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In-vitro antileishmanial and trypanocidal activities of arsonoliposomes and preliminary in-vivo distribution in BALB/c mice

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Abstract

We have studied the antiprotozoal activity of some recently prepared and characterized arsonoliposome formulations. Plain arsonoliposomes and phosphatidylcholine arsonoliposomes prepared with palmitoyl- (C16) or lauroyl-(C12) acyl side chain arsonolipids showed in-vitro antileishmanial activity after a 72-h incubation period against wild-type promastigote forms of *Leishmania donovani*. The IC50 values ranged from 0.40 to 11.6 μM arsonolipid. Interestingly, all preparations tested were found to be significantly more potent against amphotericin B- or miltefosine-resistant promastigote forms of *L. donovani*, with IC50 values ranging between 0.21- and 2.33-μM arsonolipid. When tested in-vitro against *Trypanosoma brucei brucei*, all arsonoliposome formulations were found to have anti-trypanosomal activity after a 24-h incubation period. The fact that the corresponding arsonolipids (dissolved in dimethyl sulfoxide) were found not to be potent against the *Leishmania* promastigotes or the trypanosomes tested suggested that the formation of liposomes possibly influenced the mode of interaction between the active lipid and the parasites modulating their potency. In addition, a preliminary in-vivo study in BALB/c mice was performed for the initial evaluation of the biodistribution of arsonoliposomes. The accumulation of arsenic in the BALB/c mouse liver in relatively high amounts was an additional advantage of this approach for anti-protozoal therapy, especially for visceral leishmaniasis where parasites are located mainly in the liver.

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Introduction

Infections with parasitic protozoa have always been a problem for the developing world and with the ease of international travel they are becoming of greater importance elsewhere (Barrett 1999). Leishmaniasis and African trypanosomiasis are major parasitic diseases for which the classical drugs are toxic or of low activity and their use is commonly responsible for drug resistance in the field. Thus, for the treatment of visceral leishmaniasis, the resistance to pentavalent antimonial drugs is increasing, while amphotericin B and pentamidine have toxic side effects in therapeutic doses (Balana-Fouce et al 1998). Trivalent arsenical agents such as melarsoprol (Arsobal), which is used to treat late-stage African trypanosomiasis, is also responsible for toxicity and drug resistance (Denise & Barrett 2001). Therefore there is an urgent need for new antiprotozoal therapeutic agents.

Recently, we prepared and characterized arsonolipid-containing vesicles, the arsonoliposomes (Fatouros et al 2001). Arsonolipids (Figure 1) are arsonate containing lipids, analogues of phosphonolipids with arsenic(V) replacing phosphorus(P), that have been previously synthesized and characterized (Tsivgoulis et al 1991a, b; Serves et al 1992, 1993). The vesicles prepared by these lipids have been demonstrated to possess differential cytotoxicity against various cancer cell types in culture (Gortzi et al 2002). The acyl chain length of the arsonolipid used for vesicle preparation has an effect on several vesicle physicochemical properties (membrane integrity, tendency for aggregation etc.) as well as on their cancerostatic efficiency and undesirable normal cell toxicity (Gortzi et al 2003). Since many arsenic(III) and arsenic(V)-containing compounds are known to demonstrate antiprotozoal activity, it is interesting to evaluate the antiprotozoal activity of arsonoliposomes against *Leishmania* and *Trypanosoma*, to

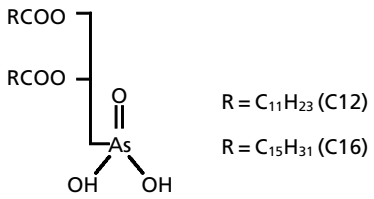

Figure 1 Structure of arsonolipids.

explore their potential for relevant applications. The fact that the arsonolipids are formulated as arsonoliposomes is an additional potential advantage for antileishmanial therapy, since liposomes varying in composition and size accumulate within the liver (Gregoriadis 1995), where parasites replicate after intravenous or intraperitoneal administration (Chang & Dwyer 1976).

In this study we have examined the trypanocidal and the antileishmanial effect of different types of arsonolipid-containing liposomes i.e. liposomes composed of arsonolipid and cholesterol (termed plain arsonoliposomes) and liposomes composed of arsonolipid mixed with phosphatidylcholine and cholesterol (termed mixed arsonoliposomes). Two arsonolipids with different acyl side chains were evaluated, since it has been demonstrated that the acyl chain length of the arsonolipid affects the biophysical (Fatouros et al 2001) and anticancer properties of the arsonoliposomes formed (Gortzi et al 2003). In addition, a preliminary in-vivo study for the initial evaluation of the biodistribution of arsonoliposomes was performed in BALB/c mice. This was to check the validity of the assumption that arsonoliposomes as vesicles will most probably accumulate in the liver after in-vivo administration.

Materials and Methods

Reagents

Phosphatidylcholine (PC) (grade 1) was purchased from Lipid Products (Nutfield, UK) and was found to be pure by TLC analysis. The *rac*-arsonolipids (2,3-diacyloxypropylarsonic acids) (Figure 1) with a lauroyl (C12) or a palmitoyl (C16) side chain were synthesized and characterized as described previously (Tsivgoulis et al 1991a, b; Serves et al 1992, 1993).

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

Liposome preparation and characterization

Liposomes containing plain arsonolipid (C12/Chol or C14/Chol, 20:10 mol/mol, L2 or P2, respectively; defined as plain arsonoliposomes) or mixtures of arsonolipid with PC (C12 or C16/PC/Chol, 8:12:10 mol/mol/mol, L1 or P1, respectively; defined as mixed arsonoliposomes) were prepared by sonication after initial dispersion of the lipids in phosphate-buffered saline (PBS), which was achieved by magnetically stirring at 70 °C (above the arsonolipid transition temperature). In all cases cholesterol was included in the liposomes prepared (at a 2:1 lipid/Chol, mol/mol ratio), as this has been found to increase membrane integrity (Fatouros et al 2001). Sonication was performed (two 5-min runs separated by a 10-min interval) using a probe type sonicator (Sonics and Materials, UK). After sonication the liposomes were centrifuged for 10 min at 10 000 rev min^{-1} to remove titanium pieces and liposome aggregates which could be present in the samples, and left undisturbed for 1 h for annealing of any structural defects (at 70 °C i.e. above the arsonolipid transition temperature).

After preparation the liposomes were filtered with a 0.22-μm filter (Waters). The lipid content of the samples was routinely determined using a colorimetric technique, the Stewart assay (Stewart 1980), which is widely applied for phospholipids. This assay was found to detect arsonolipids (at the high concentrations of the initial dispersions). In brief, liposome samples (20 μL) were vortexed with 2 mL of a solution of ammonium ferrothiocyanate (0.1 M) and 2 mL chloroform. The OD-485 nm of the chloroform phase was measured and the lipid concentration of samples was calculated by comparison with the standard curve (prepared with known concentrations of phosphatidylcholine).

As reported by Gortzi et al (2002), to be sure that all the amount of arsonolipid used during liposome preparation was incorporated in the liposomes, the arsenic content of some samples was determined by EDXRF spectroscopy (Energy Dispersive X-ray Fluorescence). An XRF Instrument (QuanX, TN Spectrace) with a thick (0.13 mm) Pd filter, equipped with a solid state Si(Li) liquid nitrogen cooled detector and an air cooled (with a side window Bremsstrahlung Rh target) X-ray tube (50 W) was used. After measuring the arsenic 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. Sonicated arsonoliposomes have been found to remain stable (in respect to physical stability – aggregation) during storage at 5 °C, for one month at least (Gortzi et al unpublished results). No aggregation was observed in any of the arsonoliposome samples at the time they were used in the experiments.

The liposomes prepared were characterized by measuring their size distribution and surface charge by photon correlation spectroscopy (Fatouros et al 2001; Gortzi et al 2002). In brief, liposome dispersions were diluted with filtered PBS 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 vesicle ζ-potential was calculated directly from the Helmhotz-Smoluchowski equation.

Biological evaluation

In-vitro antileishmanial activity

Four strains were tested in this study. *Leishmania donovani* (MHOM/IN/80/DD8) wild-type and amphotericin B-resistant promastigotes were cultured in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% foetal calf serum (FCS) and 50 μg mL^{-1} gentamicin at 26 °C in a dark environment. *L. donovani* (MHOM/ET/67/L82) wild-type and hexadecylphosphocholine (HePC)-resistant promastigotes were cultured in M199 medium supplemented with HEPES (400 mM), adenosine (100 μM) and haemin (0.1% of 25 μg mL^{-1} stock) (Sigma, Saint-Quentin Fallavier, France) and 10% heat-inactivated FCS at 26 °C in a dark environment. The antileishmanial screening was performed by the method described by Mbongo et al (1997). The screening was performed in flat-bottomed 96-well plastic tissue culture trays maintained at 26 °C in an atmosphere of 95% air/5% CO_2. Promastigote forms from a logarithmic phase culture were suspended to yield 10^6 cells mL^{-1} after haemocytometer counting. Each well was filled with 100 μL of the parasite suspension, and plates were incubated at 26 °C for 1 h before drug addition. The arsonolipids to be tested (C12 and C16) were dissolved in DMSO and then added to each well. DMSO at up to 2% (v/v) had no effect on parasite growth. Each concentration was screened in triplicate. The viability of promastigotes was checked using the tetrazolium-dye (MTT) colorimetric method.

The MTT Cell Proliferation Assay is a colorimetric assay system, which measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. After incubation of the cells with the MTT reagent, a detergent solution was added to lyse the cells and solubilize the coloured crystals. The samples were read using an ELISA plate reader at a wavelength of 570 nm. The amount of colour produced was directly proportional to the number of viable cells. The results were expressed as the concentrations inhibiting parasite growth by 50% (IC50) after a three-day incubation period.

In-vitro trypanocidal activity

Trypanosoma brucei brucei GVR 35, kindly supplied by Prof. F. W. Jennings (Glasgow, UK) was used for the in-vitro screening.

The test was conducted according to the method described by Loiseau et al (2000). Briefly, the bloodstream forms of *T. brucei brucei* GVR were maintained in-vitro without loss of infectivity for 48 h in the dark at 37 °C in a 5% CO_2 atmosphere. Screening was performed in 96-well tissue culture plates in a volume of 200 μL containing 2×10^4 parasites, in minimum essential medium (Gibco BRL), and the compounds to be tested (arsonolipids diluted in H_2O–DMSO). Drug concentrations were evaluated in triplicate. The minimum effective concentration (MEC) was defined as the minimum concentration at which no motile parasite was observed microscopically.

Prelinimary in-vivo study for the evaluation of the biodistribution of arsonoliposomes in BALB/c mice

Four female BALB/c mice (mean weight 19.5 ± 1.2 g) were obtained from the University Hospital animal facility (Patras, Greece), housed in an approved animal facility and maintained according to the National Institutes of Health "Guideline on the Care and Use of Laboratory Animals". The animals were provided with Rodent Chow and water was freely available. Mice were intraperitoneally injected with 200 μL of the P2 (C16/PC/Chol 8:12:10 (mol/mol/mol)) arsonoliposome preparation (corresponding to 0.54 mg arsonolipid). Five hours post-injection the mice were killed, and the livers, kidneys, spleens, blood, intestines, stomachs, lungs and carcass (plus skin) were removed and weighed. The arsenic content of each tissue sample was measured by placing the sample in the centre of an appropriate sample holder and applying the XRF technique described above. The arsenic content of each tissue was calculated as the mean percent of total arsenic measured in all tissues taken from each animal.

Statistical analysis

In the physicochemical characterization experiments the values calculated were mean values from at least five measurements of two independent samples. In the in-vitro antileishmanial activity evaluation all results were expressed as 50% inhibitory concentration by linear regression analysis (mean ± s.d. from three independent experiments). The values of 95% confidence limits were calculated and the P values were calculated using the Student's t-test.

Results

The physicochemical characteristics of the arsonoliposomes are presented in Table 1. The mean diameter of the vesicles ranged from 102.5 to 110.8 and 72.6 to 90.1 nm, for the plain arsonoliposomes and the mixed

Table 1 Mean diameter and ζ-potential values of the arsonoliposomes prepared. Liposomes containing plain arsonolipid (C12 or C16/Chol 20:10, mol/mol; L2 and P2, respectively) or mixtures of arsonolipid with PC (Ars/PC/Chol 8:12:10) (L1 and P1, respectively) were prepared by sonication. Measurements were performed as described in Materials and Methods immediately after vesicle preparation.

Lipid composition	Diameter (nm)	ζ-Potential (mV)
L1 (C12/PC/Chol, 8:12:10)	72.6 ± 8.4	−42.0 ± 2.8
L2 (C12/Chol, 20:10)	102.5 ± 8.5	−63.5 ± 6.3
P1 (C16/PC/Chol, 8:12:10)	90.1 ± 6.6	−50.3 ± 1.0
P2 (C16/Chol, 20:10)	110.8 ± 8.7	−69.5 ± 2.3

Each value is the mean ± s.d. of five subsequent measurements from at least two different samples.

with PC arsonoliposomes, respectively. The surface charge of all the vesicles was highly negative ranging from −63.5 to −69.5 and −42.0 to −50.3 mV, for the plain and the mixed with PC arsonoliposomes, respectively. As previously observed (Fatouros et al 2001; Gortzi et al 2002), the arsonoliposome mean diameters and ζ-potential values (absolute values) increased significantly ($P < 0.05$) when the arsonolipid content of the arsonoliposomes increased. In accordance with previous measurements, these values were not significantly affected ($P > 0.05$) by the arsonolipid (C12 or C16) used for arsonoliposome preparation (differences between formulations L1 and P1 as well as L2 and P2 were not significant ($P = 0.057$ and $P = 0.3$, respectively)).

The results of the in-vitro antileishmanial and trypanocidal activities of the arsonolipids (C12 and C16) used for liposome preparation (that was tested after dissolving the lipids in DMSO), and for the different types of arsonoliposomes studied, are presented in Table 2. Considering the antileishmanial activity, the parasites were observed 3 h after treatment to detect a possible rapid effect, but no activity was found at this time. However, as seen in Table 2, antileishmanial activity was observed after 72 h for all arsonoliposomes, against both *Leishmania* strains tested. In addition, two drug-resistant lines of *Leishmania* were tested. The first one was resistant to amphotericin B and the second one was resistant to hexadecylphosphocholine (HePC = miltefosine), a new drug in development. It was clear from the results that the resistant strains were significantly more sensitive to arsonoliposomes than the wild types.

When comparing the antileishmanial activity of the arsonoliposomes prepared using the two different arsonolipids (L1, L2 and P1, P2), it was concluded that arsonoliposomes prepared using the C16 arsonolipid (P1 and P2) were approximately two-times more active than the corresponding arsonoliposomes prepared using the C12 arsonolipid (L1 and L2), for all the strains tested ($P < 0.05$). The one exception was the amphotericin B-resistant strain, for which both types of arsonoliposomes displayed similar activity ($P \gg 0.05$).

Considering the arsonoliposome membrane lipid composition, the antileishmanial activity results revealed that for almost all the strains studied, mixed arsonoliposomes (L1 and P1), which contained PC in their membranes, were approximately 2- to 5-times (in one case) more active compared with the corresponding plain arsonoliposomes (L2 and P2) ($P < 0.05$).

It was concluded that after 24-h incubation, all arsonoliposome samples showed a trypanocidal activity against *T. brucei brucei* GVR 35 with MEC (minimum effective concentration) values ranging from 0.20 to 0.87 μM. Again the arsonoliposomes prepared using the C16 arsonolipid (P1 and P2) had more than two-times increased activity ($P < 0.05$) compared with the other arsonoliposome types tested (L1 and L2). However in this case the liposomal membrane composition (plain or mixed arsonoliposomes) did not seem to have any effect on their activity ($P \gg 0.05$).

To have some proof that after arsonoliposome administration a high proportion of the active moiety will be located in the liver (where parasites replicate), a preliminary in-vivo distribution study was carried out. Although the XRF analytical technique was not sensitive enough to measure the arsenic content of all tissues (data not shown), more than 15% of the total arsenic determined in all the body was found in the liver. This can serve as good preliminary proof that, as anticipated, arsonoliposomes accumulate in significant amounts in the liver after in-vivo administration.

Discussion

One of the first conclusions to be drawn from the results (Table 2) was that the formulation of arsonolipids in the form of arsonoliposomes was a basic prerequisite for any antiprotozoal activity. Indeed, when the arsonolipids used for arsonoliposome preparation (C12 and C16) were tested as such (dissolved in DMSO), their activity was very low in all cases of parasites studied (more than

Table 2 In-vitro antileishmanial (IC50[a], μM) after 72 h incubation, and trypanocidal (MEC[b] μM) after 24 h incubation, activities of the arsonoliposomes (L1, L2, P1 and P2 arsonoliposome compositions) and arsonolipids (C12 and C16) tested. Activity of arsonoliposomes is expressed as arsonolipid concentration (μM) of each liposomal preparation tested. Activity of arsonolipids is expressed as $\mu g\,mL^{-1}$, and was evaluated after dispersion in DMSO or DMSO and H_2O, as described in Materials and Methods.

Formulation tested Parasite	C12	C16	L1 (C12/PC/Chol, 8:12:10)	L2 (C12/Chol, 20:10)	P1 (C16/PC/Chol, 8:12:10)	P2 (C16/Chol, 20:10)
L. donovani DD8 wild type	>100	>100	11.60 ± 1.41	9.25 ± 0.78	4.72 ± 0.54	5.88 ± 0.67
L. donovani DD8 amphotericin B-resistant	>100	>100	0.87 ± 0.06	2.33 ± 0.15	0.81 ± 0.06	1.93 ± 0.09
L. donovani L82 wild type	>100	>100	0.88 ± 0.05	2.31 ± 0.20	0.40 ± 0.03	1.42 ± 0.17
L. donovani L82-HePC-R[c]	>100	>100	0.43 ± 0.03	1.70 ± 0.15	0.21 ± 0.03	0.97 ± 0.08
T. brucei brucei	>100	>100	0.87	0.58	0.20	0.24

[a]IC50: 50% growth inhibiting concentration. [b]MEC: minimum effective concentration. [c]HePC-R: hexadecylphosphocholine (miltefosine)-resistant. Each value is the mean ± s.d. from three independent experiments.

12-times lower compared with the activity of the less active liposome preparation).

When the antileishmanial activity of arsonoliposomes was considered, the arsonolipid used for liposome preparation, as well as the arsonoliposome membrane composition, seemed to have a significant impact on activity values. More analytically, when considering the effect of the arsonolipid, it was obvious from the results that in most cases the activity of arsonoliposomes containing the C16 arsonolipid (P1 and P2) was significantly higher ($P < 0.05$). This may be connected to the fact that the parasite plasma membrane is rich in palmitoyl moieties and the presence of palmitoyl residues on arsonoliposomes was probably in favour of a flip-flop mechanism promoting arsonolipid uptake within the parasite. Whatever the explanation, the assumption that the palmitoyl and lauroyl arsonoliposomes interacted differently with different parasites may be supported by the recently observed different cytotoxicity between these two types of arsonoliposomes towards HL-60 cancer cells and human umbilical cord endothelial cells (Gortzi et al 2003). The difference was – at least partially – attributed to the different uptake of the arsonoliposomes by the corresponding cells.

In most cases mixed arsonoliposomes (L1 and P1) were found to be more active ($P < 0.05$) compared with plain arsonoliposomes (L2 and P2). Since the arsonolipid moiety of each arsonoliposome type was – most possibly – the active ingredient of the liposomes, a possible explanation of the observed enhanced activity of mixed arsonoliposomes was that most of the arsonolipid molecules were located on the outer layer of the liposome membrane (bilayer) and were thus more accessible to interact with the parasites. This assumption is logical, since the larger polar head group of the arsonolipids possibly fits better on the outer layer of the membrane bilayer. Thus, for a given number of arsonolipid molecules more molecules will be located on the outer surface of mixed arsonoliposomes compared with plain arsonoliposomes. In addition, perhaps the slightly smaller size of the mixed arsonoliposomes may contribute to their higher activity. It is well known that size is a basic physicochemical characteristic of liposomes that highly affects their in-vivo kinetics and distribution, and thereby the activity of encapsulated drugs (Gregoriadis 1995). Although no definitive conclusion could be made at this time, the design of smaller arsonoliposomes (< 30 nm) could be hopeful because of the increased possibility of endocytosis of smaller particles by the flagellar pocket of the promastigote.

The observed (Table 2) higher activity of arsonoliposomes towards the amphotericin B-resistant strain may be connected with the higher fluidity of their plasma membranes compared with those of the wild-type strain (Mbongo et al 1998). Thereby, interaction of arsonoliposomes with plasma membranes of the resistant strain was probably enhanced releasing more arsonolipid molecules within the parasites. Nevertheless, further experiments are needed to assess this interesting possibility.

The demonstrated high accumulation of arsenic in the liver after intraperitoneal administration of arsonoliposomes in the in-vivo BALB/c mouse experiment was an additional advantage for the application of arsonoliposomes in parasitic disease therapy, where parasites are located mainly in the liver, as in visceral leishmaniasis.

Conclusions

The results suggested that arsonoliposome formulations might be a promising approach for developing new antiparasitic drugs. In-vivo evaluation on animal models is necessary to evaluate the opportunity for further studies. In the case of in-vivo positive results, electron microscopy and arsonolipid activity on parasite phospholipase systems (because of the replacement of phosphorus by arsenic) are required to determine the mechanism by which arsonoliposome formulations exert their antitrypanosomal and antileishmanial effects.

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