Arsonoliposomes, a Novel Class of Arsenic-Containing Liposomes: Effect of Palmitoyl-Arsonolipid-Containing Liposomes on the Viability of Cancer and Normal Cells in Culture

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Purpose. Arsonolipid-containing liposomes have been recently prepared. The demonstrated antileukemic action of arsenic trioxide prompted us to study their effect on the viability of several types of cancer cells to investigate the possibility of relevant applications. Five different cell types, three malignant (HL-60, C6, and GH3) and two non-malignant (HUVEC and RAME), were used.

Methods. Liposomes containing the palmitoyl side chain arsonolipid (with different lipid compositions) were incubated with a given number of cells. Cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide assay. Morphologic studies were also performed.

Results. Our results reveal that arsonoliposomes cause a dose (initiated at 10^{-6} M)- and time-dependent inhibition of survival in all three malignant cell lines studied. No significant effect on the survival of the normal cells studied was observed at these, as well as at 10-fold higher, concentrations, although arsenic trioxide was toxic to HUVEC cells at equivalent arsenic concentrations. Microscopy studies reveal that although morphologic changes were initiated in HL-60 and C6 cells after incubation with arsonoliposomes, no changes in HUVEC and RAME cells were observed.

Conclusions. Considering the numerous advantages of liposomal systems in therapeutics, it is concluded that the arsonoliposome system is very interesting and future applications should be exploited by further studies.

KEY WORDS: arsenic compound; arsonolipid; liposome; cell viability; cell line.

INTRODUCTION

Arsenic trioxide has been used to treat human diseases for centuries, especially in traditional Chinese medicine. It has been identified as a very effective antileukemic agent (1–3) and has been demonstrated to substantially reduce the viability and growth and/or cause apoptosis of several types of cancer cells in culture (4–10). Clinical reports from two Chinese groups, confirmed by a study in the United States (1–3), indicate that As_2O_3 is extremely effective for inducing complete remission in patients with acute promyelocytic leukemia (APL) who are resistant to both cytotoxic chemotherapy and all-*trans* retinoic acid. A significant drawback for the broader use of arsenic(III)-based compounds in other than salvage therapy applications is their high toxicity (11, 12). Recently, arsenic trioxide has been demonstrated to reduce viability of endothelial cells at relevantly low concentrations in a time- and dose-dependent manner (13). Data from several studies suggest that arsenic trioxide is active in APL at doses ranging from 0.06 to 0.20 mg per kilogram. Within this dose range, no relation between dose and efficacy was obvious; however, severe toxic reactions, including flaccid paralysis and renal failure, have been observed after attempts to increase the dose beyond this range (12).

In general, arsenic (III) toxicity may be reduced by altering the oxidation state to arsenic (V). One method of reducing drug toxicity that has worked well in several instances (especially with cytotoxic drugs) is the inclusion of the highly toxic substance in stable liposomal formulations (14, 15). This may result in reduced toxicity and simultaneous enhanced activity of the drug substance, if of course the kinetics and distribution of the drug is altered, which is the case when the drug is sufficiently retained by the vesicular carrier.

In an attempt to combine the anticancer activity of arsenic-containing compounds with the reduced toxicity and enhanced activity of liposomal formulations of cytotoxic agents, we have recently prepared and characterized liposomes that contain arsonolipids (16). Arsonolipids (1,2-diacyloxypropyl-3-arsonic acids) are lipidic analogues of phosphonolipids in which As is replacing P in their polar head group (17, 18). The integrity of liposomal membranes composed of mixtures of arsonolipids with cholesterol or cholesterol and phosphatidylcholine (mixed arsonoliposomes) was found to be sufficiently high, even in the presence of serum proteins (80% fetal calf serum; FCS). The morphology and physicochemic properties of plain and mixed arsonoliposomes were demonstrated to be affected by the length of the acyl chains of the arsonolipids and the inclusion of cholesterol in the vesicle membrane (16). In addition, the *in vivo* distribution and toxicity of arsonoliposomes is currently being investigated in our lab, whereas arsonolipids have been demonstrated to efficiently transport Ca^{2+} and Mg^{2+} through organic phases (19).

Herein, we studied the effect of this novel class of liposomes, the arsonoliposomes, on the viability of different cell types to investigate their potential for future applications in therapeutics. The arsonolipid with the palmitoyl acyl chain, which was previously demonstrated to form relevantly stable sonicated vesicles (16), was used. Three types of liposomes, one containing 100% arsonolipid and two others that contain 15 or 40% arsonolipid mixed with egg lecithin and cholesterol, were prepared. Different doses of the liposomes were incubated with different types of cells in culture, and their effects on the viability and growth of these cells were followed. Additionally, the morphology of the cells before and after arsonoliposome treatment was observed to gain preliminary knowledge on the mechanism of action of the proposed novel arsenic-containing liposomal system.

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MATERIALS AND METHODS

Reagents

Phosphatidylcholine (PC) (grade 1) was purchased from Lipid Products (Nutfield, UK) and was found to be pure by thin-layer chromatography analysis. The rac-arsonolipid (Ars) (2,3-diacyloxypropylarsonic acid) with a palmitic side chain ($R = C_{15} H_{31}$) was synthesized and characterized as described in detail before (18).

Cholesterol (Chol) was of analytical grade and was purchased from Sigma-Aldrich Ltd. (Athens, Greece). All other reagents and solvents used throughout the study were of analytical grade and were purchased from Sigma-Aldrich Ltd All media used for cell growth and handling were purchased from Biochrom (Berlin, Germany) and were of cell culture grade.

Human leukemia HL60, rat brain glioma C6, and rat pituitary tumor GH3 cells were from American Type Culture Collection (Rockville, MD). 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).

Liposome Preparation and Characterization

Liposomes containing plain arsonolipid (100%, Ars/Chol 20:10) or mixtures of Ars with PC (15 or 40%, Ars/PC/Chol 3:17:10 or 8:12:10) were prepared by sonication after initial dispersion of the lipids in phosphate buffer saline (PBS), which was achieved by magnetical stirring at 70°C (above the Ars transition temperature). In all cases, Chol was included in the liposomes prepared (at a 2:1 lipid/Chol, mol/mol ratio). Sonication was performed by two 5-min runs, separated by a 10-min interval, by a probe type sonicator (Sonics and Materials, UK). After sonication, the liposomes were centrifuged for 10 min at 18.000 g to remove titanium pieces and liposome aggregates that could be present in the samples and left for 1 h for annealing of any structural defects. 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 used (20). This assay was found to also detect arsonolipids (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-485nm of the chloroform phase is measured and the lipid concentration of samples is calculated by comparison with the standard curve.

To be sure that all arsonolipid used during liposome preparation was indeed incorporated in the liposome dispersions, the As content of some samples was also determined by Energy Dispersive X-ray Fluorescence spectroscopy analysis (QuanX, TN Spectrace). A thick (0.13 mm) Pd filter was used, while the instrument is equipped with a solid-state Si(Li) liquid nitrogen cooled detector and the X-ray tube was air cooled with a side window Bremsstrahlung with Rh target, 50 watt. After measuring the As content of the samples, it was calculated that all the arsonolipid added initially was incorporated in the liposomes.

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 by photon correlation spectroscopy with a Malvern Zetasizer 5000 (Malvern, UK), as described before (21). In brief, liposome dispersions were diluted with filtered tris-buffered saline, pH 7.4, 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. The ζ-potentials were calculated directly from the Helmhotz–Smoluchowski equation.

Morphologic observations of these vesicles were also performed recently by electron microscopy (16), and it was demonstrated that the arsonoliposomes used throughout this study had a disc shape.

Cell Culture

HUVEC 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. RAME cells were cultured in DMEM supplemented with 10% FCS and used at passages 19–21. HL60 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. Cultures were maintained at 37°C, 5% CO_2, and 100% humidity.

Cell Growth-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 incubations 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 (22). 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 HL60, the medium was removed, the cells were washed with PBS, pH 7.4, and 100 μL of acidified isopropanol (0.33 mL of HCl in 100 mL of 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, Hercules, CA) at a wavelength of 490 nm. For HL60 cells that were grown in suspension, an 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. Growth-inhibitory concentrations of 50%t (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.).

Morphologic Studies

Morphologic studies were performed with HUVEC, RAME, HL60, and C6 cells. After incubation of the cells with arsonoliposomes, the cells were harvested: The HL60 cells by centrifugation at 500 × g for 4 min and the HUVEC, RAME, and C6 cells after trypsinization and centrifugation. In the latter case, both, detached cells as well as those still adhering were collected. 10 μL of the cell suspension were mixed with an equal volume of a solution of acridine orange (10μg/mL) and examined under a Leica DMLS, fluorescent microscope (equipped with a MPS28 photographic controller).

Preliminary Liposome—Cell Interaction Studies

Liposomes were incubated as described previously (23) with confluent monolayers of HUVEC (250–2500 μg of liposomal lipid/10^5 cells) or suspensions of HL60 cells (250–2500 μg of liposomal lipid/10^6 cells) for a period of 2 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 at a concentration of 10^5 HUVEC or 10^6 HL60 cells in 2 mL of PBS. HUVEC cells were completely detached from the plate by mild treatment with trypsin (0.005%)–EDTA in PBS pH 7.4. Fluorescence intensities of 8-hydroxypyrene trisulfonic acid trisodium salt (HPTS) in the cell samples were measured (excitation 413 nm–emission 512 nm). Liposome uptake (cell-associated liposomes) was determined by comparison to the fluorescence of the liposome preparations. Fluorescence intensities of some HPTS-containing samples were also measured after disrupting vesicles and/or cells with Triton X-100 (1% v/v final concentration). No quenching of HPTS was detected. In all cases, leakage of HPTS from both liposome types studied was below 10% of the initially encapsulated amount (16), and control experiments in which the extent of interaction between cells and free HPTS was evaluated, were carried out and considered (23).

RESULTS

The physicochemic characteristics of the liposomes used are presented in Table I. The liposome mean diameter ranged between 74.8 and 116 nm, depending on the amount of Ars substituted by PC. As the amount of PC in the liposomes increased their size decreased, a fact probably linked with the smaller polar head group of PC (P is smaller than As), which allows better packing of the lipids. As demonstrated also previously (16), arsonolipids give a negative surface charge to vesicles. Indeed, negative ζ-potential values increased linearly with the amount of Ars incorporated in the liposomes (Table I).

Table I. Mean Diameter and ζ-Potential Values of Sonicated Ars/PC/Chol Arsonoliposomes Prepared in Phosphate-Buffered Saline (pH 7.4)

Lipid content of arsonoliposome	Ars/Chol 20:10 mol/mol	Ars/PC/Chol 8:12:10 mol/mol/mol	Ars/PC/Chol 3:17:10 mol/mol/mol
Diameter (nm)[a]	116 ± 11	83.1 ± 6.9	74.8 ± 7.3
ζ-Potential (mV)[a]	−69.5 ± 2.3	−50.3 ± 1.0	−42.1 ± 2.9

[a] Each value is the mean ± SD of five subsequent measurements from at least four different samples.

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 Figure 1. In addition, the IC_{50} values calculated for each preparation on each cell type studied are listed in Table II. As demonstrated by these experimental results, the viability of all cancer cells (Fig. 1, upper panels) was rapidly decreasing in an exponential manner as the arsonolipid concentration increased. The effect on cell viability was greatest for HL-60 cells, whereas the other two cancer cell types studied demonstrated a more or less similar decrease in cell viability when treated with equivalent arsonoliposome concentrations, the C6 cells being affected the least (with the exception of the Ars/Chol vesicle effect). In all the cases of cancer cells, cell viability was significantly decreased ($P \leq 0.01$) after treating cells for 24 h with 10^{-5} M arsonolipid in liposomes, whereas a 10-fold increase in arsonolipid concentration (10–$^{-4}$ M arsonolipid in liposomes) almost always resulted in viabilities below 50% (except for the C6 cells with the mixed arsonoliposomes). In contrast, these arsonolipid concentrations (10^{-5}–10^{-4} M arsonolipid) were practically ineffective (with survival of 85–90 ± 10% of the treated cells) in the two non-cancer cell types studied under identical conditions (Fig. 1, lower panels). Even higher concentrations of arsonoliposomes (up to 10^{-3} M arsonolipid/ 10^5 cells) had minimal effects on the viability of the normal cells studied, which in all cases is above 50% (Fig. 1, lower panels). This can be also concluded from the IC_{50} values calculated (from interpolations of graphical data) for all cells studied (Table II). Indeed, the lowest ratio between IC_{50} values of normal and cancer cells was ~10 [demonstrated between RAME and C6 cells when the Ars/PC/Chol (8:12:10) preparation was used] and the highest is ~300 (demonstrated between HUVEC and HL-60 cells when the Ars/Chol (20:10) preparation was used).

Considering the results of control experiments with PC/Chol liposomes, it was demonstrated that such vesicles had no effect on the viability or growth of the cells used in this study (when incubated at concentrations up to 3×10^{-4} M lipid/10^5 cells) because the final cell number after incubation for 24 h was always equal to that of the control sample incubated only with PBS (not shown).

When the effects of the different arsonoliposome compositions tested on cancer cell viability were compared, it was clear from the graphs (Fig. 1) and the values in Table II that in the cases of HL-60 and C6 cells (Fig. 1), there is a significant difference between the plain Ars liposomes (Ars/Chol) and the other two compositions tested (mixed arsonoliposomes) whereas practically no effect of liposome composition was observed in the cases of HUVEC, RAME, and GH3 cells (Fig. 1). This effect may be linked with the amount of As located on the outer layer of the liposome bilayer, which may influence the extent and mechanism of liposome–cell interaction.

To have a control experiment that would allow us to compare our results with those of others, we also studied the effect of arsenic trioxide on two of the cell types used here (which were also studied by others) HUVEC and HL-60 cells (Fig. 2). In agreement with recent studies of other groups (5, 8–10) As_2O_3 was found to significantly reduce the number of

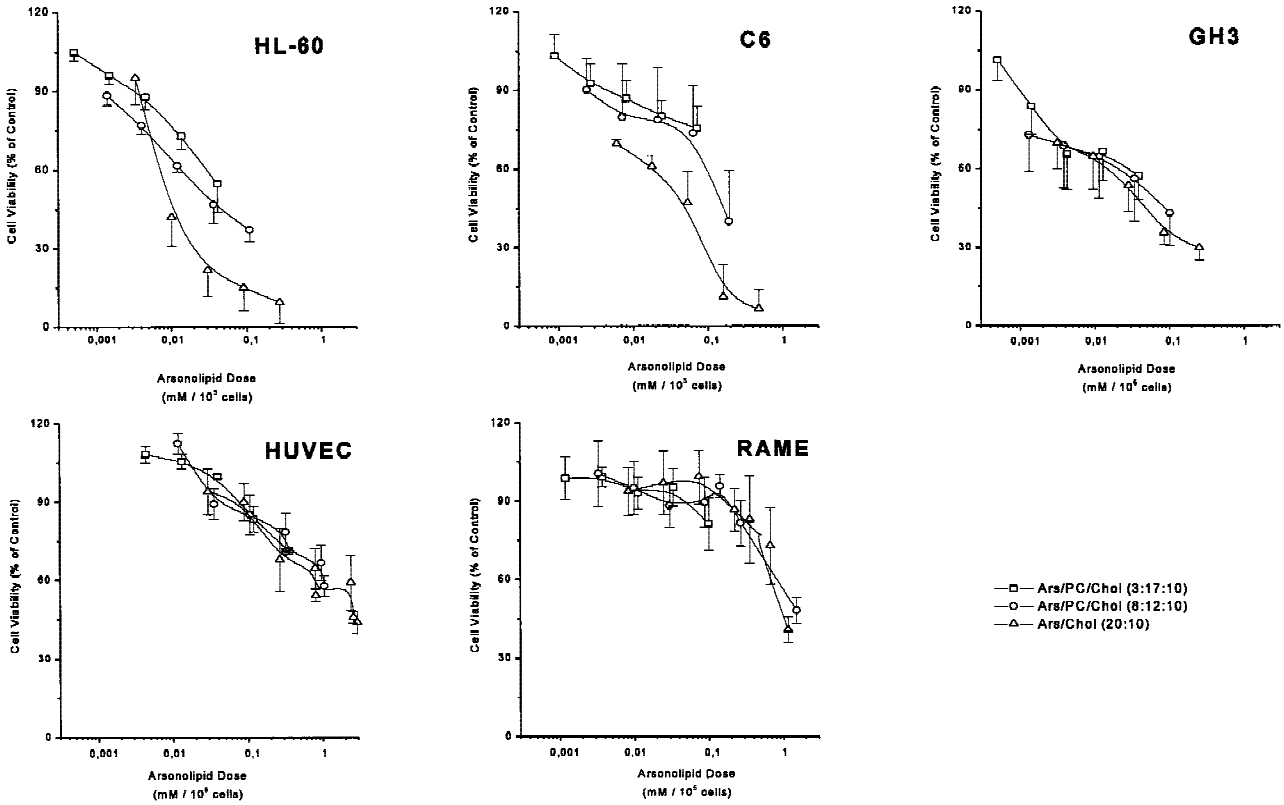

Fig. 1. Effect of arsonoliposomes on the viability of tumor (upper panels) and normal (lower panels) cell lines. Cells were incubated with various concentrations of arsonoliposomes for 24 h. Results are expressed as viability (percent of viable cells in comparison with the control cells) versus arsonolipid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

viable HL-60 cells when incubated for 24 h at concentrations higher than 2×10^{-5} M (Fig. 2). Surprisingly, arsenic trioxide is much more toxic against HUVEC cells reducing cell viability to approximately 70% at concentrations of 2×10^{-6} M, or else 10 times higher than the corresponding active concentration against HL-60 cells. These later results are absolutely comparable with those derived in another very recent study (13).

The time dependence of arsonoliposome effects (for the liposome formulation containing 40% arsonolipid) on cell viability was also studied in the two human cell types used, one cancer (HL-60) and one normal (HUVEC) for treatment periods up to 96 h. As demonstrated by the results (Fig. 3), there was a remarkable effect of time of exposure of cells on cell viability for both HL-60 and HUVEC cells. This experimental setup was performed by both methods, trypan blue exclusion and MTT assay, and their results concerning cell viability were similar. When comparing time dependence on cell vi-

Table II. 50% Growth-Inhibitory Concentrations (IC_{50}) of Arsonoliposomes (Expressed as the Arsonolipid Content of Liposomes in Each Case) for the Various Cell Types Studied

Lipid content of arsonoliposomes	IC_{50} ($\times 10^5$ M)[a]				
	HL-60 cells	C6 cells	GH3 cells	HUVEC cells	RAME cells
Ars/Chol (20:10)	0.85	3.71	3.14	253	100
Ars/PC/Chol (8:12:10)	2.89	14.59	5.45	ND	147
Ars/PC/Chol (3:17:10)	6.14	ND	ND	ND	ND

The values were calculated from interpolations of the graphical data presented in Figure 1 using the Microcal Origin Program (Version 5).
[a] SD values for the IC_{50} values were not calculated because these values were estimated from graphical interpolations.
ND = not determined.

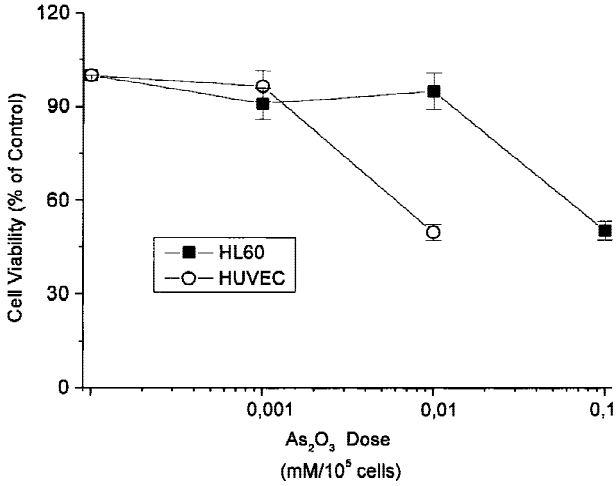

Fig. 2. Effect of arsenic trioxide on the viability of HL-60 (■) and HUVEC cells (○) at various concentrations of As_2O_3 for 24 h. Results are expressed as percent of viable cells in comparison with the control. Each experiment was performed in triplicate and the bars represent standard error values.

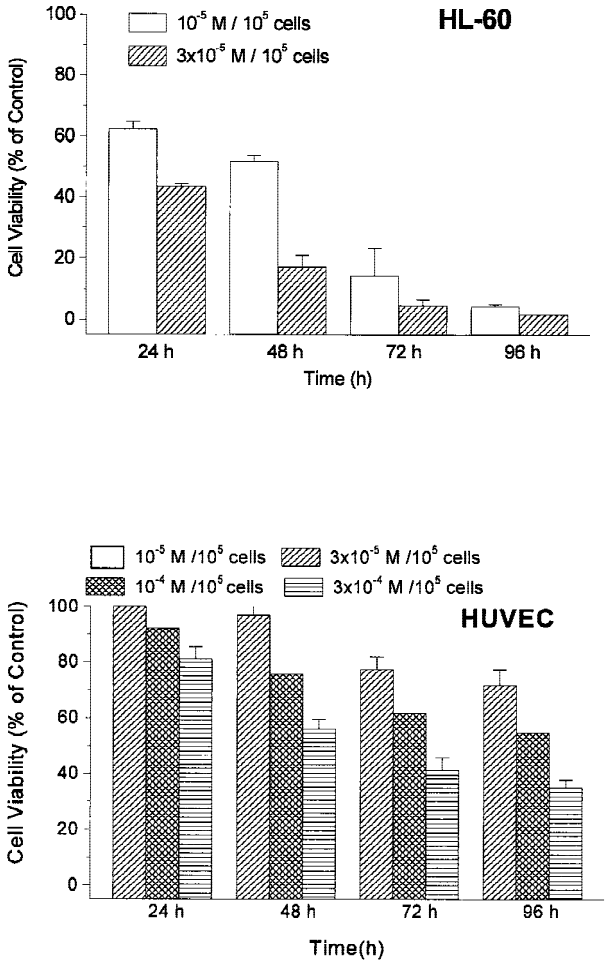

Fig. 3. Effect of arsonoliposomes on the viability of HL-60 cells (upper panel) and HUVEC cells (lower panel). Cells (10^5 cells/mL) were incubated for 24, 48, 72, and 96 h with various concentrations of arsonoliposomes. Arsonoliposomes with lipid composition of Ars/PC/Chol 8:12:20 were used in these experiments. Results are expressed as viability (percent of viable cells in comparison with the control cells) versus arsonolipid content of the liposomal formulation. Each experiment was performed in triplicate and the bars represent standard error values.

ability of HUVEC and HL-60 cells, it is evident that the rate of decrease in cell viability was higher for the cancer cells when the effect of the same arsonolipid concentration (3×10^{-5} M) is considered. Nevertheless, HUVEC viability also decreases linearly with the time of exposure (up to 72 h) when a 10 times higher arsonolipid concentration is applied (3×10^{-4} M), whereas this arsonolipid concentration is practically ineffective on these normal cells after only 24 h of incubation (Fig. 3, lower panel).

In morphologic studies, HL-60, C6, HUVEC, and RAME cells were treated with two intermediate arsonoliposome concentrations to observe possible arsonoliposome-induced changes in cell morphology. However, after staining the treated as well as the control cells with acridine orange and observing them by a fluorescence microscope, no obvious morphologic changes that are characteristic of apoptosis, such as cell shrinkage or cell membrane blebbing, were visible (Fig. 4), at least under the specific conditions applied. Nevertheless, in the cases of the cancer cells studied, HL-60 (Fig. 4, A–C) and C6 cells (Fig. 4, J–L), it is obvious that their morphology was significantly affected after incubation with the highest dose of arsonoliposomes used for these experiments (Fig. 4, C and L). Indeed, in the HL-60 cells significant cell and nucleus swelling (a preliminary step to membrane rupture during cell necrosis) was observed (Fig. 4C), whereas in some of the C6 cells, acridine fluorescence of the cell nucleus was significantly higher when compared with the control cells (Fig. 4L). On the other hand, no obvious effect on cell morphology was observed in the HUVEC and RAME cells (Fig. 4, D–E, G–I), with the exception of some small change in the shape of the RAME cells (Fig. 4I), related perhaps to the way they adhere to the slide surface. In contrast to this, high concentrations (10^{-4} M) of AS_2O_3 were clearly demonstrated to cause apoptosis to HL-60 cells after staining the cells under the same conditions (Fig. 5B).

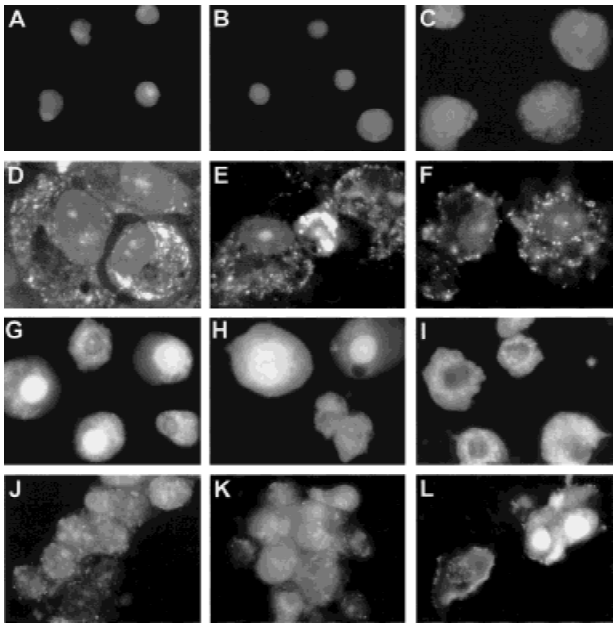

Fig. 4. Morphologic characteristics of HL-60 (A–C), HUVEC (D–F), RAME (G–I), and C6 cells (J–L) before (A, D, G, and J) and after treatment with various concentrations of arsonoliposomes for 24 h, and staining with acridine orange as described under Materials and Methods. The lipid composition of arsonoliposomes 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}, and F = 10^{-3} M.

To further investigate the mechanism of the differential toxicity of arsonoliposomes towards normal and cancer cells,

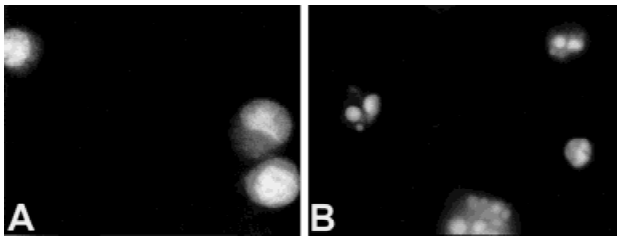

Fig. 5. As_2O_3-induced morphologic changes, characteristic of apoptosis, in HL-60 cells. Cells were treated with 10^{-4} As_2O_3 (B) or without As_2O_3 (A) for 24 h and then stained with acridine orange as described in Materials and Methods.

we performed some preliminary arsonoliposome–cell interaction studies. For this, we used HL-60 cells as a cancer cell model and HUVEC cells as a normal cell model because the interaction of these cells with conventional liposomes [PC/Chol and PS/Chol (PS = Phosphatidyl-serine)] were previously thoroughly studied in our lab (23). The results of these preliminary studies are very interesting (Fig. 6), revealing that arsonoliposomes interact highly with the HL-60 cells (Fig 6, upper panel), whereas their interaction with HUVEC cells is minimal (Fig. 6, lower panel). This behavior of arsonoliposomes is completely opposite of that demonstrated previously with conventional liposomes (23), which interact quantitatively higher with HUVEC cells in comparison with HL-60 cells. The negative surface charge of the arsonoliposomes could not be related with this difference between arsonoliposomes and conventional liposomes because PS/Chol liposomes also have highly negative ζ-potential values (23). This conclusion, in addition to the fact that the interaction of arsonoliposomes with HL-60 cells was demonstrated to significantly increase with the arsonolipid content of these liposomes (Fig 6, upper panel), suggest that the increased interaction with cancer cells is related to the presence of As on the liposome membrane.

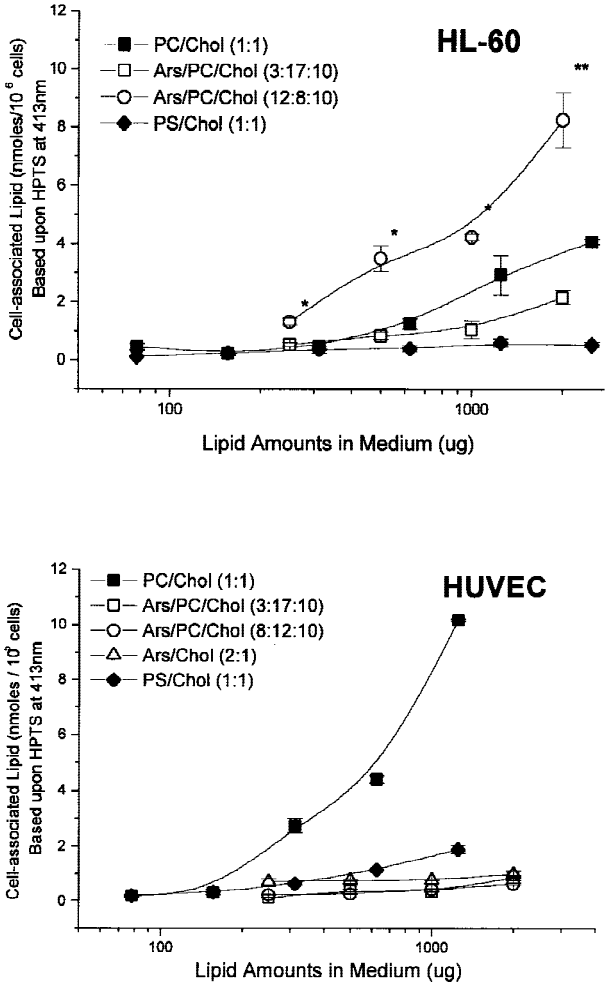

Fig. 6. Interaction of arsonoliposomes with HL-60 cells (upper panel) and HUVEC cells (lower panel) for 2 h at 37°C. The cellular uptake of liposomes (expressed as nmoles liposomal lipid) was estimated from the 8-hydroxypyrene trisulfonic acid trisodium salt (HPTS) fluorescence of cells by comparison with the fluorescence of the initial liposome dispersion used in each case, as described in detail previously (23). Results are expressed as mean ± standard error of the mean from at least three independent experiments. For direct comparison, the values estimated previously (23) for conventional phospholipid vesicles are incorporated in the graphs. $*P < 0.05$, $**P < 0.01$ (after comparison with results from PC/Chol versicles).

DISCUSSION

Recent advances related to the use of arsenic trioxide as a very potent antileukemic agent have prompted us to study the possibility of arsonoliposome applications in therapeutics. As a preliminary investigation, the effect of different doses of these novel, recently prepared and characterized arsonolipid-containing liposomes (16) on the growth and viability of five different cell types, three malignant cell lines (HL-60, C6 and GH3) and two normal (HUVEC and RAME), was evaluated. In addition, the interaction of some arsonoliposomes with one normal (HUVEC) and one cancer (HL-60) cell type, were investigated and the results of this liposome–cell interaction study were compared to those previously reported, for conventional (phospholipid) liposomes (23).

In this study, we demonstrated that arsonolipid-containing liposomes significantly decrease the viability of several different cancer cells while being more or less ineffective towards normal cells under identical exposure conditions. When comparing the results of the different liposomal formulations tested (Fig. 1), it is clear that for all formulations there is an increase in toxicity towards cancer cells when increasing the amount of co-incubated arsonoliposomes. Under identical experimental conditions, plain phospholipid (PC/Chol 2:1 mol/mol) liposomes (up to a concentration of 3×10^{-4}M / 10^5 cells) had no effect on cell viability (not shown). This indicates that the arsonolipid that is present in the vesicle membranes is the active substance. Furthermore, in two of the three cancer-cell types studied, the liposomes containing plain arsonolipid, without PC, were found to be significantly more toxic ($P \leq 0.01$), even when results were expressed as cell viability versus arsonolipid content of liposomes. This is possibly linked with the availability of As on the outer layer of the vesicle bilayer [because these vesicles were previously observed to be unilamellar (16)], which probably affects the interaction of the liposomes with the cells (degree of interaction or velocity of interaction or both). Indeed, it was clearly demonstrated (Fig. 6, upper panel) that the interaction between arsonoliposomes and HL-60 cells is substantially higher (when the liposome lipid uptake is considered) as the arsonolipid content of liposomes increases. Furthermore, the fact that arsonoliposomes interact highly with HL-60 cells (Fig. 6, upper panel) compared to HUVEC cells (Fig. 6, lower panel) may serve as a serious indication that possibly the demonstrated differential toxicity of arsonoliposomes is connected with a different mode and extent of interaction of these liposomes with cancer cells. Nevertheless, further studies are needed to clarify the mechanism by which arsonoliposomes interact with each cell type (internalization or fusion with the cell membrane).

The results of the morphologic study are in good correlation with the cytotoxicity values estimated by the MTT assay (Fig. 1 and Table II) and the trypan blue exclusion test (not shown) because significant morphologic changes were in-

duced by arsonoliposomes on the cancer cells but not the normal ones (Fig. 5). The morphologic changes induced by arsonoliposomes in HL-60 and C6 cells provide indications that perhaps arsonoliposomes cause necrosis in HL-60 and apoptosis in C6 cells. However, only indications can be provided by these preliminary experiments, and further studies are required to gain any definite proof about the mechanism by which arsonoliposomes affect the viability of cancer cells. Nevertheless, the mechanism of the demonstrated antileukemic effect of arsenic trioxide also remains uncertain. Although it is known that in APL cells arsenic targets promyelocyte proteins (PML) onto nuclear bodies and induces degradation of the PML/RAR-a fusion protein (7, 8), whereas in non-APL cells, it causes degradation of only the PML protein (7), others suggests that arsenic-induced apoptosis is independent of PML and PML/RAR-a (5). In addition, several alternative mechanisms of action of arsenic trioxide have been proposed, as modulation of the intracellular glutathione redox system (24). Recently, a combination between direct toxicity against leukemia cells and antiangiogenic activity has been also proposed (13). Whatever the mechanism of action, the use of As_2O_3 as a potent antileukemic agent is currently being proposed by several clinicians (1–3, 25), whereas others are still concerned about the indubitable toxicity related to arsenic compounds (11, 12). Thereby, an arsenic-containing liposomal formulation that may be tailored in such a way to target specific cells may be of great therapeutic value. Furthermore, the fact that the novel system proposed herein may be less toxic towards normal cells is undoubtedly an additional advantage. Considering the *in vivo* toxicity of arsonoliposomes, although their LD_{50} values have not been determined, in preliminary toxicity studies (unpublished results), when arsonoliposomes were administered to Balb-c mice at arsonolipid doses equivalent to 5 mg As_2O_3 /kg body weight, no obvious acute or prolonged (up to 4 weeks) toxicity was observed, whereas the morphology of different organs is currently being evaluated for signs of toxicity.

When comparing the arsonoliposome results of this study with results of the effects on cells induced by arsenic trioxide, gained by others and us (Figs. 2 and 6), it is evident that arsonoliposomes are substantially more potent against HL-60 cells when the As content of the liposomal formulations tested is taken into account. In addition, although As_2O_3 was found to be highly toxic against HUVEC cells, equivalent amounts of arsonoliposomes had practically no effect on these normal cells. These results may serve as good indications that the proposed arsenic-containing system is specific, affecting only the cancer cell types studied and thereby it possibly interacts with the cells through a different mechanism, or combination of mechanisms.

In conclusion, although only suggestions can be made about the mechanism of action of the novel arsenic-containing liposomal system proposed, the results of this study reveal that it is indeed a very interesting system justifying continuation of studies *in vitro* as well as *in vivo*.

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