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Vesicular Systems (Niosomes and Liposomes) for Delivery of Sodium Stibogluconate in Experimental Murine Visceral Leishmaniasis

C. A. HUNTER, T. F. DOLAN* G. H. COOMBS AND A. J. BAILLIE*

*Department of Zoology, University of Glasgow, Glasgow G12 8QQ, and * Department of Pharmacy, School of Pharmaceutical Sciences, University of Strathclyde, Glasgow G1 1XW, UK*

Abstract—Suppression of *Leishmania donovani* liver amastigotes by sodium stibogluconate has been determined in a murine model of experimental visceral leishmaniasis. Niosomal and liposomal drug formulations were equiactive and both increased drug efficacy by an order of magnitude compared with that of free drug. Niosomes containing 30 mol % cholesterol were prepared from three different non-ionic surfactants and no significant difference in activity was detected among the different drug-loaded niosomes. Both negatively charged and neutral vesicles were found to be equally effective. However, vesicle cholesterol content had a slight influence on the antiparasitic activity of the drug-loaded niosomes. Empty vesicles produced a dose-dependent parasite suppression for all vesicles studied. Studies of antimony distribution in the mouse using neutron activation analysis showed high liver levels after i.v. administration of the carrier forms of the drug.

Non-ionic surfactant vesicles, niosomes, have been shown (Azmin et al 1984) to be capable of acting as a delivery system for methotrexate, with potential applications in cancer chemotherapy, and more recently (Baillie et al 1986) for sodium stibogluconate, the drug of choice in the treatment of visceral leishmaniasis. The latter disease is of interest because carrier-mediated therapy may ultimately offer real therapeutic advantages in man. In addition, however, animal models of visceral leishmaniasis provide a means of comparing different delivery systems in a functional way, that is on the basis of parasite suppression.

This paper extends the preliminary findings with niosomal stibogluconate (Baillie et al 1986) which were concerned with vesicles formed from the single alkyl chain surfactant, I. We have now varied the proportion of cholesterol and dicetyl phosphate in the vesicles, but more importantly have used two other surfactants, II and III as vesiculogens, vesicle forming amphiphiles. Surfactant II has a dialkyl hydrophobic moiety, thereby more closely resembling a phospholipid, and might be expected to form vesicles which were less permeable to entrapped solute than monoalkyl surfactant I niosomes. Surfactant III is of interest because, unlike surfactants I and II which are ether-linked, the hydrophobic and hydrophilic portions of the molecule are ester-linked and may be degraded by esterases in-vivo to triglyceride and a fatty acid (palmitic). Such biodegradability is an important property for any carrier system and without it carrier material in-vivo may accumulate intracellularly in the lysosomal compartment of phagocytic cells.

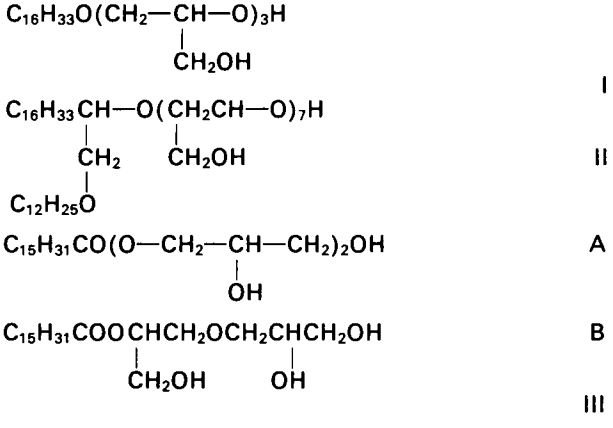
The proven ability of liposomes to enhance the antileishmanial activity of various drugs is shown here to be a property that is not unique to phospholipid vesicles. Vesicles formed from three different synthetic amphiphiles can be used to achieve the same effect.

Correspondence to: A. J. Baillie, Dept of Pharmacy, University of Strathclyde, George Street, Glasgow G1 1XW, UK.

Materials and Methods

Materials

Sodium stibogluconate (Pentostam) equivalent to 0·32 mg Sb mg^{-1} was obtained from the Wellcome Foundation, UK. The three non-ionic surfactants (I, II and III) were obtained from L'Oreal, France and used as received. They are the vesicle-forming, non-ionic surfactants synthesized by Vanlerberghe et al (1978); in each case the number of units in the hydrophilic portion is a number average value. Surfactant III is not ether-linked, and contains the isomers in the proportion A:B, 92:8. Synthetic (>99% pure) L-α-phosphatidylcholine, dipalmitoyl (DPPC), ash-free cholesterol (CHOL) and dicetyl phosphate (DCP), approx. 97% pure by GC, were used as received from Sigma. All other reagents were of analytical grade.



I

II

A

B

III

Vesicle preparation

Niosomes were prepared by an ether injection method (Baillie et al 1985) in which 450 μmol of surfactant or surfactant-CHOL or surfactant-CHOL-DCP mixture dissolved in diethyl ether was injected slowly into 5 mL 300 mg mL^{-1} aqueous sodium stibogluconate solution at 60°C. Liposomes were prepared by hydrating, under a N_2 atmosphere, 114 μmol of DPPC-CHOL or DPPC-CHOL-DCP

mixture with 8 mL 300 mg mL^{-1} aqueous sodium stibogluconate solution at 45–50°C for 2 h, with gentle agitation. Vesicles were either 'drug-loaded' and contained stibogluconate or were 'empty' and contained 300 mM glucose solution. The mean size of the diameter of the niosomes was 333–381 nm (s.d. typically ±80) and of liposomes it was 860 ± 50 nm (s.d.).

Unentrapped drug was removed from vesicle suspensions by dialysis (Visking tubing) against 300 mM glucose solution. Dialysed vesicle preparations were adjusted to the required drug concentration (on the basis of antimony content) by dilution with 300 mM glucose solution.

Antimony determination

Flame atomic spectrometry (Pye Unicam SP90 Atomic absorptiometer) was used to determine the antimony content of vesicle preparations which were disrupted in propanol before assay. The antimony standard was a solution of $SbCl_3$ in dilute HCl and the sensitivity of the method 1 μg Sb mL^{-1}.

Parasites

Leishmania donovani (strain MHOM/ET/67/L82, obtained from The London School of Hygiene and Tropical Medicine) was maintained by passage through female, 10–14 week old, Golden Syrian hamsters (*Mesocricetus auratus*). A suspension of *L. donovani* (0·4 mL, $\simeq 2 \times 10^8$ amastigotes) was administered intraperitoneally to the hamsters which were killed 8–14 weeks after inoculation, the infected spleen being removed and homogenized in PSGEMKA buffer (Hart et al 1981). The released amastigotes were washed and resuspended in PSGEMKA buffer and used to infect further hamsters or to infect mice by tail vein inoculation. Mice were given 0·2 mL parasite suspension containing 2×10^7 *L. donovani* amastigotes.

Parasite clearance studies

Female BALB/c mice (Department of Zoology, University of Glasgow), of about 20 g, with free access to food and water were used. Mice were infected on day 1 as described above.

Table 1. The reduction of liver parasite burden, expressed as % suppression, achieved by the intravenous administration (tail vein) of sodium stibogluconate in several vesicular forms. Vesicles comprised amphiphile, non-ionic surfactant or phospholipid, with and without admixture of cholesterol (CHOL) and dicetyl phosphate (DCP). Molar composition is shown. Mice were infected on day 1 with 2×10^7 amastigotes of *L. donovani* and treated on days 7 and 8 with the various carrier forms of the stibogluconate. The total dose of drug (expressed as antimony) given per mouse is indicated. Liver parasite burdens were determined on day 14 and % suppression calculated for individual mice relative to the mean parasite burden for an appropriate untreated control group. For any treatment, the mean % suppression ± standard error of the mean with numbers of animals in parenthesis, is given.

Sb dose (μg/mouse)	Amphiphile	Reduction in liver parasite burden, % suppression, and type of vesicular carrier used. Vesicle composition			
	Surfactant I	100%	+CHOL 30%	+CHOL 50%	+CHOL 20%, DCP 10%
10		n.d.	−27·4 ± 6·6 (4)	n.d.	14·2 ± 8·8 (5)
20		24·5 ± 7·1 (10)	−31·9 ± 10·4 (4)	38·7 ± 7·6 (6)	5·3 ± 6·1 (8)
40		69·5 ± 1·9 (8)	30·2 ± 5·3 (4)	79·1 ± 5·8 (8)	26·5 ± 11·5 (8)
80		92·9 ± 2·1 (9)	76·5 ± 2·3 (5)	88·1 ± 3·4 (3)	65·2 ± 1·6 (7)
160		96·5 ± 1·4 (7)	91·4 ± 2·7 (4)	96·8 ± 0·9 (7)	84·5 ± 1·9 (8)
320		99·1 ± 0·3 (10)	n.d.	99·2 ± 0·2 (9)	97·6 ± 0·6 (7)
	Surfactant II				
20		20·2 ± 14·3 (4)	41·0 ± 5·3 (7)	53·2 ± 7·7 (9)	n.d.
40		7·2 ± 7·2 (4)	68·0 ± 4·0 (9)	77·8 ± 4·3 (6)	
80		37·2 n.a. (2)	83·9 ± 3·8 (8)	91·0 ± 1·6 (8)	
160		53·7 ± 13·3 (4)	96·5 ± 1·1 (8)	96·6 ± 0·9 (9)	
320		95·4 ± 0·9 (4)	99·2 ± 0·1 (9)	99·2 ± 0·1 (9)	
640		99·0 ± 0 (4)	n.d.	n.d.	
	Surfactant III				
10		n.d.	13·9 ± 14·2 (6)	n.d.	n.d.
20			45·0 ± 11·7 (15)		
40			57·6 ± 10·4 (12)		
80			86·6 ± 3·5 (12)		
160			93·1 ± 1·9 (12)		
320			97·4 ± 0·8 (10)		
	Phospholipid				
8		n.d.	n.d.	n.d.	1·2 ± 11·9 (4)
10			n.d.		4·2 ± 7·6 (6)
16			n.d.		0 ± 10·5 (4)
20			63·4 ± 6·2 (4)		18·1 ± 5·1 (3)
32			n.d.		33·3 ± 3·7 (4)
40			59·1 ± 11·4 (5)		90·1 ± 2·7 (5)
64			n.d.		69·1 ± 6·3 (5)
80			89·7 ± 1·8 (5)		89·9 ± 1·4 (5)
160			98·0 ± 0·3 (4)		90·6 ± 4·1 (4)
320			99·3 ± 0·4 (5)		n.d.

n.a. = not applicable, n.d. = not determined.

Treatment, using free drug solution, niosomal and liposomal drug suspensions and empty niosomes and liposomes was carried out on days 7 and 8 by tail vein administration of 0·2 mL volumes. The mice were killed on day 14 and multiple liver impression smears prepared and stained using Giemsa's stain. Liver parasite burden was assessed by counting ≤ 2 smears per animal and was based on the number of parasites per 100 host cell nuclei. For all treatment groups of animals, there was an untreated control group which had been infected with the same amastigote suspension. The reduction in liver parasite burden achieved in an animal by a particular treatment was calculated relative to the mean parasite burden (n = 5 or 6) for the control group and expressed as the % parasite suppression. In all cases, the mean parasite suppression in the treatment group is shown.

Tissue distribution of antimony

The distribution of antimony in various tissues of mice after intravenous administration of free or vesicular (amphiphile-:CHOL 7:3 molar ratio) stibogluconate was determined by neutron activation analysis.

Mice were injected via the tail vein with 1 mg stibogluconate, that is an equivalent antimony dose of 320 μg, in the form of 0·2 mL free drug solution or liposome or niosome suspension. After 24 h, the animals were killed and various organs and tissues removed and weighed into small polythene vials. These tissue samples were then freeze-dried, the vials and contents reweighed and the lids of the vials sealed shut by melting the plastic.

The sealed vials were irradiated for a total of 54 h at a nominal flux of 3×10^{12} neutrons $cm^{-2}s^{-1}$ in the Argonaut UTR 300 reactor at the Scottish Universities Reactor Centre, East Kilbride. Neutron flux was monitored by small ($\simeq 10$ mg) pieces of pure iron wire attached to the exterior of each vial and the actual flux received by each vial was based on the activity of ^{59}Fe, 1·099 MeV, in the iron. Triplicate antimony standards each comprising 20 μL antimony trioxide (99·9%, Analar) dissolved in HCl (99·9% analytical) were pipetted into vials and dried in-situ. These vials were sealed and irradiated with the samples as above.

21 days after irradiation, to allow decay of sodium, potassium, manganese and chlorine, the irradiated samples were counted on a shielded 130 cm^3 Ge-Li detector. γ-Ray spectra were recorded in 4096 channels and peak count rates calculated. Antimony content was based on the activity of ^{124}Sb, $t_{\frac{1}{2}}$ 62·5 days, 1·691 MeV.

The entire irradiation/counting procedure was validated using samples of standard reference bovine liver (SRM 1577, National Bureau of Standards).

Results

The data of Tables 1, 2 and Fig. 1 show that the vesicular forms of stibogluconate were more active than the free form in this murine model of visceral leishmaniasis. This confirms our earlier, preliminary, findings with surfactant I niosomes (Baillie et al 1986). In general it can be seen that vesicular formulations of the drug were at least an order of magnitude more active than free drug solution in this respect. As carriers of stibogluconate, no significant difference between liposomes and niosomes prepared from double or single aliphatic chain surfactants (II and III) could be discerned (Fig. 1).

There was some difference detected among the various types of niosome tested (Table 1) and from Fig. 1 it is evident that at low dosage in particular, Surfactant I niosomes, containing 30 mol % CHOL, were less effective than niosomes with the same CHOL content but prepared from Surfactants II or III. Based on the Sb dose required per mouse for 50% suppression (ED50) and an ED50 for free

Table 2. The reduction of liver parasite burden, expressed as % suppression, achieved by dosing infected mice via the tail vein with free stibogluconate solution. Mice were infected on day 1 with 2×10^7 amastigotes of *L. donovani* and treated on days 7 and 8. The total dose of drug (expressed as antimony) given per mouse is shown. Liver parasite burdens were determined on day 14 and % suppression calculated for individual drug treated animals relative to the mean parasite burden for an appropriate untreated control group.

Sb dose (μg/mouse)	% suppression*
66	10·4 ± 7·7 (5)
200	35·6 ± 16·3 (5)
400	65·6 ± 5·8 (13)
660	72·2 ± 7·9 (11)
800	91·0 ± 3·7 (16)
1320	97·6 ± 0·8 (30)
1600	90·7 ± 4·3 (24)
2000	100 n.a.(20)

* Mean ± standard error of mean, number of animals used is shown in parentheses.
n.a. = not applicable.

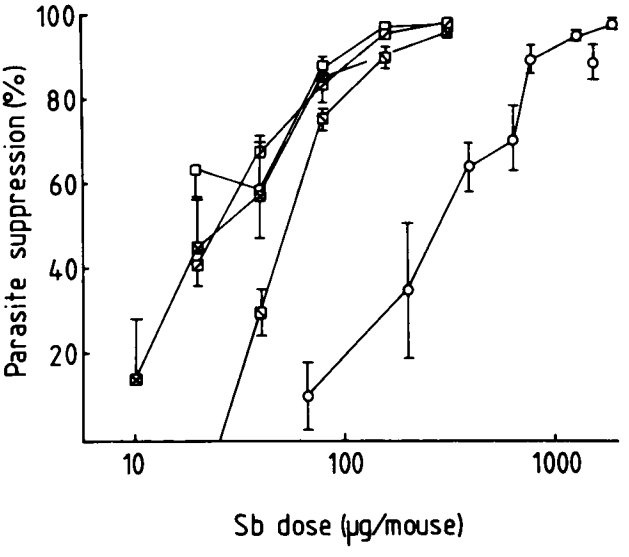


FIG. 1. The reduction of liver parasite burden, expressed as % suppression, achieved by the intravenous administration (tail vein) of sodium stibogluconate as free solution or in several vesicular forms. Vesicles comprised amphiphile, non-ionic surfactant or phospholipid, with the admixture of 30 mol % cholesterol. Mice were infected on day 1 with 2×10^7 amastigotes of *L. donovani* and treated on days 7 and 8 with the various formulations of stibogluconate. The total dose of drug (expressed as antimony) given per mouse is indicated. Liver parasite burdens were determined on day 14 and % suppression calculated for individual mice relative to the mean parasite burden for an appropriate untreated control group. For any treatment, the mean %suppression ± standard error of the mean is shown. Formulation used; ○, free drug solution; □, liposomes; ⊠, Surfactant I niosomes; ⧄, Surfactant II niosomes; ⊠, Surfactant III niosomes.

Table 3. Efficiency of entrapment of sodium stibogluconate in niosomes and liposomes. Vesicles were disrupted by dilution in propanol and the antimony content determined by atomic absorptiometry. It has been assumed that the intravesicular stibogluconate concentration was the same (300 mg mL^{-1}) as that used for hydration of the amphiphiles.

Vesicular type and molar composition	Entrapment efficiency* l mol^{-1} of vesicle constituents
Surfactant I 100%	0·91 ± 0·09 (5)
Surfactant I + CHOL 30%	0·45 ± 0·12 (5)
Surfactant I + CHOL 50%	0·39 ± 0·08 (4)
Surfactant II + CHOL 30%	0·50 ± 0·06 (3)
Surfactant III + CHOL 30%	0·42 ± 0·08 (3)
Phospholipid + CHOL 30%	0·42 ± 0·11 (3)
Phospholipid + CHOL 20%, DCP 10%	0·93 ± 0·09 (3)

* Mean value ± standard error, number of observations in parentheses.

Table 5. Tissue levels of antimony in the uninfected mouse 24 h after administration (tail vein) of 1 mg sodium stibogluconate (320 μg antimony) in 0·2 mL volumes as free solution (F) or liposomal (L) and niosomal (N) formulations. For each organ, the whole tissue was freeze-dried, irradiated and the antimony content determined as ^{124}Sb. For muscle (gastrocnemius, sample wet weight ≃ 0·5 g) it was assumed that 40% of the body weight was muscle and the value for Sb per organ for this tissue is the calculated content for total skeletal muscle.

Tissue	Tissue Sb concentration (ppm)			Sb per organ (μg)		
	N	L	F	N	L	F
Liver	54·07	43·30	7·41	14·11	15·41	2·76
Lung	5·14	0	0·56	0·29	—	0·02
Kidney	1·60	0·10	0	0·21	0·12	—
Heart	0·10	0·89	0	0·01	0·02	—
Spleen	7·83	0	7·40	0·67	—	0·60
Muscle	n.d.	0·27	0·31	n.d.	0·78	0·76

n.d. = not determined.

drug solution of 300 μg, the increased efficacy of these niosomal formulations were: Surfactant I niosomes, ED50 = 50 μg, a 6× increase; Surfactant II niosomes, ED50 = 24 μg, a 12·5× increase; Surfactant III niosomes, ED50 = 20 μg, a 15× increase. The basis for these between vesicle differences is not known. Entrapment efficiencies (Table 3) for the types of surfactant vesicle described in Fig. 1 were similar. At higher doses of drug, in the range 160–320 μg Sb per mouse, the different non-ionic surfactant vesicle formulations were equally effective.

For vesicles comprising Surfactant I and 30 mol % CHOL, the negative % suppression found (Table 1) at antimony doses of 10 and 20 μg per mouse apparently indicates an increase in liver infestation. This was not observed with any other type of treatment and has not been investigated further. This observation may well be simply a consequence of the variability inherent in the type of experiments described here.

Some parasite suppression could also be achieved by administration of empty vesicles (Table 4). Empty niosomes could be ranked Surfactant I > Surfactant II > Surfactant III in terms of this inhibitory activity. The empty liposomes used in these experiments were as active as empty Surfactant III niosomes against the liver parasites. The mechanism for this apparent antiparasitic activity of empty vesicles is not known.

Although the antimony distribution data from neutron activation analysis (Table 5) must be regarded as preliminary, they support our earlier results (Baillie et al 1986) for liver antimony levels based on atomic absorptiometry. That is, liver antimony levels were much higher (5–6× at 24 h) when the drug was given in vesicular form. Within the constraints of the limited number of observations, the clearest difference between liposomes and niosomes was that higher levels of antimony were detected in the lungs and spleen with the latter type of vesicle. There was no apparent difference, however, between the ability of the two types of vesicle to deliver drug to the liver. This is in accord with passive drug delivery to the infected liver as the basis for the high activity of the vesicular formulations.

Table 4. The reduction of liver parasite burden, expressed as % suppression, achieved by dosing infected mice via the tail vein with a suspension of 'empty' vesicles. In all cases, non-ionic surfactant vesicles comprised 70% amphiphile and 30% cholesterol and liposomes, 70% phospholipid, 20% cholesterol and 10% dicetyl phosphate (molar compositions). Vesicles were given at two dose levels, high and low, equivalent to the amount of vesicular material administered to achieve a total dose of 320 and 160 μg Sb per mouse in niosomal or liposomal form.

Vesicle type	Dose level	% suppression*
Surfactant I	high	41·6 ± 4·9 (17)
	low	30·5 ± 5·1 (8)
Surfactant II	high	39·1 ± 4·1 (13)
	low	11·3 ± 10·4 (11)
Surfactant III	high	14·4 ± 9·5 (7)
	low	n.d.
Phospholipid	high	17·6 ± 9·4 (18)
	low	8·1 ± 4·9 (12)

* Mean ± standard error of the mean, number of animals used is shown in parentheses.
n.d. = not determined

Discussion

The high efficacy of several drugs in liposomal form against visceral leishmaniasis in animal models has been well established. Such studies include sodium stibogluconate in a mouse model (Black et al 1977), sodium stibogluconate and meglumine antimonate in a hamster model (Alving et al 1978), potassium antimony tartrate and sodium stibogluconate in a mouse model (New et al 1978), 8-aminoquinolines in a hamster model (Alving et al 1980), amphotericin B and other antifungal drugs in a mouse model (New et al 1981) and, more recently, meglumine antimonate and amphotericin B in a hamster and a primate model (Berman et al 1986). This work has been reviewed by Alving (1986) as has the use of liposomal drug carrier systems in parasitic diseases (Croft 1986). It is apparent that the magnitude of the observed increase in drug efficacy occasioned by the use of a carrier system is very much dependent on the experimental conditions used. Important variables include the size of the infective inoculum and hence challenge to the host, route of administration, volume inoculated, the timing of the treatment in relation to the infection and the strain of parasite and host animal used. In addition, variables associated with the experimental therapy, such as number of doses, liposome

type and phospholipid concentration, can also be expected to influence the experimental outcome.

The diversity of experimental conditions used makes comparisons of the various studies and drug formulations extremely difficult. The most important aspect of our findings is the demonstration of an alternative vesicular carrier strategy to a phospholipid based system and that these novel vesicular systems are as effective as liposomes in delivering drug in experimental visceral leishmaniasis.

We have in our model closely defined the responses to free and carrier forms of stibogluconate and so established the relative efficacies of the vesicular formulations. Surprisingly, however, we have been unable to achieve the reported several hundred-fold increase in drug efficacy brought about by the use of liposomes (for reviews see Alving 1986, 1983). This (apparent) discrepancy can, however, be readily explained on the basis of methodological differences.

It would appear that all of the vesicular stibogluconate systems tested in this study fall within the same activity decade, that is between 10 to 100 μg antimony per mouse. It is interesting to speculate that this would represent the range of activity of any vesicular system carrying the highly water-soluble stibogluconate. For drugs with such solubility characteristics it must be assumed that they are entrapped in the internal aqueous space of the vesicle, liposome or niosome, and the ability of this compartment to accommodate drug may be a limiting aspect of vesicular carriers. Any manipulation of vesicle composition, such as changing the vesicle forming amphiphile and/or the proportion of other lipids such as cholesterol or dicetyl phosphate appear only to shift the efficacy of the carrier within this range. Further support for this concept may be obtained by examination of the data of Black et al (1977), Alving et al (1978) and New et al (1978) for liposomal stibogluconate. Their preparations were all active in the range 1 to 16 mg Sb kg^{-1}. For the 20 g animals in this study, this is equivalent to 20 to 320 μg Sb per mouse and is thus similar to the activities we found for liposomal and niosomal forms of the drug. An implication of this observation is that a rational choice of a particular vesicular carrier system would be predicated by considerations such as lack of toxicity, cost and stability rather than carrier efficacy. In these respects, niosomes may have some advantages over liposomes and so we suggest that their potential as carriers of antileishmanial drugs should be pursued.

Acknowledgements

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