Fig. 8) decreases rapidly, while for NB4 (Fig. 6A) and MDA-MB-468 (Fig. 5) a more gradual cell viability decrease is observed.

Concerning the effect of lipid composition on the anticancer activity of arsonoliposomes, as seen in Figs. 5–8 and in Table 2, the different types of cancer cells studied exhibit different behaviours; For MDA-MB-468 (which were the least affected by arsonoliposomes, cancer cells, from the ones studied herein) (Fig. 5, Table 2) arsonoliposomes are more cytotoxic in the order DSPC-based > PEGylated PC > PEGylated DSPC > PC-based (and differences between corresponding values are always statistically significant ($p < 0.05$)). For MT-4 cells (Fig. 8, Table 2) the order is: DSPC-based > PC-based > PEGylated DSPC > PEGylated PC (again differences are significant ($p < 0.05$)). For PC3 cells (Fig. 7A, Table 2) the order is: PC-based ≥ PEGylated DSPC > DSPC-based > PEGylated PC, and difference between the two first arsonoliposome types is not significant. And last, for NB4 cells (Fig. 6A and Table 2), the order is PEGylated PC ≥ DSPC-based > PC-based > PEGylated DSPC (practically opposite to the order observed in PC3 cells) and again the first two are not significantly different.

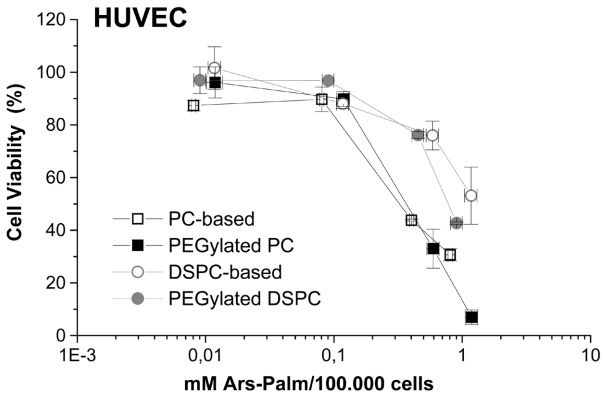

Figure 4. Effect of arsonoliposomes on the viability of HUVEC (normal cells). Cells were incubated with various concentrations of arsonoliposomes for 24 h. Arsonoliposomes containing 27 mol% Ars were evaluated. Results are expressed as viability (per cent of viable cells in comparison with the control cells) *versus* arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate, and the bars represent SD values.

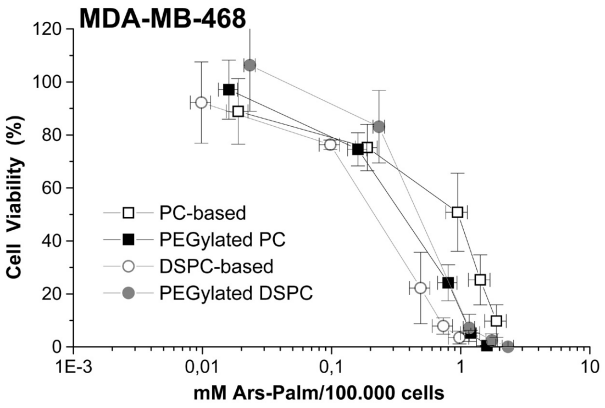

Figure 5. Effect of arsonoliposomes on the viability of MDA-MB-468 (breast cancer cells). Cells were incubated with various concentrations of arsonoliposomes for 24 h. Arsonoliposomes containing 27 mol% Ars were evaluated. Results are expressed as viability (per cent of viable cells in comparison with the control cells) *versus* arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate, and the bars represent SD values.

Arsonoliposomes containing 10 mol% Ars in their lipid membranes, instead of 27 mol%, were also evaluated in terms of arsonoliposome-induced growth inhibition of NB4 and PC3 cells. As seen in Figs. 6B and 7B, and also from the corresponding IC-50 values (Table 2), these arsonoliposomes are substantially less toxic towards both of the cancer cell-types evaluated. Similar results were obtained previously [3] in the cases of HL-60 leukaemic and C6 glioma cells. Thus, our current results strengthen the suggestion made before (to explain the results obtained with HL-60 and C6 cells), that as the Ars content of arsonoliposomes increases, more Ars molecules are present on the outer

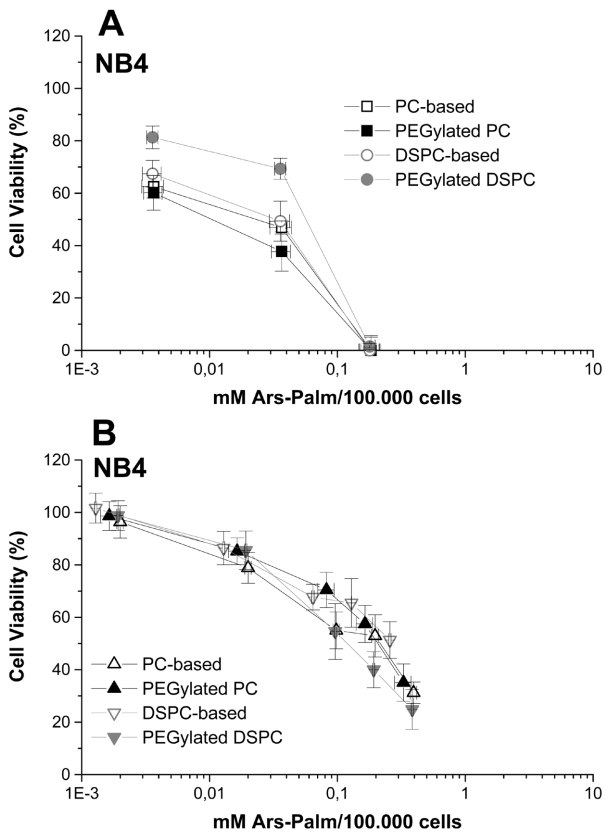

Figure 6. Effect of arsonoliposomes on the viability of NB4 (leukaemic cells). Cells were incubated with various concentrations of arsonoliposomes for 24 h. (A) Arsonoliposomes containing 27 mol% Ars were evaluated. (B) Arsonoliposomes containing 10 mol% Ars were evaluated. Results are expressed as viability (per cent of viable cells in comparison with the control cells) *versus* arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate, and the bars represent SD values.

bilayer of the vesicles, being readily available for interaction with cancer cells.

4 Discussion

The mechanism of arsonoliposome anticancer activity is not known however it has been postulated [2] that the reduction of Ars As(V) to the more toxic As(III) by glutathione (Fig. 2), which is known to be present in higher concentrations in some cancer cell types (compared to normal glutathione levels) [11] may be implicated. Recently it was noticed that arsonoliposomes are sensitive to glutathione and their integrity decreases when they are incubated in glutathione-rich solutions [12]. However, some arsonoliposome types with high integrity (as DSPC-based and PEGylated arsonoliposomes) [6, 7] which were lately demonstrated to have better bioavailabity after *in vivo* injection (compared to arsonoliposomes with low integrity (PC-

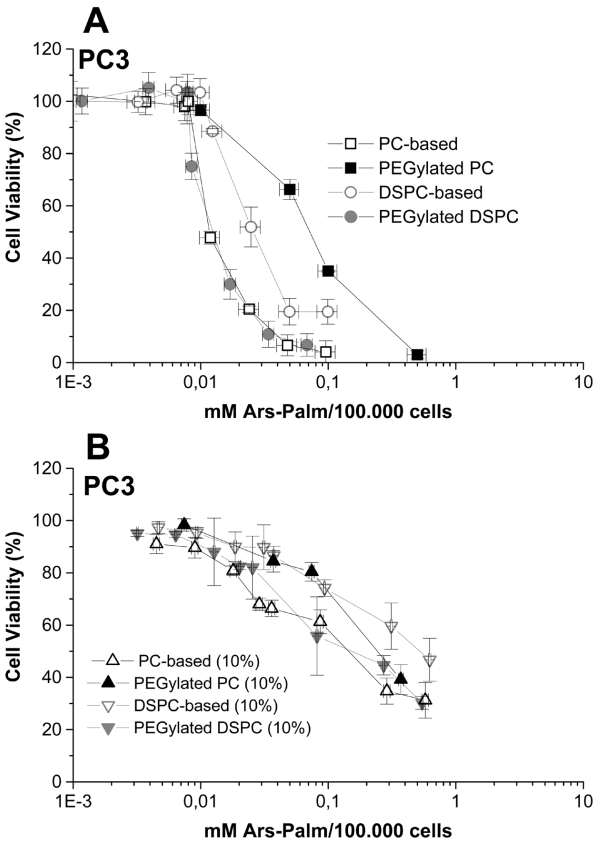

Figure 7. Effect of arsonoliposomes on the viability of PC3 (prostate cancer cells). Cells were incubated with various concentrations of arsonoliposomes for 24 h. (A) Arsonoliposomes containing 27 mol% Ars were evaluated. (B) Arsonoliposomes containing 10 mol% Ars were evaluated. Results are expressed as viability (per cent of viable cells in comparison with the control cells) *versus* arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate, and the bars represent SD values.

based)) [9], were not glutathione-sensitive [12]. In order to evaluate if the previously demonstrated anticancer activity of the more leaky PC-based arsonoliposomes [3, 4] is not abolished in the case of more stable arsonoliposomes and to study the general effect of arsonoliposome lipid composition on their activity, we investigated herein the anticancer activity of various types of stable arsonoliposomes on different types of cancer cells, in culture.

In addition to arsonoliposome types that have been previously formulated and characterized (as DSPC-based, non-PEGylated and PEGylated arsonoliposomes) PEGylated PC-based arsonoliposomes (PEGylated PC) were also constructed and studied. As mentioned above (in the results section), the integrity of this newly constructed arsonoliposome type (Fig. 3) is intermediate between that of the PC-based arsonoliposomes and DSPC-based or PEGylated DSPC ones, proving that PEGylation increases the stability of PC-based arsonoliposomes, as it was seen before for the DSPC-based ones [7].

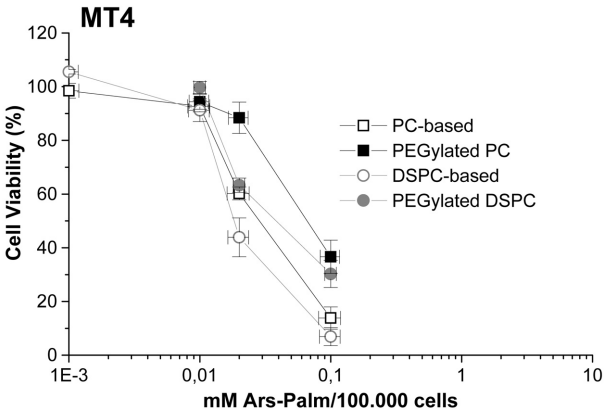

Figure 8. Effect of arsonoliposomes on the viability of MT4 (human T-lymphocyte cells). Cells were incubated with various concentrations of arsonoliposomes for 24 h. Arsonoliposomes containing 27 mol% Ars were evaluated. Results are expressed as viability (per cent of viable cells in comparison with the control cells) *versus* arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate, and the bars represent SD values.

Nevertheless, an interesting outcome of this study is that arsonoliposome lipid composition does not have a common straightforward effect on arsonoliposome-induced cell growth inhibition of cancer cells, for the various cell types evaluated. For some cell types (of those studied) the less stable arsonoliposomes are more toxic while for others more stable arsonoliposomes induce higher toxicity. As a matter of fact, the arsonoliposome activity is not in rank order correlation with their stability (PEGylated DSPC > DSPC-based > PEGylated PC > PC-based) for any of the cancer cells evaluated, with the minor exception of NB4 cells for which the order of decreasing toxicity formulations is almost similar with the decreasing stability order. This suggests that membrane integrity or rigidity is not a determining factor for arsonoliposome-induced cancer cell growth inhibition, and that the 24 h incubation period is adequate for all types of arsonoliposomes to induce a substantial reduction in cancer cell viability at lower Ars concentrations, compared to HUVEC cells, with the exception of the MDA-MB-468, breast cancer cells. In fact, the latter human breast cancer cell type was seen to be minimally affected by all types of arsonoliposomes at lower arsonolipid concentrations (at which the other cancer cell types studied demonstrated substantial reduction of cell growth) while the IC_{50} values calculated are very similar to those calculated for HUVEC cells.

Although the elucidation of the mechanism of arsonoliposome anticancer activity is not in the scope of the current investigation, it is interesting that in a previous study this specific (breast cancer) cell type was found to be the one most affected by arsenic trioxide – and at the same time less resistant-[24]. Nevertheless, there is no certainty that arsonoliposomes have similar mechanism of action with arsenic

trioxide – in terms of their anticancer activity – and it is thus difficult to comment on the mechanism of the demonstrated herein resistance of breast cancer cells towards arsonoliposomes.

What is the most important outcome and conclusion of this study is that the more stable (less leaky) arsonoliposome-types, that were evaluated for the first time for their anticancer activity, are equivalently active towards cancer cells (compared with the more leaky PC-based arsonoliposomes studied before [2 – 4]). This finding proves that all types of arsonoliposomes may be used for the development of cancer-specific carriers of cytotoxic agents (that can be incorporated or encapsulated in the vesicles). Depending on the specific cancer type targeted, and its distribution in the body, the appropriate arsonoliposome-type can be selected, based on its *in vivo* pharmacokinetics, *in vitro* cytotoxicity and drug retention capacity.

The authors thank Professor P. Klepetsanis for his valuable help with the atomic absorption measurements of arsenic concentration of arsonoliposomes. S. G. A. is especially grateful to Professor M. Lanotte for kindly providing NB4 cells. This work is a partial requirement for the Ph.D. thesis of P. Z.

The authors have declared no conflict of interest.

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