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Research Paper

Arsonoliposomes for the Potential Treatment of Medulloblastoma

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Purpose. To investigate the arsonoliposome effect on medulloblastoma cells (VC312Rs) related to uptake, endocytotic mechanism and cell viability.
Methods. VC312R viability in presence of either arsonoliposomes or stealth liposomes was studied using MTT assay for 1–4 days. Fibroblasts (3T3) were used as control. Apoptosis was studied for 2 h, 5 h and 24 h. Bodipy-labelled arsonoliposome uptake (time- and dose-dependent) was estimated using FACS analysis. The endocytotic mechanism was investigated using inhibitors of clathrin- (chlorpromazine) and caveolae-mediated endocytosis (filipin).
Results. Arsonoliposomes affected significantly the VC312R viability compared to 3T3 cells and induced apoptosis to VC312Rs after 2 h of incubation. Apoptosis was not observed for 3T3 cells. Liposome uptake *versus* time showed a bimodal pattern. Clathrin-mediated endocytosis was the main endocytotic mechanism at low lipid concentrations and caveolae at higher ones; thus, dose-dependent uptake did not show a plateau at increased lipid concentrations.
Conclusions. Arsonoliposomes showed “selective” toxicity towards medulloblastoma cells inducing apoptosis after 2 hs of incubation. Therefore, arsonoliposomes are promising anticancer vehicles for brain tumour treatment.

KEY WORDS: apoptosis; arsonoliposomes; cellular uptake; liposomes; medulloblastoma.

INTRODUCTION

Medulloblastoma is the most common malignant tumour of central nervous system in children (1), representing 20% of all intracranial tumours. Although they are classified as paediatric tumours, around 30% of medulloblastoma cases are reported in adults (2). Medulloblastoma arises from remnants of the neuroectoderm in the fourth ventricle, and grows in cerebellar vermis and often fills the ventricle, causing hydrocephalus (3). The clinical treatment of these tumours is highly aggressive: surgery is needed to remove the medulloblastoma, and both radiotherapy and chemotherapy are used to avoid recurrences (4). Furthermore, chemotherapeutic agents like dibromodulcitol and carboplatin exhibit some important side effects, such as anaemia (5), leucopenia and thrombocytopenia (6). To reduce the inconveniences introduced by these side effects, chemotherapeutic agents can be encapsulated in drug delivery systems, such as liposomes.

Arsonoliposomes (7) are a novel class of liposomes that are composed of arsonolipids with phospholipids and cholesterol. Arsonolipids are analogues of phospholipids where the phosphonic head has been replaced by an arsonic head. It has been reported (8,9) that small unilamellar arsonoliposomes demonstrate high toxicity against various cancer cell lines, while at the same concentrations they are non-toxic to normal cells. These characteristics make arsonoliposomes suitable for cancer therapy as drug delivery systems and chemotherapeutic agents at the same time. Thus, in this study we investigated for the first time the interaction of the arsonoliposomes with a medulloblastoma cell line *in vitro*, evaluating the uptake and the toxicity towards VC312R cells in monolayers.

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ABBREVIATIONS: ABB, annexin binding buffer; Bodipy, boron-dipyrromethene; C_{16}-As, 2,3-dipalmitoyloxypropylarsonic acid; Chol, cholesterol; CPZ, chlorpromazine hydrochloride; DAPI, 4′,6-diamidino-2-phenylindole; DHPE, 1,2-dihexadecanoyl-SN-glycero-3-phosphoethanolamine; DMSO, dimethylsulfoxide; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; MEM, minimum essential medium; PBS, phosphate buffer saline; PFA, paraformaldehyde; PI, propidium iodide; PS, phosphatidyl serine; Rhod, rhodamine; SUPW, sterile ultra pure water; TNF-α, tumoural necrosis factor.

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MATERIALS AND METHODS

Materials

Distearoyl-phosphatidylcholine (DSPC) was obtained from Lipoid; arsonolipids were kindly provided by Prof. P. Ioannou (Department of Chemistry, University of Patras, Greece). Chol, thiazolyl blue tetrazolium, Sephadex G50, all buffer salts and organic solvents were purchased from Sigma. Rhod, Bodipy-DHPE and annexin V were obtained from Invitrogen; all components used for cell culture were from Gibco. DAPI and PI containing mounting media were purchased from VectaShield.

Cell Culture

The VC312R medulloblastoma tumour cells were grown as monolayers in MEM supplemented with 10% FBS, 200 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 7.5% sodium bicarbonate solution and SUPW, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated either in 75 cm^2 flasks or in 24-well plates at a density of 2×10^6 cells/mL/well. After 4–5 days, the cells were passaged at about 70–80% sub-confluency. The normal fibroblast cells (3T3) were cultured in monolayers in 75 cm^2 culture flasks in a medium composed of 10% FBS, 7.5% sodium bicarbonate, L-glutamine, MEM and SUPW.

Liposomes Preparation and Characterization

Plain liposomes were prepared using the thin film method. In brief, liposomes were prepared from DSPC and Chol at a molar ratio 2:1. The mixture of DSPC and Chol previously dissolved in chloroform methanol (2:1) was dried to a thin film using nitrogen stream. PBS buffer (pH 7.4) at 60°C was added to a final lipid concentration of 5 mg lipid/mL. Liposomes were sonicated in a bath sonicator for 20 min and left for annealing of any structural defects at 60°C for 1 h. Then, liposomes were sonicated using a probe sonicator (Soniprep 150) in two 5-minute cycles with a 1 min interval, left for annealing at 60°C for 1 h and centrifuged for 5 min at 3,000 rpm to precipitate titanium fragments.

Arsonoliposomes were prepared from DSPC, C_{16}-As and Chol at the molar ratio 12:8:10. The mixture of lipids previously dissolved in chloroform: methanol (2:1) was dried under a nitrogen stream. PBS (pH 7.4) at 80°C was added to a final lipid concentration of 5 mg/mL. The samples were kept at 80°C and vigorously stirred for 4 h. Then, the liposomes were sonicated using a probe sonicator for a cycle of 2×5 min with a 1 min interval, left for annealing and centrifuged for 5 min at 3,000 rpm.

The fluorescence-labelled arsonoliposomes were prepared in the same way by adding the fluorescent dye (Bodipy-DHPE or Rhod-DHPE) in the mixture of the lipids dissolved in the organic solvent at the very initial step of liposome preparation in 0.2% molar ratio of the total lipids used to prepare the liposomes. After centrifugation, the samples were passed through a Sephadex G50 column (1 mm×40 mm) to eliminate the free dye.

Liposome size distribution was measured at 25°C using photon correlation spectroscopy at a fixed angle of 90° using PCS (Malvern Instruments Ltd., Malvern, UK). Zeta potential was measured using Laser Doppler Anemometry by Malvern Zetasizer IV (Malvern Instruments Ltd., UK).

Liposome morphology was observed by transmission electron microscopy (JEOL JEM 1010, Jeol UK Ltd., Welwyn, UK) using 1% phototungstic acid as contrast agent.

Uptake Studies

Qualitative Liposome Uptake Studies

Uptake of arsonoliposomes by VC312R and 3T3 cells was studied qualitatively using Rhod-labelled liposomes. The cells were seeded overnight at 37°C on 13 mm coverslips at concentration of 2×10^4 cells/mL/well in 24-well plates and incubated with Rhod-labelled arsonoliposomes (200 μg/10^6 cells/mL/well) for 2 h. Then, the cells were fixed with 2% PFA and washed three times with PBS before mounting with a drop of mounting medium containing DAPI. The samples were examined on fluorescent microscope using Improvision Openlab software.

Quantitative Liposome Uptake Studies

The amount of arsonoliposome uptake by VC312R cells was studied using FACS analysis.

Cells were incubated with Bodipy-labelled arsonoliposomes (200 μg/10^6 cells/mL/well) at 37°C, for 15 and 30 min, 1, 2, 3, 4, 5, 12 and 24 h. Cells non-treated with arsonoliposomes were used as negative control to set the area of interest on the FACS analysis instrument. Following incubation, the medium was discarded and the cells washed twice with PBS to remove any liposomes that had not entered the cells. Then, the cells were fixed with freshly prepared 0.5% PFA solution in PBS for 15 min at room temperature in the dark. Then, the cells were washed twice with PBS, resuspended in PBS and transferred to appropriate tubes for the flow cytometry analysis. The samples were stored at 4°C in the dark and analysed the same day of the preparation.

Dose-dependent uptake was investigated by incubating the cells with Bodipy-labelled arsonoliposomes (100, 200, 400, 800 μg/10^6 cells/mL/well) for 2 h.

Uptake Inhibition Studies

The cells were incubated for 2 h with chlorpromazine (10 μg/mL) and filipin (5 μg/mL) as described by Rejman *et al*. (10). Then, the cells were incubated for 2 h with Bodipy-labelled arsonoliposomes at the concentrations of 200, 400 and 800 μg/10^6 cells/mL/well. Cells incubated in absence of inhibitors were used as control. All raw data were analysed using Weasel software (WEHI Biotechnology Centre).

Cytotoxicity Studies (MTT Assay)

Cell viability of both VC312R and 3T3 cells was evaluated using MTT assay. The MTT assay is based on the ability of viable cells to convert thiazolyl blue tetrazolium bromide solution to the blue formazan crystals in their

mitochondria (11). Briefly, the cells were seeded at a concentration of 5×10^4 cells/mL/well in 24-well plates and were incubated with liposomes at increasing concentrations (20 μg/mL to 1,000 μg/mL) for a time period from 1–4 days. The cells in the first three wells were incubated in absence of liposomes. Additionally, DMSO (0.5 mL/well) was used as positive control for cell death.

At the specified period of incubation time, 100 μl of MTT solution (concentration 5 mg/mL) was added to the wells and incubated for 2 h at 37°C. The blue formazan salts were dissolved in 100 μl of acidified isopropanol (0.33 μl HCl in 100 mL isopropanol), which was transferred to 96-well plates, and the absorbance was read on a microplate reader (Bio-Tec Instruments) at wavelength of 490 nm. Viability of the cells was calculated by comparing the number of viable cells in the liposome-treated wells to the non-liposome-treated cells.

Apoptosis Studies

Early Apoptosis Studies

VC312R cells were seeded in a 24-well plate at the concentration of 2×10^4 cells/mL/well and incubated with arsonoliposomes (200 μg/10^6 cells) in 1% serum containing medium at 37°C in an atmosphere containing 5% CO_2 for 2, 5 and 24 h. TNF-α 50 ng/mL was added as positive control for apoptosis induction, and DSPC/Chol plain liposomes at 200 μg/10^6 cells were used as negative control. Then, cells were washed with cold PBS and re-suspended in 200 μl ABB (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). After the addition of 10 μl of Alexa Fluor 488 annexin V conjugate, the cells were incubated at room temperature in the dark for 15 min. Then, the cells were washed with ABB and the coverslips mounted on the slides using a PI-containing mounting medium to stain the dead or apoptotic nuclei.

The same protocol was used to estimate the apoptotic effect of the arsonoliposomes on the fibroblasts.

Late Apoptosis and Necrosis Studies

Cells (2×10^4 /mL/well) were incubated with arsonoliposomes (200 μg/10^6 cells/mL) in 1% serum containing medium at 37°C in an atmosphere containing 5% CO_2 for 2, 5 and 24 h. TNF-α 50 ng/mL was added as positive control for apoptosis induction and DSPC/Chol plain liposomes (200 μg/10^6 cells) were used as negative control. Following incubation, the cells were washed with cold PBS and 10 μl of PI solution was added and the coverslips mounted on the slides using a DAPI-containing mounting medium.

Statistical Analysis

All the results have been expressed as the mean of at least three experiments ± standard error. One-way ANOVA was used for statistical analysis of the inhibition of the uptake studies and the viability of cells (either VCRs or 3T3s) treated with a certain liposomal formulation over the incubation time of 1–4 days. Two-way ANOVA was used to compare the viability of cells incubated with conventional liposomes *versus* APLs over the incubation period of 4 days. The confidence interval was set at 99%.

RESULTS

Characterisation of Liposomes

Arsonoliposomes and conventional, plain liposomes prepared with probe sonication were of spherical shape (Fig. 1) and similar size of approximately 90 nm (Table I). The liposome formulation polydispersity index was quite low, indicating a dispersion of a relatively narrow size distribution despite the fact that liposomes were prepared using a probe sonicator.

Liposome size should be small (under 200 nm) so the vesicles would avoid rapid blood clearance and travel through tissue fenestrae. As expected, arsonoliposomes exhibit more negative zeta-potential in comparison to plain liposomes (Table I), and this is attributed to the presence of OH-groups on the arsonic polar head.

Uptake Studies

Qualitative Studies

Qualitative uptake studies were carried out using Rhod-labelled arsonoliposomes. 200 μg/mL of arsonoliposomes were incubated for 2 h with either VC312Rs or 3T3s. Fig. 2 shows that arsonoliposomes were taken up by both cell lines.

Quantitative Studies

In these experiments, the cellular uptake of arsonoliposomes was determined by measuring the fluorescence intensity of the fluorescently labelled lipid, Bodipy-DHPE, which had been incorporated in the liposomal bilayer. The amount of liposomes taken up by the cells was measured using median of the fluorescence intensity of the samples.

Initially, the cells were incubated with arsonoliposomes at the concentration of 200 μg/10^6 cells/mL/well in order to explore the cellular uptake in response to the incubation time.

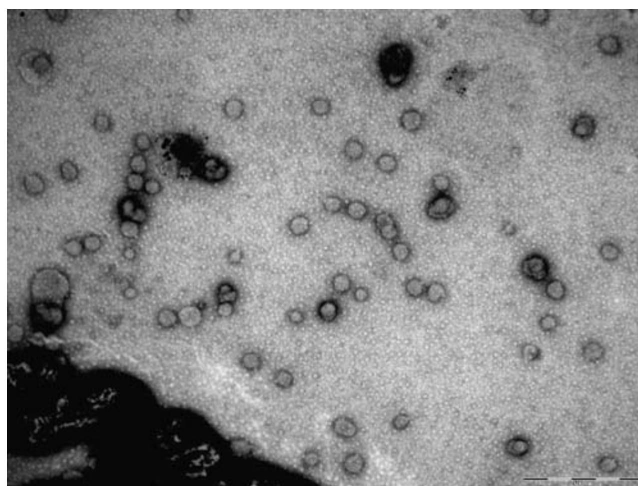


Fig. 1. TEM image of arsonoliposomes (× 50 k magnification). Bar: 500 nm.

Table I. Physicochemical Properties of Conventional Liposomes and Arsonoliposomes

Liposome type	Size (nm) (sd) [PDI]	Zeta-potential (mV) (sd)
DSPC/Chol	75.5 (2.41) [0.288]	−4.8 (2.4)
DSPC/C_{16}-As/Chol	87.0 (3.80) [0.271]	−36.23 (2.32)
DSPC/C_{16}-As/Chol in VCR culture medium	120.1 (2.85) [0.281]	−32.46 (1.86)

The results suggested that the uptake occurred at two plateaus (Fig. 3a). The first one seemed to have taken place within the first 3 h of incubation, while after 4–5 h the fluorescence noticeably increased and the second one was obtained between 5 and 12 h. Moreover, the fluorescence shifted to higher values 24 h later. It is important to highlight that arsonoliposomes were taken up rapidly by the cells, and the uptake increased as incubation time increased, too. These results show that there was a very high interaction of the arsonoliposomes with the cancer cells, and there was progressive increase in the level of fluorescent intensity concomitant with prolongation of incubation of the cells with the arsonoliposomes.

Furthermore, we investigated the effect of the lipid dose on the uptake by incubating the cells with arsonoliposomes (100, 200, 400, 800 μg/10^6 cells/mL) for 2 h. The plots, obtained with the method described before, show that the results can be significantly affected by the dose of arsonoliposomes used. The uptake seemed to have reached a plateau at a lipid concentration of 200 μg/10^6 cells/mL (Fig. 3b). However, at higher lipid concentrations (400–800 μg/10^6 cells/mL) the uptake increased quite significantly.

The results obtained from those experiments can be compared to the PGA-nanoparticle uptake by DAOY cells (12) and indicate that nano-scale particles can be taken up by different cell lines in the same pattern, which may involve similar mechanisms of endocytosis. Therefore, we investigated the mechanism of uptake in relation to lipid dose and incubation time.

Uptake Inhibition Studies

The mechanism of uptake was studied by incubating the cells with the arsonoliposomes in either the absence or the presence of two inhibitors using flow cytometry.

According to Rejman *et al.* (10), the internalization of particles with a diameter less than 200 nm involve clathrin-coated pits. CPZ is known to interact with clathrin and causes clathrin loss from the surface membrane (13). Therefore, we used CPZ to investigate any correlation between the arsonoliposome uptake and the clathrin-mediated endocytosis. Fig. 3c shows that the arsonoliposome uptake in presence of CPZ (10 μg/mL) decreased at low concentration (200, 400 μg/10^6 cells/mL/well) of lipid, while it was slightly enhanced at higher concentrations (800 μg/10^6 cells/mL). This result suggested that at least another mechanism is involved in the internalization of arsonoliposomes at a higher lipid concentration. Huth *et al.* (14) reported that caveolae-mediated endocytosis also plays an important role in liposome uptake. In order to confirm that arsonoliposomes are taken up also via this pathway, we studied the liposome uptake in presence of filipin (5 μg/mL), an inhibitor of the caveolae-mediated endocytosis (10, 15, 16). As shown in Fig. 3c, the uptake at lower concentrations remained unchanged, while it was inhibited at lipid concentration of 800 μg/10^6 cells/mL.

Our studies show that the arsonoliposome uptake is strongly inhibited after treatment with CPZ and filipin, and this inhibition is related to the arsonoliposome concentration.

Cytotoxicity Studies

The influence of various concentrations of either arsonoliposomes or conventional liposomes on both VC312R medulloblastoma cells and 3T3 normal fibroblast cells was studied using the MTT cell viability assay.

Cell viability was studied over 4 days of cell incubation with liposomes (Fig. 4). The results represent the means and standard errors around the means of three to four sets of experiments.

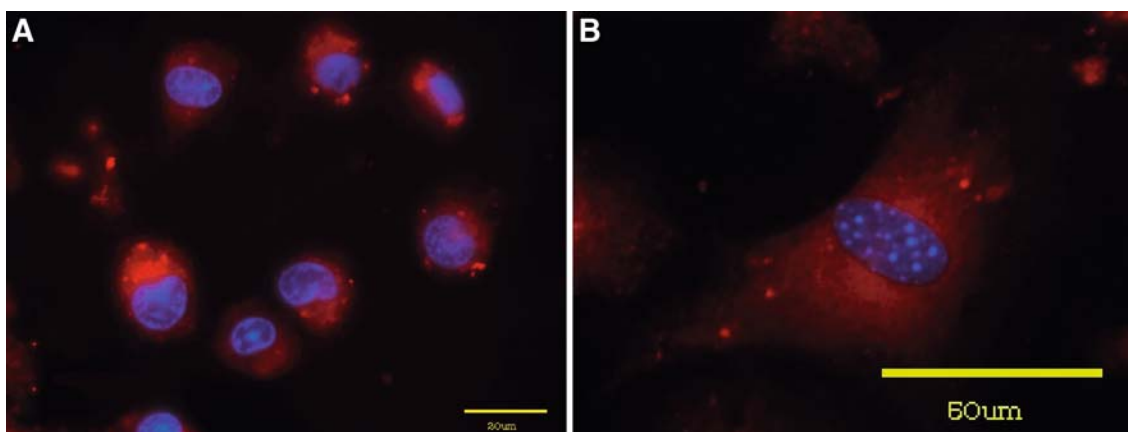


Fig. 2. Uptake of Rhod-labelled arsonoliposomes (*red*) after 2 h incubation by **A** VC312R and **B** 3T3 cells. Nuclei were stained *blue* by DAPI (× 100 magnification).

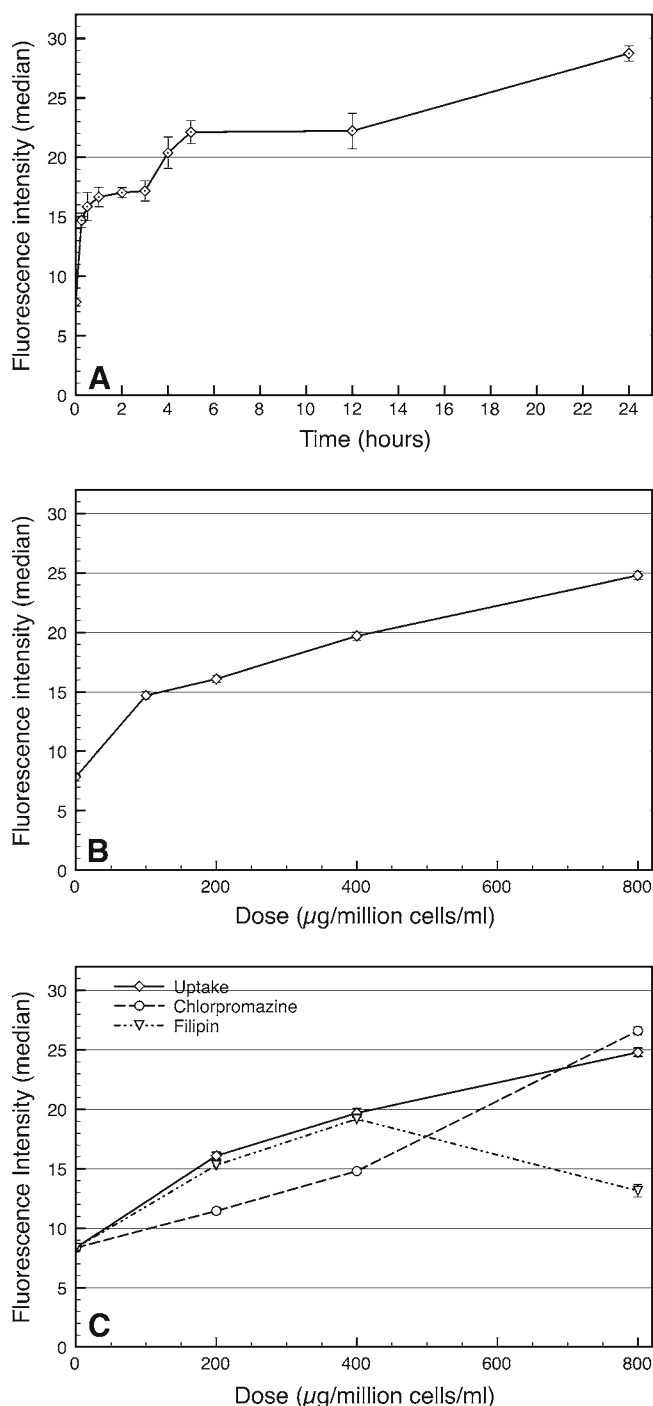


Fig. 3. Bodipy-labelled arsonoliposomes uptake by VC312R cells. **A** time dependence: VC312R cells were incubated with 200 μg/mL dose for different time intervals. **B** dose dependence: VC312R cells were incubated with different concentrations of arsonoliposomes for 2 h. **C** dose dependence in presence of inhibitors: VC312 cells were incubated with different concentrations of arsonoliposomes in presence of CPZ (10 μg/mL) and filipin (5 μg/mL). Mean ± SEM (n=3).

Fig. 4a and c show that plain liposomes were not toxic to any of the two cell lines tested over the 4-day period. This was expected, as DSPC and Chol are FDA-approved lipids without showing any toxicity and immunogenicity reactions. Also, they are lipids used in already licensed products in the market, such as Doxil® and Caelyx®.

On the other hand, the effect of arsonoliposomes on VC312R cells was significantly higher in comparison to 3T3 cells for the same number of days (Fig. 4b and d) (p<0.01). Fig. 4b shows clearly that as the liposome concentration increased, the cell viability decreased for all days studied, and the arsonoliposome influence was detrimental after 4 days (p< 0.01). For example, cell viability was approximately 74% after 1-day incubation with 200 μg/mL of arsonoliposomes, whereas it dropped to 25% after 4 days incubation with the same liposome concentration. It was obvious that the arsonoliposomes were less toxic to the normal fibroblast cells under identical experimental conditions as illustrated in Fig. 4d. For instance, during the second day of the experiment, the percentage viability of the VC312R cells following the addition of 400 μg/mL of arsonoliposomes was only 36% compared to 69% of the 3T3 cells at the same liposome concentration (p<0.01). Therefore, it was concluded that the arsonoliposomes were more toxic to the cancer cells than to the non-cancer 3T3 cells. This outcome agrees with previously reported data by Gortzi *et al.* on the toxicity of arsonoliposomes on two non-malignant cell lines (HUVEC, RAME) and three cancer cell lines (C6, HL-60, Gh3) (8). DMSO used as positive control for cell death reduced the viability of both cell lines to approximately 0% over the incubation time (1–4 days).

The fact that arsonoliposomes interact highly with VC312R cells (Fig. 4b), compared to 3T3 cells (Fig. 4d), may explain the notion that the demonstrated differential toxicity of arsonoliposomes possibly is a reflection on a diverse mode and extent of interaction of these liposomes with cancer cells compared to normal cells (9). Previous experiments have shown that arsonoliposomes can induce morphological changes on the cancer cells due to cell apoptosis (8,9).

A possible mechanism for arsonoliposome toxicity involves the glutathione redox system, which is highly expressed in cancer cells (17,18). Furthermore, arsenic induces apoptosis due to a number of other mechanisms like destabilization of mitochondrial membrane and activation of caspases (19).

In addition, it has been reported elsewhere that in acute promyelocytic leukaemia, arsenic in the form of arsenic trioxide targets promyelocyte proteins and results in degradation of the promyelocytic leukemia locus/retinoic acid receptor (PmL/RAR) fusion protein, while in the non-APL tumours, arsenic causes destruction of only the PmL protein (20). On the other hand, previously reported data have concluded that arsenic-induced apoptosis of the cells is independent of PmL or PmL/RAR (21).

Based on the specific toxicity of arsonoliposomes towards the cancer cells, modification of the surface of the arsonoliposome formulation to achieve specific targeting to cancer cells would be the next step for promising cancer therapy. So, further studies are essential to clarify the exact mechanism of interaction of the arsonoliposomes with the cancer cells and to explain differences in the ways these liposomes behave with different cell types.

Apoptosis Studies

Early Apoptosis Studies

Gortzi *et al.* (8) have reported that arsonoliposomes can cause apoptosis in cancer cells. In our experiments, we

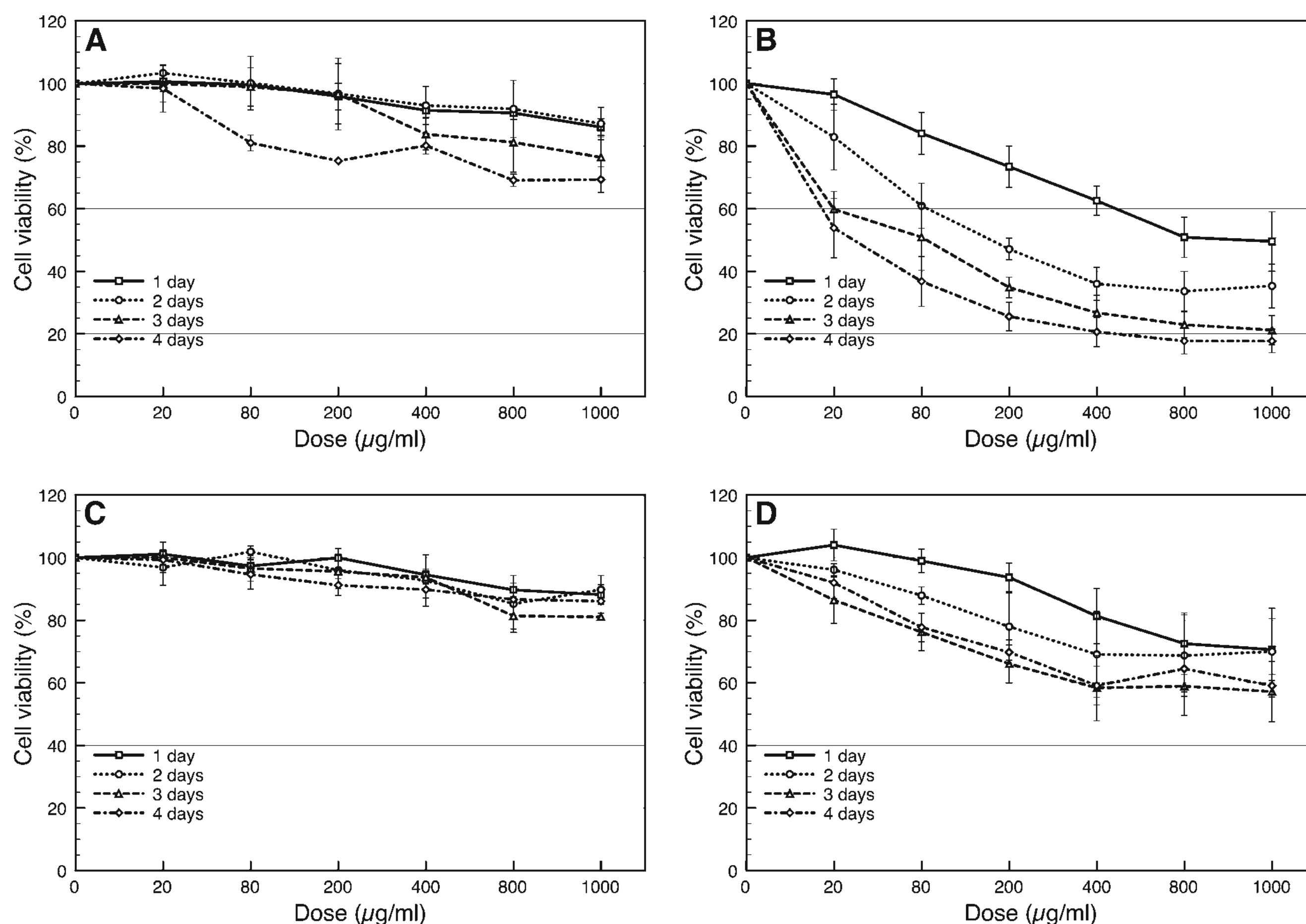


Fig. 4. MTT assay on VC312R (**A**, **B**) and 3T3 (**C**, **D**) cells using DSPC liposomes (**A**, **C**) and arsonoliposomes (**B**, **D**) respectively. Mean ± SEM (n=3).

observed the stage of the apoptotic effect in terms of time as well. Apoptosis was induced using TNF-α, while DSPC/Chol plain liposomes were used as negative control, since they have been proven not toxic to cancer cells. The cells were incubated for 2, 5 and 24 h with arsonoliposomes at the concentration of 200 μg/10^6 cells/mL. The samples were incubated with Alexa Fluor 488 annexin V fluorescent conjugate; in the apoptotic cells, PS is translocated to the outer leaflet of the plasma membrane, and it is exposed to the extracellular environment (22). Annexin V is a calcium-dependent phospholipid binding protein that has a high affinity for PS (23). In order to create the proper condition for annexin V to bind the PS, a calcium-containing buffer was needed. A PI-containing mounting medium was used to investigate the morphology of the nuclei. This fluorescence dye stains only the late apoptotic or necrotic nuclei, while the early apoptotic nuclei are not affected. Thus, by the double-staining technique it is possible to distinguish the healthy cells (not stained) from the early apoptotic (green) and late apoptotic/necrotic cells (green and red).

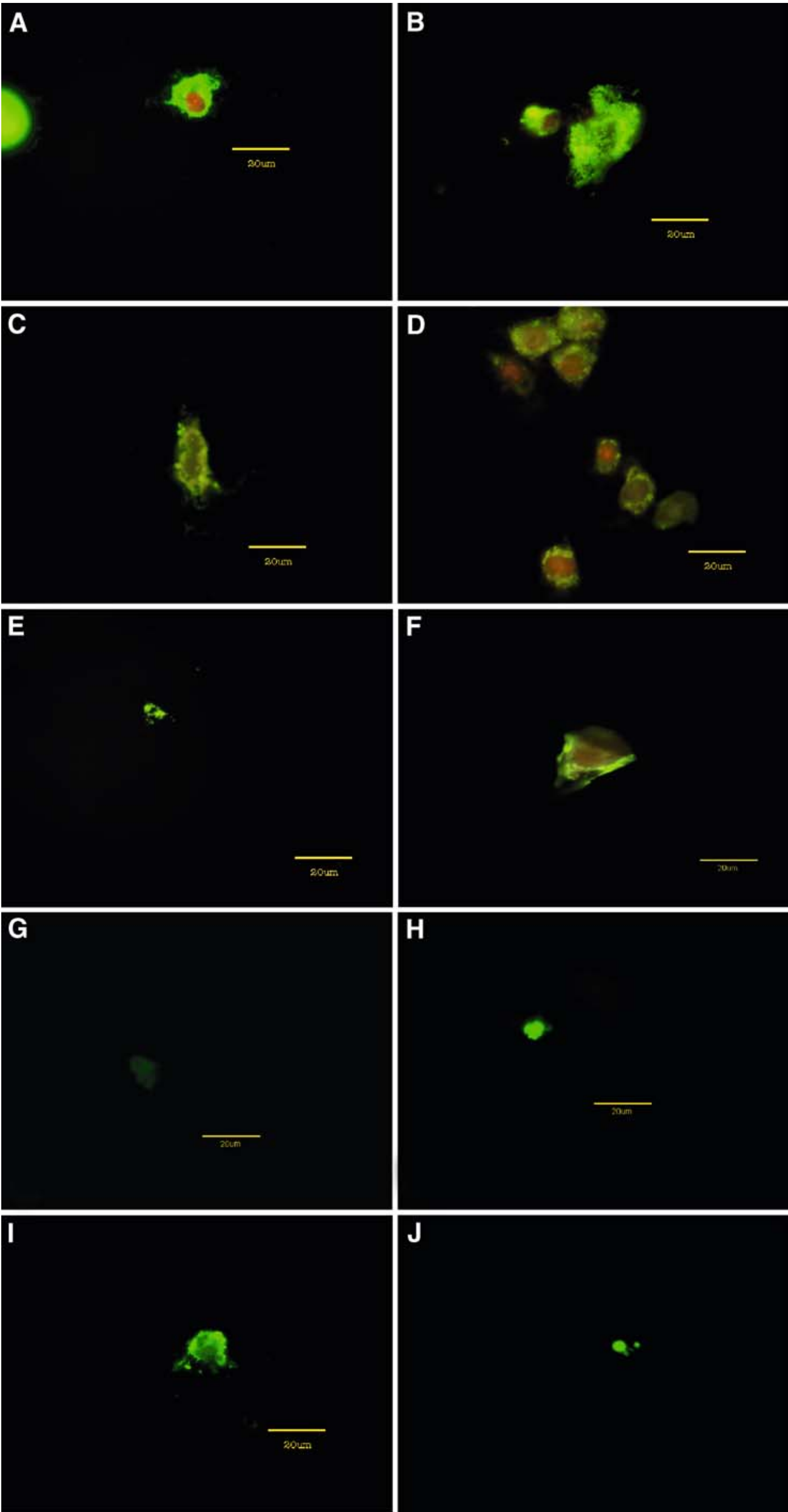
Fig. 5e shows that DSPC/Chol liposomes are not toxic to the cancer cells. Fig. 5b–d shows the progress of cell apoptosis in presence of arsonoliposomes for incubation times 2 to 24 h. After 2 h, the cells did undergo early apoptosis, while after 5 h cells showed signs of late apoptosis. That process was completed after 24 h as most of the population was double stained, which showed necrotic cells.

Furthermore, we tested whether arsonoliposomes were not toxic towards 3T3 cells as indicated by the data from the MTT assay. The fibroblasts were treated in the same way as VC312R cells. Not surprisingly, no sign of apoptosis was observed after 2 and 5 h of incubation with arsonoliposomes (Fig. 5g, h), while only a few cells underwent apoptosis after 24 h (Fig. 5i). No red staining was detected, suggesting that arsonoliposomes did not induce necrosis to 3T3 cells (Fig. 5g–i).

Late Apoptosis and Necrosis Studies

The studies on cell death induction by arsonoliposomes led us to investigate the morphological changes related to apoptosis, particularly the chromatin modification, which is characteristic for late apoptosis. To get information on the kind of cell death, the cells were incubated for 2, 5 and 24 h

Fig. 5. Fluorescence microscope images of VC321R cells after incubation with **A** TNF-α **B** 200 μg/mL of arsonoliposomes for 2 h, **C** 200 μg/mL of arsonoliposomes for 5 h, **D** 200 μg/mL of arsonoliposomes for 24 h and **E** 200 μg/mL of DSPC liposomes for 5 h. Fluorescence microscope images of 3T3 cells after incubation with **F** TNF-α **G** 200 μg/mL of arsonoliposomes for 2 h, **H** 200 μg/mL of arsonoliposomes for 5 h, **i** 200 μg/mL of arsonoliposomes for 24 h and **J** 200 μg/mL of DSPC liposomes for 5 h. Dead cell nuclei are stained *red* by PI, early apoptotic cells are stained *green* (Annexin V). ▶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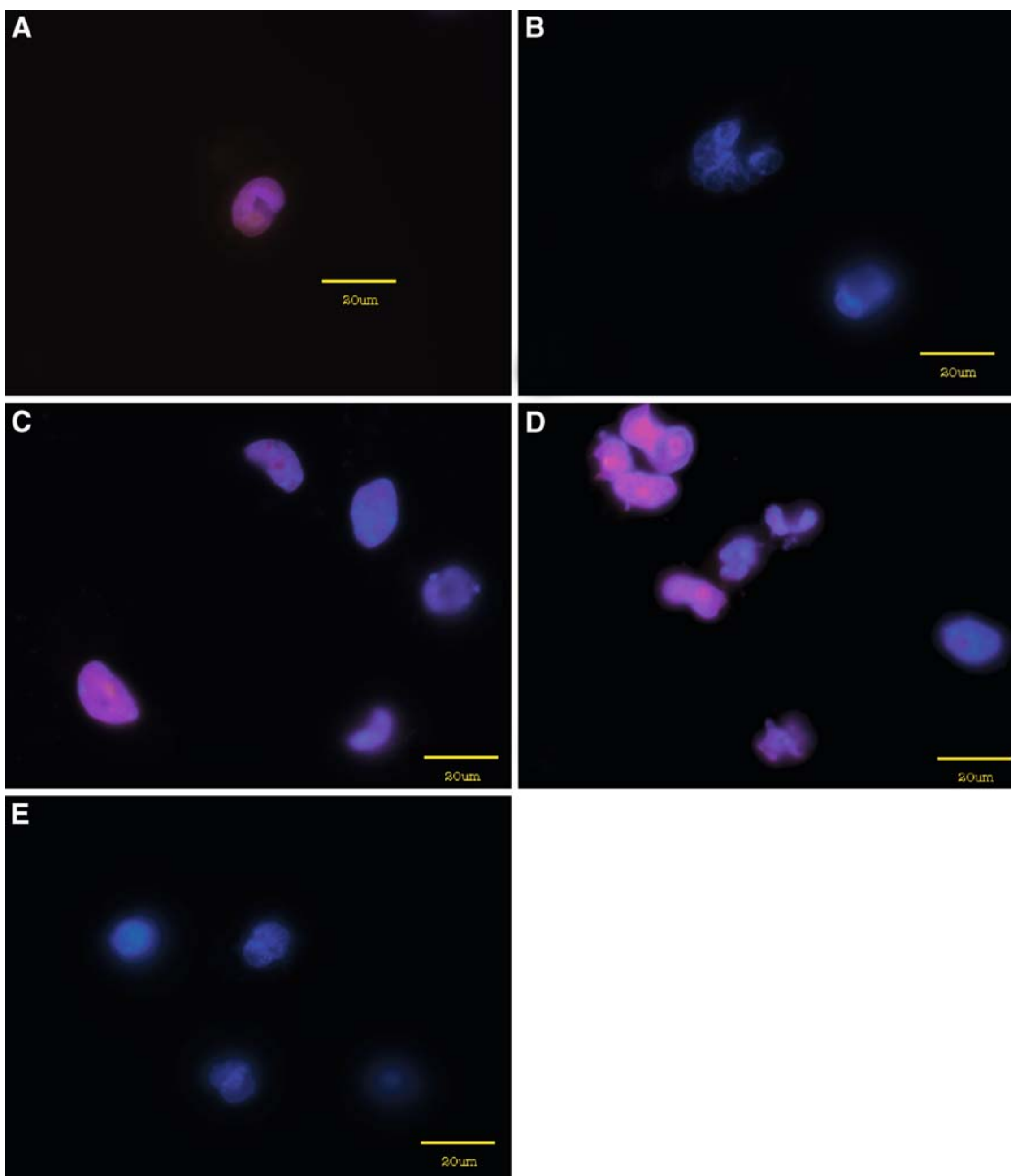

Fig. 6. Fluorescence microscope images of VC321R cells after incubation with **A** TNF-α **B** 200 μg/mL of arsonoliposomes for 2 h, **C** 200 μg/mL of arsonoliposomes for 5 h, **D** 200 μg/mL of arsonoliposomes for 24 h and **E** 200 μg/mL of DSPC liposomes for 5 h. Nuclei are stained *blue* by DAPI, dead cells nuclei are stained *red* by PI.

with arsonoliposomes at the concentration of 200 μg/10^6 cells/mL. TNF-α and DSPC/Chol liposomes were used as positive and negative control for cell apoptosis/necrosis, respectively (Fig. 6a, e). We used DAPI (blue) to analyze the morphology of the nuclei and PI (red) to identify the necrotic cells.

Fig. 6 shows that the chromatin started condensing after 2 h of incubation (Fig. 6b), while after 5 h most of the cells were double stained and the nuclei appeared "bubbly" (Fig. 6c); this clearly indicates that the cells were in advanced apoptotic stage and would have undergone necrotic death. After 24 h of incubation, cells were fragmented and all of them were intensely double stained (Fig. 6d). As expected, DSPC/Chol liposomes did not affect cell viability (Fig. 6e).

DISCUSSION

In our studies, we sought to investigate the effect of the arsonoliposomes towards a brain tumour cell line and evaluate their potential as a novel class of drug delivery system and/or anticancer agent. It is worth emphasizing that liposomes have been so far used as carriers for anticancer agents. In our case, the carrier (arsonoliposome) itself exhibits

cytotoxic activity as it has been shown by previous and current studies using a variety of cancer (HeLa, $HepG_2$, HL-60, VC321Rs) and normal cells (HUVEC, RAME, 3T3) (8,9).

The particular liposomal composition was chosen based on data already published on the effect of those liposomes on cell lines. Gortzi *et al.* showed that the C_{16}-arsonoliposomes exhibited higher cytotoxicity towards the cancer cells in comparison to normal cells (8, 9). The authors studied the effect of arsonoliposomes composed of arsonolipids of various acyl chain lengths (C_{12}, C_{14}, C_{16}, C_{18}) on the cell viability of normal and cancer cells. C_{16}-arsonoliposomes exhibited best performance in comparison to C_{14}-containing ones. C_{12}- and C_{18}- liposomes tended to aggregate (indicating low stability), while the former formulation was highly toxic to normal cells. The same study showed that the cell uptake of the C_{16}-As/PC/Chol (8/12/10) was higher among the other liposomal compositions tested (C_{16}-As/PC/Chol: 3/17/10 molar ratio, PC/Chol: 1/1 molar ratio). Additionally, DSPC increased the liposomal membrane stability in serum as demonstrated by relevant studies by Haikou *et al.* (18). Therefore, the formulation with composition C_{16}-As/DSPC/Chol (8/12/10 molar ratio) has shown the most promising results for further investigation in *in vitro* and *in vivo* systems.

The first set of our experiments was focused on the evaluation of the liposome uptake by the VC312R cells in a monolayer. Using the fluorescence microscope and FACS techniques, we have demonstrated that the internalization of arsonoliposomes actually took place in a time-dependent and dose-dependent pattern. The results obtained from the quantitative analysis were consistent with the data obtained from a similar set of experiments carried out using PGA-nanoparticles on another medulloblastoma cell line (DAOY) and suggested that similar mechanisms can be involved in the uptake of both drug delivery systems (12). Moreover, the profile of the internalization graphs suggested that arsonoliposomes could enter the cells via two or more pathways. We showed that the clathrin-coated pits were involved in the endocytosis at low concentrations of arsonoliposomes, while the caveolae-mediated mechanism was the prominent mechanism of uptake when the concentration increased. According to Rejman and coworkers (10), caveolae-mediated endocytosis is a size-dependent mechanism involved in the internalization of particles larger than 500 nm. Arsonoliposome size, measured after 2 h of incubation in VC312R medium at 800 μg/mL, was only 120 nm with low polydisperisty index of 0.281 (Table I), substantially lower than the threshold (500 nm) required for activation of caveolar uptake. Thus, further studies are needed in order to fully understand the mechanisms that underlie arsonoliposome internalization via caveolae-mediated endocytosis.

The distinctive feature of arsonolipid-containing liposomes is the high selectivity towards the cancer cells in terms of toxicity, which makes them good candidates as anticancer agents. In our cytotoxicity studies, arsonoliposomes showed to be significantly effective against VC312R cells; after 4 days of incubation the viability of the latter was 25% using 200 μg/mL of lipid concentration. At the same time, arsonoliposomes showed only a moderate effect towards fibroblasts: in this case the cell viability was approximately 70%. Indeed, arsonoliposomes induced apoptosis relatively early (after 2 h at 200 μg/mL) and caused necrosis to most of the cells after 24 h of incubation. Furthermore, arsonoliposomes did not seem to induce apoptosis to fibroblasts (3T3 cells). Of course the cell interaction with the arsonoliposomes is a very important factor because As (V) is reduced to As (III) inside the cells, thus cell death is induced (9, 24). *In situ*, liposomes will have to diffuse through tumour extracellular matrix proteins to become available to the cancer cells. Thus, arsonoliposome penetration will need to be studied further in 3-D aggregate models of VC312R grown in culture.

In conclusion, we demonstrated that arsonoliposomes exhibited selective toxicity towards the medulloblastoma cell line in comparison to the fibroblasts (normal cells) tested. Induction of apoptosis due to the presence of arsonoliposomes was time-dependent, while uptake relied on both dose and time. Interestingly, the arsonoliposome uptake seemed to take place via clathrin-coated pits at low lipid concentrations, while caveolae-mediated endocytosis was the prominent mechanism at higher lipid dose. Moreover, normal cells were not affected by the treatment, and thus arsonoliposomes may represent a potentially useful strategy for cancer treatment with limited side effects to normal tissues.

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