Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmaceutical Sciences 18 (2003) 175–183
EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES
www.elsevier.com/locate/ejps

Arsonoliposomes: effect of arsonolipid acyl chain length and vesicle composition on their toxicity towards cancer and normal cells in culture

Olga Gortzi[a], Sophia G. Antimisiaris[a,*], Pavlos Klepetsanis[a], Evangelia Papadimitriou[b], Panayiotis V. Ioannou[c]

[a]*Laboratory of Pharmaceutical Technology, Department of Pharmacy, University of Patras, Rio 26500, Patras, Greece*
[b]*Molecular Pharmacology, Department of Pharmacy, University of Patras, Rio 26500, Patras, Greece*
[c]*Department of Chemistry, University of Patras, Rio 26500, Patras, Greece*

Received 30 July 2002; received in revised form 8 November 2002; accepted 27 November 2002

Abstract

Arsonolipid-containing liposomes were investigated in order to characterize the influence of the lipid acyl-chain length and liposome composition on cytotoxicity. Three types of cancer cells (HL-60, C6 and GH3), and two types of normal cells (HUVEC and RAME) were used. Liposomes containing the lauroyl, myristoyl and stearoyl side chain arsonolipids (with different lipid compositions) were incubated with a given number of cells and cell viability was estimated (MTT assay and trypan blue exclusion). Morphological studies were also performed in some cases. In addition, the interaction between some of the prepared arsonoliposomes and HUVEC cells was assessed. Results reveal that all the studied arsonoliposomes cause a dose dependent inhibition of survival in all three malignant cell lines studied (initiated at 10^{-6} M). The corresponding toxicity against normal cells (HUVEC and RAME) is much lower for all arsonoliposomes, except for the lauroyl side chain arsonoliposomes which were demonstrated to be relatively toxic towards normal cells, especially RAME. The microscopic observations that these vesicles possibly cause apoptosis of most cell types studied, as well as the different speed of their cytotoxic activity, imply a different mechanism of action for this arsonoliposome type. Taking the results of this study in conjunction with our previous results on arsonoliposome physical stability and cytotoxicity, it is recommended that palmitoyl-arsonolipid arsonoliposomes be used for further investigations in vivo towards the development of an anticancer product.

Keywords: Arsenic; Arsonoliposome; Liposome; Cell viability; Cytotoxicity; Arsonolipid

1. Introduction

In an attempt to prepare a system that may combine the therapeutic potential of arsenic trioxide (ATO) (Soignet et al., 1998; Akao et al., 1999; Lu et al., 1999; Roboz et al., 2000; reviewed by Bode and Dong, 2002), with the side-effect and toxicity reducing benefits of liposomal formulations (Gregoriadis, 1993; Lasic and Papahadjopoulos, 1995, 1998), we recently exploited the possibility of preparing ATO-encapsulating liposomes (Kallinteri and Antimisiaris, unpublished results). However, very rapid leakage of ATO through the liposomal membrane was observed, a phenomenon possibly connected to the high in vivo activity of this compound. As an alternative we used arsonate containing lipids or *rac*-arsonolipids (Ars) (2,3-diacyloxypropylarsonic acid), which were recently synthesized and characterized (Tsivgoulis et al., 1991a,b; Serves et al., 1992, 1993) in order to prepare As(V)-containing vesicles, the arsonoliposomes (Fatouros et al., 2001).

These vesicles have been preliminarly characterized with respect to their physical stability, size distribution and morphology as well as membrane integrity during incubation in the presence of serum proteins (Fatouros et al., 2001; Gortzi et al., unpublished results). From our previous studies we concluded that the size and lamellarity of arsonoliposomes, as well as the acyl chain length of the specific arsonolipid used for vesicle preparation highly influence both, vesicle stability and morphology. Recently we reported on the small unilamellar vesicles prepared from the palmitoyl-side chain arsonolipid (Gortzi et al., 2002) and demonstrated a high toxicity of these prepara-

*Corresponding author. Tel.: +30-2610-997-725; fax: +30-2610-996-302.
E-mail address: s.antimisiaris@upatras.gr (S.G. Antimisiaris).

0928-0987/02/$ – see front matter
doi:10.1016/S0928-0987(02)00259-2

tions against several types of cancer cells, while at the same concentrations they were non-toxic for normal cells. These findings were considered very interesting justifying the need for further investigation of the proposed anti-cancer system in vivo. Nevertheless, in order to choose the specific arsonoliposome preparation(s) with which we will continue our studies, it is important to have data about the in vitro cytotoxicity of all the arsonoliposome types prepared in our laboratory.

The aim of this study is to evaluate the relative cytotoxic effects of vesicles, prepared from arsonolipids available in our laboratories, which were not evaluated in our previous study (Gortzi et al., 2002). The results are discussed with respect to the toxic effects obtained with the vesicles studied herein and the previously evaluated vesicles formed by the palmitoyl acyl chain arsonolipid (Gortzi et al., 2002).

2. Materials and methods

2.1. Reagents

Egg L-α-phosphatidylcholine (PC) (grade 1) was obtained from Lipid Products, Nutfield, UK. The 99% purity of the lipid was verified (New, 1990) by thin layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany). Cholesterol (Chol) was of analytical grade and was purchased from Sigma–Aldrich (Athens, Greece). All other reagents and solvents used throughout the study were of analytical grade and were purchased from Sigma–Aldrich (Athens, Greece). All media used for cell growth and handling were purchased from Biochrom (Berlin, Germany), and were of cell culture grade.

Arsonate containing analogues of phosphonolipids, the *rac*-2,3-diacyloxypropylarsonic acids (Ars) with different side chains (R=$C_{11}H_{21}$ (C_{12}), $C_{13}H_{27}$ (C_{14}), and $C_{17}H_{35}$ (C_{18})), were synthesized as described previously (Serves et al., 1992, 1993).

Human leukemia HL-60, rat brain glioma C6 and rat pituitary tumor GH3 cells were from ATCC, USA. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords and rat adrenal medulla microvascular endothelial cells (RAME), were a kind gift of Dr P.I. Lelkes (University of Wisconsin Medical School, WI, USA).

2.2. Liposome preparation and characterization

Liposomes containing plain arsonolipid (100%-Ars/Chol 20:10) or mixtures of Ars with phosphatidylcholine (PC) (15 or 40%-Ars/PC/Chol 3:17:10 or 8:12:10) were prepared as previously described (Gortzi et al., 2002). In all cases Chol was included in the liposomes prepared (at a 2:1 lipid/Chol, mol/mol ratio), since it substantially increases arsonoliposome membrane integrity (Fatouros et al., 2001). The lipid content of the samples was routinely determined using the Stewart assay (Stewart, 1980). This assay was found to also detect arsonolipids (at the high concentrations of the initial dispersions). Liposome samples were kept at 5 °C until use in cell culture experiments.

The liposomes prepared were characterized by measuring their size distribution and surface charge as described before (Fatouros et al., 2001). In brief, liposome dispersions were diluted with filtered PBS, pH 7.40 and sized immediately by photon correlation spectroscopy (Malvern Instruments, Model 4700C). Zeta potentials (Zetasizer 5000, Malvern Instruments, UK) were measured at 25 °C by laser Doppler spectroscopy.

2.3. Cell culture

HUVEC were cultured in M199 supplemented with 20 mM Hepes pH 7.4, 10% heat-inactivated fetal calf serum (FCS), 150 μg/ml endothelial cell growth supplement (Sigma) and 5 U/ml heparin and used at passages 1–5. RAME cells were cultured in DMEM supplemented with 10% FCS and used at passages 19–21. HL-60 cells were cultured in RPMI supplemented with 10% FCS. C6 and GH3 cells were cultured in HAM's F-10 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.

2.4. 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, 48, 72 and 96 h. Cell viability after incubation was assessed by: (a) trypan blue exclusion using a hemocytometer, and (b) measuring the number of cells, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay (Mosmann, 1983). For this, MTT stock (5 mg/ml in PBS) at a volume equal to 1/10 of the medium volume was added to all wells of an assay and plates were incubated at 37 °C for 2 h. For all cells except HL-60, the medium was removed, the cells were washed with PBS, pH 7.4 and 100 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) were 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 (Bio-Rad) at a wavelength of 490 nm. For HL-60 cells that were grown in suspension, equal volume of acidified isopropanol was added to the medium of the cells after incubation with MTT. After the formazan crystals were dissolved, the samples were measured spectrophotometrically at a wavelength of 570 nm and the background absorbance at 685 nm was subtracted.

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. The 50% growth inhibition concentrations (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.

2.5. Morphological studies

Morphological evaluation of the mechanism of action of the C_{12}-arsonoliposomes was carried out with all cell types used, except GH3 cells. As previously described for the C_{16}-arsonoliposomes (Gortzi et al., 2002), following incubation of the cells with arsonoliposomes, the cells were harvested. For this, HL-60 cells were centrifuged at $500\times g$ for 4 min and HUVEC, RAME and C6 cells were centrifuged after trypsinization (in the latter case, both detached cells as well as those still adhering were collected). Finally, 10 μl of each cell suspension was mixed with an equal volume of a solution of acridine orange (10 μg/ml) and examined under a Leica DMLS fluorescent microscope (equipped with a MPS28 photographic controller).

2.6. Arsonoliposome–HUVEC interaction: quantification of intracellular As accumulation

Liposomes were incubated as described previously (Papadimitriou and Antimisiaris, 2000; Gortzi et al., 2002) with confluent monolayers of HUVEC (1000–3500 μg of liposomal lipid/2×10^5 cells) for a period of 4 h, in the corresponding culture medium at 37 °C. After incubation, the cells were washed three times in ice-cold PBS, pH 7.4 and suspended in 1 ml of PBS. Intracellular concentrations of arsenic were determined using atomic absorption spectrophotometry after digestion with nitric acid, as previously described (Carre et al., 2002).

3. Results

3.1. Studies of 24-h cytotoxicity

The liposomes used in the cytotoxicity and cell interaction studies were characterized by measuring their size distribution and surface charge. The results of these measurements are presented in Table 1. In absolute correlation with previous findings (Fatouros et al., 2001), vesicle mean diameter ranged between 62.2 and 120.3 nm, depending on the amount and type of Ars used for vesicle preparation. As also demonstrated previously, as the amount of PC in the liposomes increased vesicle mean diameter decreased, a fact that has been linked to the smaller polar head group of PC (P is smaller than As). In addition, arsonolipids give a negative surface charge to vesicles, which increases linearly with the increase of the Ars proportion in the liposomes (Table 1).

The effect of different concentrations of arsonoliposomes (expressed as arsonolipid concentration in all cases) on the viability of all the cell types studied after a 24-h incubation period, is presented in Figs. 1–3, for the three different arsonolipids used, C_{12}, C_{14} and C_{18}, respectively. Results demonstrate that cancer cell viability decreases as the arsonolipid concentration incubated with the cells increases. In addition, it is obvious that the vesicles prepared from arsonolipid C_{12} are the most toxic towards normal cells (lower panel graphs in each figure). Indeed, especially in the case of RAME cells, these arsonoliposomes are found to be relatively toxic (cell viability is significantly reduced when arsonolipid concentration is $>3\times10^{-5}$ M).

In order to have a better feel for the relative cytotoxicity

Table 1
Mean diameter and ζ-potential values of sonicated plain and mixed (Ars/PC/Chol) arsonoliposomes, prepared in PBS buffer, pH 7.4

Liposome composition	Ars/PC/Chol 3:17:10, mol/mol/mol	Ars/PC/Chol 8:12:10, mol/mol/mol	Ars/Chol 20:10, mol/mol
Arsonolipid	Vesicle mean diameter (nm)[a]		
C_{12}	62.2±5.2	74.4±6.1	106.3±4.6
C_{14}	75.4±6.1	85.6±3.4	110.3±5.2
C_{16}[b]	78.5±3.6	89.7±5.6	116±11
C_{18}	80.2±8.7	95.2±7.1	120.3±5.7
Arsonolipid	ζ-Potential (mV)[a]		
C_{12}	−23.9±1.9	−40.0±3.4	−63.5±6.3
C_{14}	−30.2±2.5	−43.5±4.6	−65.4±1.5
C_{16}[b]	−39.4±1.7	−48.5±1.8	−69.5±2.3
C_{18}	−33.9±4.3	−46.8±3.0	−59.2±3.2

[a] Each value is the mean±S.D. of five subsequent measurements from at least three different samples.
[b] The values for the C_{16} arsonolipid liposomes were taken from our previous study (Gortzi et al., 2002).

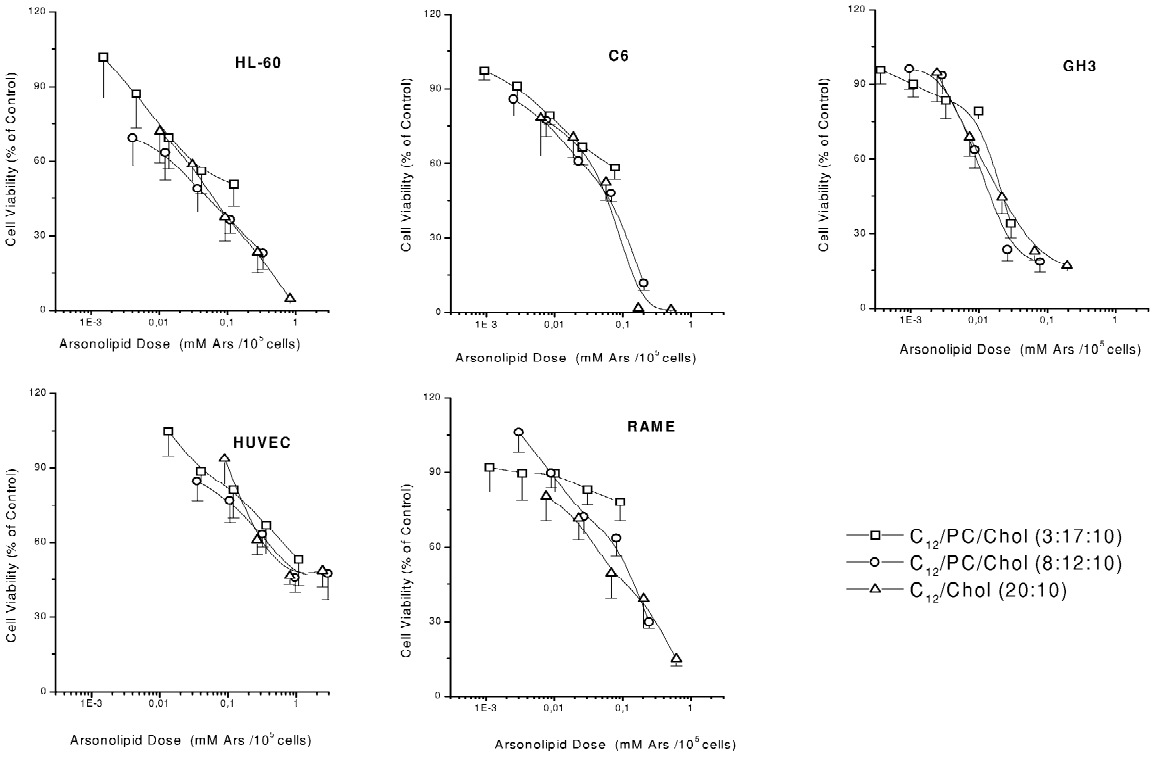

Fig. 1. Effect of C_{12} arsonoliposomes on the viability of tumor (upper panels) and normal (lower panels) cell lines. Cells were incubated with various concentrations of arsonoliposomes for 24 h. Results are expressed as viability (% viable cells in comparison with the control) versus arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate and the bars represent S.D. values.

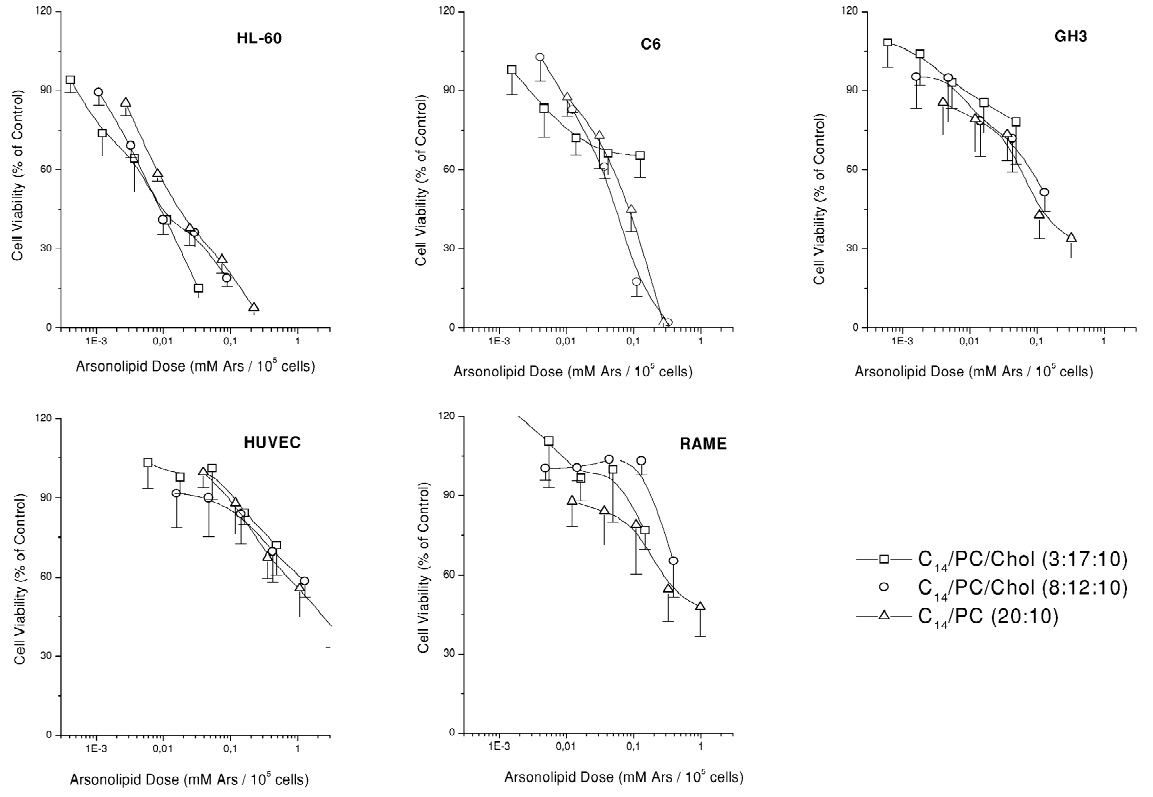

Fig. 2. Effect of C_{14} arsonoliposomes on the viability of tumor (upper panels) and normal (lower panels) cell lines. Cells were incubated with various concentrations of arsonoliposomes for 24 h. Results are expressed as viability (% viable cells in comparison with the control) versus arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate and the bars represent S.D. values.

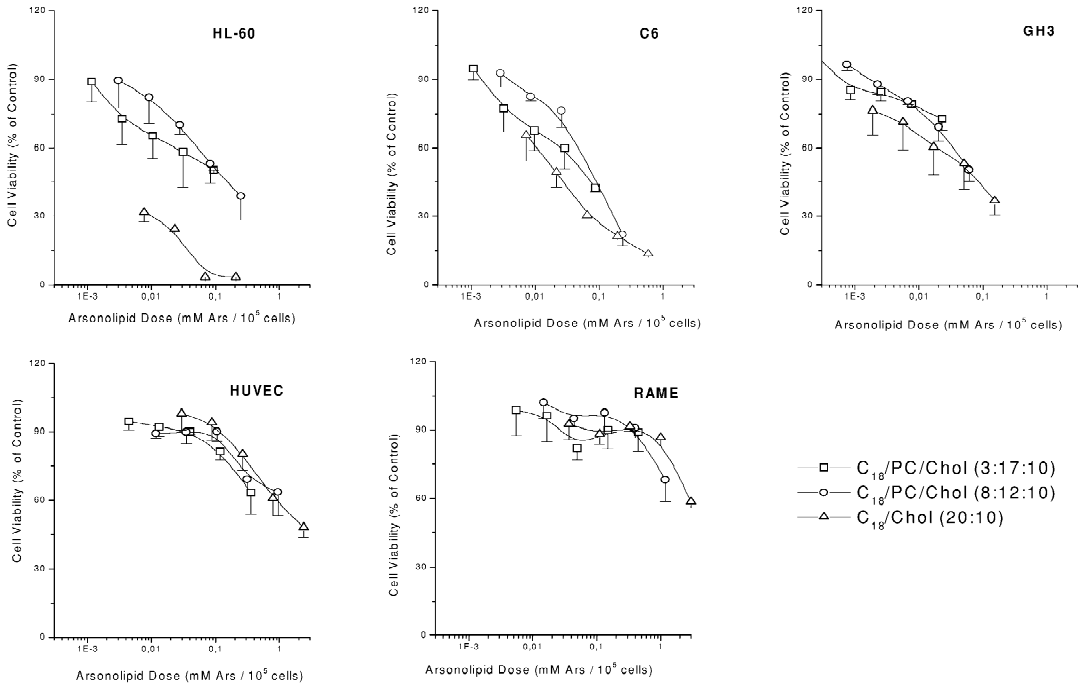

Fig. 3. Effect of C_{18} arsonoliposomes on the viability of tumor (upper panels) and normal (lower panels) cell lines. Cells were incubated with various concentrations of arsonoliposomes for 24 h. Results are expressed as viability (% viable cells in comparison with the control) versus arsonolipid content of each liposomal formulation. Each experiment was performed in triplicate and the bars represent S.D. values.

of the different vesicles studied, the IC_{50} values for each preparation and each cell type were estimated by graphical interpolations of the results presented in Figs. 1–3 (Table 2). Indeed, the relative toxicity of C_{12} arsonoliposomes is evident by comparing IC_{50} values estimated for the various types of arsonoliposomes towards the normal cell types studied (IC_{50} for RAME cells is more than ten times lower than that of the next most toxic arsonoliposome preparation). The corresponding value calculated for the HUVECs is again significantly lower (the formulation is more toxic) compared to the values calculated under identical conditions for the arsonoliposomes prepared with the other arsonolipids. This demonstrated (Fig. 1) relatively higher toxicity of the C_{12} arsonoliposomes towards normal cells (in comparison to other arsonolipids studied) is probably a disadvantage of this arsonoliposome type. Nevertheless, the striking difference between the behavior of C_{12} arsonoliposomes and all other arsonoliposome types studied prompted us to examine the time dependence of the cytotoxicity of these arsonoliposomes, as well as the mechanism of cell death, as they were previously studied for the C_{16} arsonoliposomes (Gortzi et al., 2002). The results of these studies are presented below

Considering the results from the other two arsonolipids as well as the previously derived results for the C_{16} arsonoliposomes (Gortzi et al., 2002), it is obvious that the liposomes prepared by these three arsonolipids are more or less equivalently efficient when their cytotoxicity is considered. Indeed the differences in the IC_{50} values calculated under identical conditions for these three types of arsonoliposomes are in most cases statistically insignificant ($P<0.05$).

When the effects of the different lipid compositions tested on cancer cell viability are compared, it is clear from the graphs (Figs. 1–3) that in most cases the lipid composition has no effect on the cytotoxicity. Only in the case of the C_{18} arsonoliposomes incubated with HL-60 cells (Fig. 3) is there a very big difference between the preparation containing only arsonolipid (Ars/Chol 2:1) and those consisting of mixtures of Ars with PC. A similar effect of liposome composition on HL-60 cytotoxicity was also detected for the C_{16} arsonoliposomes, as reported previously. This effect was attributed to the possibility that greater amounts of arsonolipids are on the outer side of the bilayer of vesicles consisting of plain arsonolipids compared to those formed of mixtures of PC with arsonolipids. However no proof is provided for this suggestion.

3.2. Time-dependent cytotoxicity studies and morphological studies

We examined the effect of time on the cytotoxicity of some of the prepared C_{12} arsonoliposomes. Experimental methods were identical with those used previously with the C_{16} arsonoliposomes (Gortzi et al., 2002), and results for both types of arsonoliposomes are presented in Fig. 4. From these results it is evident that the time dependency of arsonoliposome-induced cytotoxicity towards both HL-60 and HUVECs is different for the two arsonoliposomes studied. Indeed, from the 24-h point of the HL-60 cells

Table 2
The 50% growth inhibition concentrations (IC_{50}) of arsonoliposomes (expressed as the arsonolipid content of liposomes in each case) for the various cell types studied

Arsonoliposome composition	IC_{50} ($\times 10^5$ M)[a]				
	HL-60 cells	C6 cells	GH3 cells	HUVEC cells	RAME cells
C_{12} Arsonolipid					
C_{12}/Chol (20:10)	4.8 (2.2)†	4.8 (1.9)†	1.61 (0.32)†‡	70 (14)‡	7.1 (2.5)†
C_{12}/PC/Chol (8:12:10)	4.2 (1.1)†‡	4.8 (1.7)†‡	1.23 (0.22)†‡	85 (23)‡	12.3 (3.5)
C_{12}/PC/Chol (3:17:10)	11.3 (2.7)	ND	1.90 (0.41)	ND	ND
C_{14} Arsonolipid					
C_{14}/Chol (20:10)	1.10 (0.30)*†‡	6.7 (1.4)†‡	9.0 (2.1)*†‡	161 (36)*	91 (20)*
C_{14}/PC/Chol (8:12:10)	0.71 (0.12)*	5.2 (1.1)	13.6 (3.4)*	ND	ND
C_{14}/PC/Chol (3:17:10)	0.71 (0.18)*	ND	ND	ND	ND
C_{16} Arsonolipid					
C_{16}/Chol (20:10)	0.85 (0.22)*†‡	3.7 (2.1)†‡	3.1 (0.52)†‡	253 (48)*‡	100 (27)*†
C_{16}/PC/Chol (8:12:10)	2.9 (0.7)‡	14.6 (3.7)*‡	5.5 (1.1)*‡	ND	147 (84)*
C_{16}/PC/Chol (3:17:10)	6.1 (1.2)	ND	ND	ND	ND
C_{18} Arsonolipid					
C_{18}/Chol (20:10)	<0.75*	2.1 (0.6)*†‡	6.9 (1.5)*†‡	195 (31)*	ND>300*
C_{18}/PC/Chol (8:12:10)	10.6 (3.5)	6.2 (1.7)	6.4 (1.7)*	ND	ND
C_{18}/PC/Chol (3:17:10)	~9.7	5.5 (1.3)	ND	ND	ND

The values were calculated from interpolations of the graphical data presented in Figs. 1–3, using the Microcal Origin Program (Version 5). The values for the C_{16} arsonoliposomes were taken from our previous study (Gortzi et al., 2002). ND, not determined.
†Significantly different ($P<0.05$) from the corresponding IC_{50} value for HUVEC.
‡Significantly different ($P<0.05$) from the corresponding IC_{50} value for RAME cells.
*Significantly different ($P<0.05$) from the corresponding IC_{50} value of the same cells incubated with the C_{12} arsonoliposomes (same type of liposomes).
[a] S.D. values for the IC_{50} values were not calculated, since these values were estimated from graphical interpolations.

(Fig. 4, upper panel) it is observed that although at both studied concentrations the C_{16} preparations are more toxic compared to those of C_{12}, this picture is reversed at the 48-h point, where the C_{12} preparations are substantially more toxic. Indeed, time of incubation (after the first 24 h of incubation) has a significantly larger effect on the result (cytotoxicity) for the C_{12} arsonoliposomes compared to the C_{16} arsonoliposomes for this cancer cell type (HL-60 cells). For the normal cells tested (HUVEC), the time dependency of C_{12} arsonoliposome induced-cytotoxicity is completely different (Fig. 4, lower panel). At the lowest concentration tested (3×10^{-5} M) they are practically non-toxic after 24 h of incubation, while after 48 h the toxicity approaches a plateau value (of ~40% cell viability) which is also the value obtained after 24-h incubation with higher concentrations of arsonolipid ($>3\times10^{-4}$ M). However, after this point the value remains constant and independent of the time of incubation. The demonstrated differences in the dose (Figs. 1–3) and time dependence (Fig. 4) of differential cytotoxicity of the C_{12} arsonoliposomes in comparison with the other types of arsonoliposomes studied, imply that different mechanisms of cytotoxicity apply in the two cases. In order to further investigate this possibility we performed morphological studies with the C_{12} arsonoliposomes.

For this, cells were treated with two intermediate arsonoliposome concentrations for 24 h and were then stained with acridine orange, a useful probe for detecting apoptotic cells. As presented in Fig. 5, morphological changes, which are characteristic of apoptosis, such as cell shrinkage or cell membrane blebbing, were visible for the treated HL-60 (Fig. 5B,C), HUVEC (E,F) and RAME cells (H,I). In the C6 cells significant cell and nucleus swelling (a preliminary step to membrane rupture during cell necrosis) was observed (Fig. 5L). When compared to the results of the morphological studies performed before with the C_{16} arsonoliposomes (Gortzi et al., 2002), it is clear that the C_{12} arsonoliposomes demonstrate different mechanisms of cell death for all the cell types studied, at least under the experimental conditions used here.

In order to further investigate the different cytotoxicity of C_{12} and C_{16} arsonoliposomes towards HUVEC cells, we quantitatively evaluated the uptake of As in the cells by measuring As content of the cells after they were co-incubated with varying amounts of the two types of arsonoliposomes for 4 h at 37 °C. The results of this study (Fig. 6) reveal that the uptake of As from C_{12}-arsonoliposomes is always higher compared to that measured after incubation of the cells with the same concentrations of C_{16} arsonoliposomes. Higher uptake of As by HUVECs from the C_{12} arsonoliposomes may be a contributing factor of the higher toxicity observed by these vesicles towards HUVEC cells. In addition these experiments prove that As is actually taken up by cells (at least these cells), a very interesting observation linked with the mechanism of action of arsonoliposomes. Nevertheless, more interaction

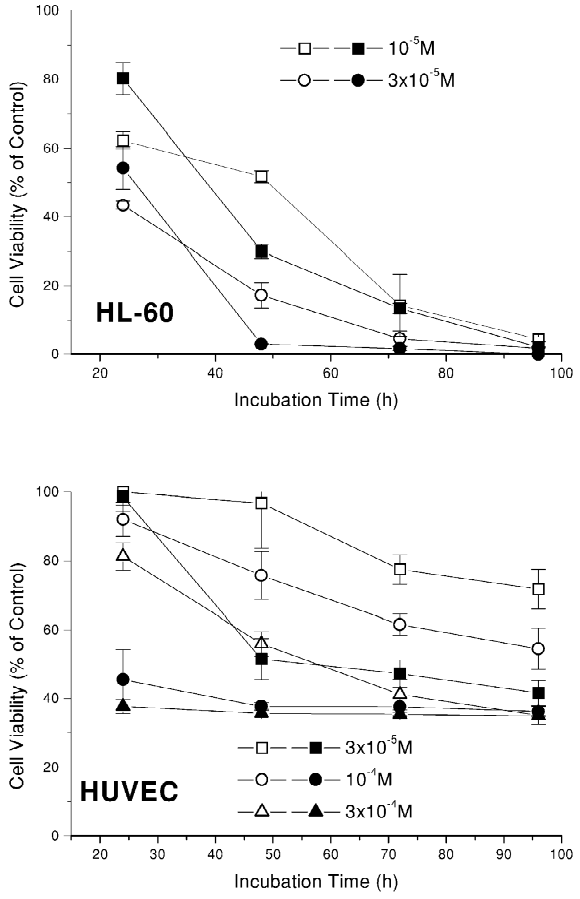

Fig. 4. Time dependency of the effect of C_{12} arsonoliposomes (closed symbols) on the viability of HL-60 cells (upper figure) and HUVECs (lower figure). Cells (10^5 cells/ml) were incubated for 24, 48, 72 and 96 h, with various concentrations of arsonoliposomes. Arsonoliposomes with lipid composition of Ars/PC/Chol 8:12:20 were used in these experiments. Results are expressed as viability (% viable cells in comparison with the control) versus arsonolipid content of the liposomal formulation. Each experiment was performed in triplicate and the bars represent S.E. values. The results of the C_{16} arsonoliposomes (open symbols) were taken from our previous study (Gortzi et al., 2002).

experiments should be carried out in order to clarify the arsonoliposome action mechanism. The fact that the method applied here can be used for accurate quantification of As uptake in cells is helpful for further studies.

4. Discussion

Arsonolipids (1,2-diacyloxypropyl-3-arsonic acids) are lipidic analogues of phosphonolipids in which As replaces P in their polar head group (Tsivgoulis et al., 1991a,b; Serves et al., 1992, 1993). The ability of arsonolipid As(V) to be reduced to As(III) by thiols (Timotheatou et al., 1996) is an interesting aspect of these lipids. In vivo, such interactions with membrane-bound or cytoplasmic thiols may alter the biochemistry of the cell, rendering the arsonolipids active against tumor cells when the latter contain elevated levels of thiols.

With the intention of preparing an anti-cancer product we used these lipids to formulate liposomes (arsonoliposomes), which recently demonstrated (Gortzi et al., 2002) differential toxicity (arsonoliposomes prepared by 1,2-dipalmitoyloxypropyl-3-arsonic acid, C_{16}), towards cancer and normal cells, justifying further study of this system.

Since several properties (morphology and physicochemical characteristics) of arsonoliposomes are affected by the acyl chain length of the arsonolipid used to prepare vesicles (Fatouros et al., 2001; Gortzi et al., unpublished results), we studied here the cytotoxicity of arsonoliposomes prepared by C_{12}, C_{14} and C_{18} arsonolipids.

The experimental results (Figs. 1–3 and Table 2) show that C_{12} arsonoliposomes behave differently from the vesicles prepared by the other two arsonolipids studied. Indeed, while the C_{14} and the C_{18} arsonoliposomes demonstrate a more or less similar behavior as that observed previously for the C_{16} arsonoliposomes, C_{12} arsonoliposomes are relatively more toxic towards the normal cells studied. However, if the cytotoxicity after 48 h of incubation (and not 24 h) is considered, the C_{12} arsonoliposomes demonstrate high differential toxicity towards HL-60 cells compared to HUVECs. In other words, the negative picture for these arsonoliposomes is reversed under different experimental conditions (longer incubation period). The results of additional studies performed (morphological observation of cells after incubation (Fig. 5) and preliminary evaluation of arsonoliposome uptake by cells; Fig. 6), provide additional indications that perhaps the C_{12} and C_{16} arsonoliposomes have a different mechanism of action and introduce further confusion considering the relative value of the two types of arsonoliposomes.

Nevertheless, differential cytotoxicity is not the only parameter that should be taken into account. Indeed, when developing a liposomal formulation as an anticancer product, it is very important to consider also the product stability from a technological point of view. Previously it has been demonstrated that non-sonicated C_{18} arsonoliposomes are considerably less stable compared to the other arsonoliposomes, due to their increased tendency to aggregate (Fatouros et al., 2001). This was confirmed for the non-sonicated vesicles and also demonstrated for sonicated C_{18} arsonoliposomes in a very recent study in which the self- and calcium-induced aggregation of the various arsonoliposomes prepared in our laboratory was evaluated (Gortzi and Antimisiaris, unpublished results). Therefore, from a pharmaceutical stability point of view, the C_{18} arsonoliposomes are of less value in comparison to the other arsonoliposomes.

Considering the physical stability of C_{12} arsonoliposomes, it has been demonstrated that the membrane integrity of these vesicles is comparably very low, a fact that was explained by the microscopic evidence that this

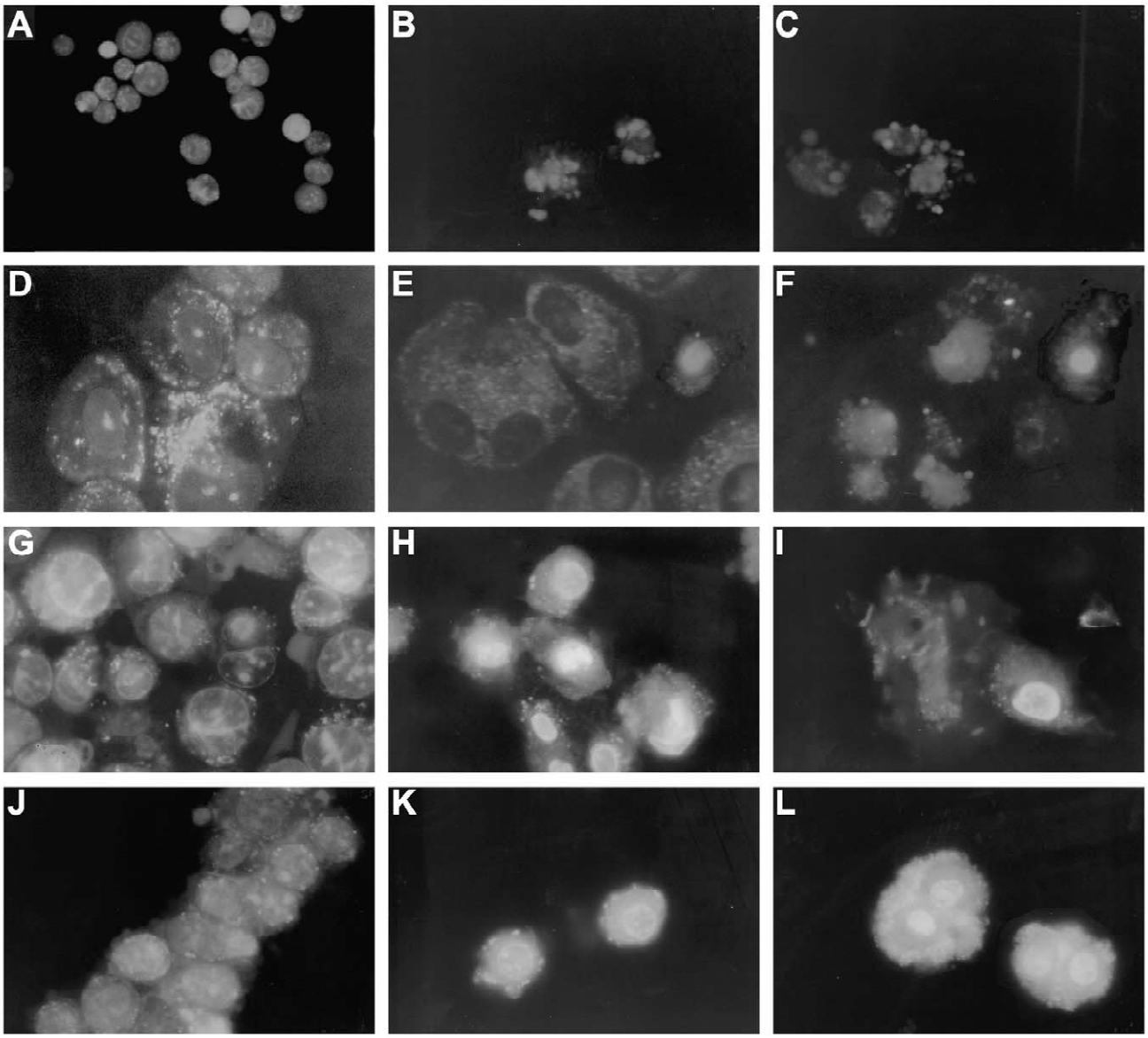

Fig. 5. Morphologic characteristics of HL-60 (A–C), HUVEC (D–F), RAME (G–I) and C6 cells (J–L) before (A,D,G,J) and after treatment with various concentrations of C_{12} arsonoliposomes for 24 h, and staining with acridine orange as described in Materials and methods. The lipid composition of arsonoliposomes used was Ars/PC/Chol 8:12:10 (mol/mol/mol) and the arsonolipid concentration in each case was: B,H,K: 2×10^{-5} M; C,I,L: 10^{-4} M; E: 5×10^{-4} M; and F: 10^{-3} M.

lipid forms long tubular structures which after sonication 'break' into cubes (Fatouros et al., 2001). A high percent of cholesterol in the vesicle membrane increases the retention of encapsulated molecules. The peculiar morphology of the structures formed by the C_{12} arsonolipids may contribute, if it is not absolutely connected, to their different behavior towards cells (Zarif, 2002). Anyhow, before any definite conclusions about their in vivo applicability can be made, further characterization of the system is required.

Therefore, from the different arsonoliposomes studied, the C_{14} and the C_{16} seem better, or at least more predictable, when their physical stability is also considered. When differential cytotoxicity that may be expressed by the ratio of IC_{50} values for normal cells over the values calculated for cancer cells ($IC_{50,\ normal\ cells}/IC_{50,\ cancer\ cells}$) is compared, the C_{16} vesicles are better compared to the C_{14}. Indeed the ratios for the C_{16} arsonoliposomes range between 68 and 298, and 27 and 118, when HUVECs or RAME are considered as normal cells, respectively, while the corresponding values for the C_{14} arsonoliposomes range between 18 and 146, and 10 and 83

Concerning the mechanism of action of arsonoliposomes, we cannot conclude much from the experiments performed up to now. Nevertheless, the toxicity of arsonoliposomes towards cancer cells is probably not con-

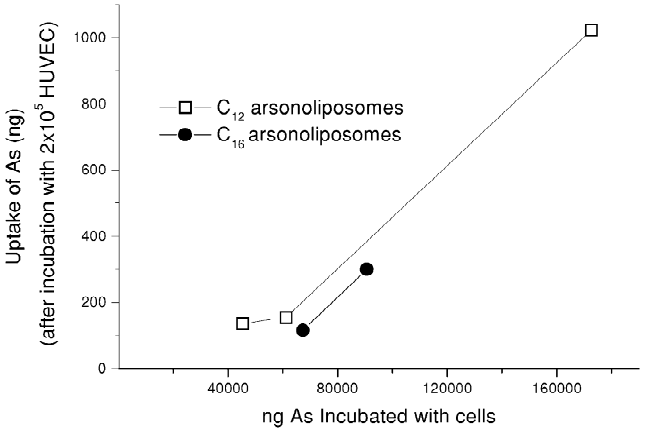

Fig. 6. Interaction of arsonoliposomes with HUVEC cells, for 4 h at 37 °C. The cellular uptake of As (ng) was estimated by atomic absorption spectrometry after digestion with nitric acid. Results are expressed as mean from four independent experiments (S.D. values, not shown, were ~10% of the mean value in all cases).

nected with the anionic nature of these vesicles, since in a previous study (Papadimitriou and Antimisiaris, 2000) we found that PS/Chol sonicated liposomes are not toxic towards HL-60 cells (at similar concentrations as those studied here). In addition, the fact that As is taken up by HUVEC cells after incubation of the cells with arsonoliposomes (C_{12} or C_{16}), is an interesting observation, however, more studies are needed in this direction.

Summarizing, the results of this study demonstrate a significant difference in the cytotoxicity only between the C_{12} and all the other arsonoliposomes (C_{14}, C_{16} and C_{18}), which demonstrate more or less similar in vitro cytotoxicity. When these results are considered in addition to the knowledge acquired from our previous studies about the physical stability of the different arsonoliposome preparations, it is recommended that the C_{16} arsonoliposomes be used for further investigations.

Acknowledgements

This work is a partial requirement for the fulfillment of the doctorate thesis of O.G.

References

Akao, Y., Nakagawa, Y., Akiyama, K., 1999. Arsenic trioxide induces apoptosis in neuroblastoma cell lines through the activation of caspase 3 in vitro. FEBS Lett. 455, 59.

Bode, A.M., Dong, Z., 2002. The paradox of arsenic: molecular mechanisms of cell transformation and chemotherapeutic effects. Crit. Rev. Oncol./Hematol. 42, 5.

Carre, M., Carles, G., Andre, N., Douillard, S., Ciccolini, C., Briand, C., Braguer, D., 2002. Involvement of microtubules and mitochondria in the antagonism of arsenic trioxide on paclitaxel-induced apoptosis. Biochem. Pharmacol. 63, 1831.

Fatouros, D., Gortzi, O., Klepetsanis, P., Antimisiaris, S.G., Stuart, M.C.A., Brisson, A., Ioannou, P.V., 2001. Preparation and properties of arsonolipid containing liposomes. Chem. Phys. Lipids 109, 75.

Gortzi, O., Papadimitriou, E., Kontoyannis, C., Antimisiaris, S.G., Ioannou, P.V., 2002. Arsonoliposomes, a novel class of arsenic-containing liposomes: effect of palmitoyl-arsonolipid-containing liposomes on the viability of cancer and normal cells in culture. Pharm. Res. 19, 79.

Gregoriadis, G., 1993. Liposome Technology, 2nd Edition. CRC Press, Boca Raton, FL.

Lasic, D.D., Papahadjopoulos, D., 1995. Liposomes revised. Science 267, 1275.

Lasic, D.D., Papahadjopoulos, D., 1998. Medical Applications of Liposomes. Elsevier Science, Amsterdam.

Lu, M., Levin, J., Sulpice, E., Sequeira-Le Grand, A., Alemany, M., Caen, J.P., Han, Z.C., 1999. Effect of arsenic trioxide on viability, proliferation, and apoptosis in human megakaryocytic leukemia cell lines. Exp. Hematol. 27, 845.

Mosmann, T.J., 1983. Rapid colorimetric assay for cellular growth and survival—application to proliferation and cytotoxicity assays. Immunol. Methods 65, 5.

New, R.R.C., 1990. In: New, R.R.C. (Ed.), Liposomes: A Practical Approach. IRL Press, Oxford University Press, Oxford, p. 134, see also p. 255.

Papadimitriou, E., Antimisiaris, S.G., 2000. Interactions of PC/Chol and PS/Chol liposomes with human cells in vitro. J. Drug Target. 8, 335.

Roboz, G.J., Diaz, S., Lam, G., Lane, W.L., Soignet, S.L., Warell, R.P., Rafii, S., 2000. Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. Blood 96, 1525.

Serves, S.V., Sotiropoulos, D.N., Ioannou, P.V., Jain, M.K., 1992. Synthesis of (*R*)- and (*S*)-1,2-diacyloxypropyl-3-arsonic acids: optically active arsonolipids. Phosphorus Sulfur Silicon 71, 99.

Serves, S.V., Sotiropoulos, D.N., Ioannou, P.V., Jain, M.K., 1993. One pot synthesis of arsonolipid via thioarsenite precursors. Phosphorus Sulfur Silicon 81, 181.

Soignet, S.L., Maslak, P., Wang, Z.G., Jhanwar, S., Calleja, E., Dardashti, L.J., Carso, D., De Blasio, A., Gabrilove, J., Scheinberg, D.A., Pandolfi, P.P., Warrell, Jr. R.P., 1998. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. New Engl. J. Med. 339, 1341.

Stewart, J.C.M., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10.

Timotheatou, D., Ioannou, P.V., Scozzafava, A., Briganti, F., Supuran, C.T., 1996. Carbonic anhydrase interaction with lipothioarsenites: a novel class of isozymes I and II inhibitors. Metal-Based Drugs 3, 263.

Tsivgoulis, G.M., Sotiropoulos, D.N., Ioannou, P.V., 1991a. 1,2-Dihydroxypropyl-3-arsonic acid: a key intermediate for arsonolipids. Phosphorus Sulfur Silicon 57, 189.

Tsivgoulis, G.M., Sotiropoulos, D.N., Ioannou, P.V., 1991b. *rac*-1,2-Diacyloxypropyl-3-arsonic acids: arsonolipid analogues of phosphonolipids. Phosphorus Sulfur Silicon 63, 329.

Zarif, L., 2002. Elongated supramolecular assemblies in drug delivery. Rev. J. Controlled Release 81, 7.