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Stearylamine-bearing cationic liposomes kill *Leishmania* parasites through surface exposed negatively charged phosphatidylserine

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***Objectives*: Lipid-associated formulations of antileishmanial agents have proved to be more effective therapies with reduced toxicities. Previous studies from our group and others revealed that liposomes bearing phosphatidylcholine and stearylamine (SA) themselves kill *Leishmania* and other protozoan parasites *in vitro* and *in vivo*, without causing any adverse effect on host. In the present study, we offer detailed insights into the mechanism of action of these liposomes.**

***Methods*: Mechanism study was carried out using fluorometric, confocal and electron microscopic methods.**

***Results*: Herein, we provide evidence for induction of membrane disruption by specific interaction with surface phosphatidylserine (PS) of *Leishmania* promastigotes and amastigotes, phospholipids normally not found on mammalian cell surface, with SA-containing liposomes. Cell surface PS on different forms of *Leishmania* facilitated liposome-induced parasite killing. The target selectivity of the liposomes was further proved through inhibition of antileishmanial activity with annexinV, and strong affinity with anionic PS rather than phosphatidic acid-containing liposomes for leishmanicidal activity.**

***Conclusions*: SA-bearing liposomes specifically kill *Leishmania*, but are non-toxic to murine peritoneal macrophages and human erythrocytes.**

Keywords: promastigotes, amastigotes, membrane, drug

Introduction

Leishmania parasites are the causative agents for cutaneous and visceral leishmaniasis (VL) with ~2 million cases annually and a prevalence rate of 12 million, respectively. VL, principally caused by *Leishmania donovani*, is fatal if left untreated.[1] Pentavalent antimonials, though toxic, remain the first-line drugs for leishmaniasis. Emergence of drug resistance has pushed in second-line drugs such as amphotericin B and pentamidine, which cause toxic side effects.[2–4] Miltefosine, the most recent oral drug for VL, is potentially teratogenic.[2] Moreover, clinical trials have identified occasional gastrointestinal toxicity that requires treatment withdrawal.[4,5]

Lipid associated formulations of antileishmanial agents provide more effective therapies with considerably larger doses and reduced dosing schedules.[6] Of these, AmBisome, a liposomal formulation of amphotericin B, is the safest and can be administered at doses (7.5 mg/kg) much higher than its free form (1.5 mg/kg). The advantage of such antileishmanial formulations is their ability to concentrate high levels of drugs in the infected target organs.[3] Earlier, we and others have reported that drug-free cationic liposomes, comprising egg phosphatidylcholine (PC) and stearylamine (SA), have *in vitro* antiprotozoan activity against *Trypanosoma cruzi*, *Trypanosoma brucei gambiense* and various species of *Leishmania*, as well as *in vivo* activity against *Toxoplasma gondii* and *L. donovani*.[7–11] Further, a single dose of suboptimal sodium antimony gluconate (SAG) entrapped in PC–SA vesicles showed profound activity against chronically infected BALB/c mice. The enhanced efficacy of SAG is due to the synergistic antileishmanial activity of the cationic liposomes,[12] the mechanism of which is not known. Here, we address the possible mode of action of PC–SA liposomes on *L. donovani* parasites. Our work supports the idea that SA-bearing liposomes can recognize phosphatidylserine (PS) on the parasite membrane and that this recognition is necessary for its ability to damage the parasite plasma membrane resulting in its ultimate death.

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Materials and methods

Reagents

All reagents were purchased from Sigma (St Louis, MO, USA) or Merck (GmBH, Germany).

Parasites and animals

Promastigotes of *L. donovani* strain AG83 (MHOM/IN/1983/AG83) were grown at 22°C in M199 supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin and 100 mg/L streptomycin (M119-S).[13] The mutant R2D2 strain, which is defective in the synthesis of the repetitive unit of lipophosphoglycan (LPG), was kindly provided by S. J. Turco (University of Kentucky, School of Medicine, Lexington, USA). *L. donovani* axenic amastigotes were grown at 37°C in M199, supplemented with 25% FCS and 10 mM Tris-succinate, pH 5.5.[14] BALB/c mice reared in institute facilities were used for experimental purposes with prior approval of the Animal Ethics Committee of IICB. Intracellular amastigotes were isolated from spleen and liver of 10–12 week infected animals and purified, as described previously,[15] and finally isolated through discontinuous percol gradient. No loss of viability, as determined by propidium iodide (PI) incorporation under fluorescence microscope (Leica DMI 4000 B), and no transformation to promastigotes occurred during the procedure of purification of amastigotes.

Preparation of liposomes

Liposomes were prepared with PC in association with cholesterol (Chol), SA (Fluka, Switzerland), phosphatidic acid (PA) or PS at a molar ratio of 7:2, as described.[13] In addition, PC–PS liposomes were prepared at a molar ratio of 1:1. Briefly, the thin dry lipid film was dispersed in PBS and the suspension was sonicated for 60 s in a Microson XL-2000 (Micronix Inc., NY, USA).

PC–SA liposomes were visualized under JEOL-100CX electron microscope. The vesicles dispersed in PBS were absorbed on a carbon-coated grid and the grid was washed and dried. Liposomes, negatively stained with an aqueous 1% phospho-tungstic acid,[16] demonstrated an average diameter of 150–200 nm.

Leishmanicidal activity

Freshly transformed *L. donovani* promastigotes, axenic amastigotes (2×10^6 cells/mL) and freshly isolated intracellular amastigotes (2×10^7 cells/mL) were incubated with different concentrations of PC–SA (8.87–213 μM) for 60 min at 22 and 37°C for promastigotes and amastigotes, respectively. Inhibition of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to insoluble formazan by mitochondrial dehydrogenase was used as a viability parameter for the parasites and compared with untreated media control.[17] Treated and untreated parasites were incubated with 2 mg/mL MTT solution for 4 h at 37°C. The reduced formazan was solubilized and A_{570} was determined in a spectrophotometer (Hitachi High Technologies, USA). ED_{50} and ED_{90} defined as the liposome concentrations that inhibited MTT reduction with respect to control parasites by 50% and 90%, respectively, were calculated using sigmoidal regression analysis (Microsoft Excel).

Cytotoxicity assay

L. donovani promastigotes and murine peritoneal macrophages were incubated with various concentrations of PC–SA or PC–PS liposomes or media alone for 60 min at 22 and 37°C, respectively. The DNA-binding dye PI (final concentration of 5 mg/L) was added to the cells and incubated further for 5 min at room temperature in the dark. PI-stained cells were counted on a confocal microscope (TCS-SP Leica) with laser beam excitation at 568 nm. Cytotoxicity ($\%_{cytox}$) was calculated from the percentage of PI-positive cells in media alone (PI_M) and in PC–SA or PC–PS liposomes (PI_{exp}): $\%_{cytox} = 100 \times (PI_{exp} - PI_M)$.[18]

Determination of plasma membrane potential ($\Delta\Psi$)

The change of $\Delta\Psi$ was estimated by bisoxonol, whose fluorescence intensity increases on insertion into the membrane once the cell is depolarized. Assays were performed as described.[19] Briefly, parasites containing 0.2 μM bisoxonol were resuspended in *N*-methylglucamine-Cl media, pH 7.4, for calibration and Cl^- medium for $\Delta\Psi$ determination of samples. Liposomes were added at different concentrations and changes in fluorescence monitored for 15 min using a spectrofluorometer (Hitachi), equipped with λ_{exc} and λ_{ems} 540 and 580 nm filters, respectively.

Modification of bioenergetics parameters

The following assays were carried out:

(i) The ATP content was determined by the luciferin-luciferase method.[20] The assay is based on the requirement of luciferase for ATP in producing light. Briefly, PC–SA and PC–PS liposomes-treated and untreated cells (2×10^7) were harvested and resuspended in PBS. ATP extracted in boiling extraction buffer, pH 7.4, was measured using Sigma luciferase ATP assay kit. The amount of ATP in experimental samples was calculated from a standard curve prepared with ATP and expressed as nmol/10^7 cells.

(ii) The oxygen consumption rate was determined,[21] using a Clarke-type oxygen electrode. Briefly, PC–SA and PC–PS liposome-treated promastigotes were resuspended in respiration medium (2×10^9 cells/mL) at 4°C. Measurements were performed at 1×10^8 parasites/mL after dilution in the same medium previously equilibrated with air. Total cellular protein was measured, so that the rate of oxygen consumption could be normalized to the protein content of the cells.

Electron microscopy

Surface morphology of PC–SA liposome-treated parasites was studied by scanning electron microscopy (SEM). Cells quick-fixed in 2.5% glutaraldehyde and post-fixed with 1% OsO_4 were gradually dehydrated and air-dried. They were then sputter-coated with gold palladium and examined in a JEOL JSM-5200 electron microscope using an accelerating voltage of 20 kV.

Transmission electron microscopy (TEM) was performed with liposome-treated and -untreated cells.[22] Briefly, cells were fixed in 3% glutaraldehyde in PBS, post-fixed with 1% OsO_4 for 16–20 h, gradually dehydrated in ethanol and finally embedded in SPURRT resin. Thin cut sections were stained with uranyl-acetate and lead acetate and were observed in a JEOL-100CX electron microscope.

AnnexinV-binding assay by flow cytometry

For surface PS detection, AG83 promastigotes, freshly isolated intracellular amastigotes, axenic amastigotes and normal murine peritoneal macrophages were washed and resuspended in binding buffer

(10 mM HEPES, 150 mM NaCl and 2.5 mM $CaCl_2$) at pH 7.3. Cells (2×10^6) were incubated at room temperature for 15 min with annexinV-fluorescein isothiocyanate (FITC) following the manufacturer's instructions (Apoptosis Detection Kit, BD Biosciences) and analysed on a flow cytometer (BD LSR, Becton Dickinson, San Jose, CA, USA) by Cell Quest software. Ten thousand-gated events were harvested from each sample.

Effect of annexinV on PC–SA leishmanicidal activity

Cell suspensions in binding buffer were incubated with or without annexinV at 26°C for 30 min.[23] Cells were incubated with PC–SA liposomes (213 μM) for an another hour. Viability of the promastigotes was assayed by MTT reduction.

Effect of PC–PS liposomes on leishmanicidal activity of PC–SA liposomes

PC–SA liposomes (213 μM) were incubated for 30 min with different concentrations of PC–PS, PC–PA and PC–Chol liposomes (5–132 mg/L). Promastigotes (2×10^6/mL) were added and viability assayed after 1 h by MTT reduction.

Statistical calculations

All data comparisons were tested for significance by using two-tailed Student's *t*-test using GraphPad software; P values <0.05 were considered significant.

Results

Inhibition of MTT reduction in promastigotes and amastigotes indicating potency of SA-bearing liposomes

PC–SA liposomes (Figure 1a) were tested for their ability to kill parasites by inhibition of MTT reduction in stationary phase promastigotes of AG83 and R2D2 strains, and axenic and freshly isolated intracellular amastigotes of AG83, at a concentration range of 8.87–213 μM. Liposomes showed leishmanicidal activity in the micromolar range with ED_{50} and ED_{90} doses at 124 μM (±2) and 213 μM (±3.5) against AG83 promastigotes. PC–SA liposomes were most active against freshly isolated intracellular AG83 amastigotes followed by equipotency towards promastigotes of AG83 and R2D2, and lastly by AG83 axenic amastigotes (Figure 1b and c). Comparative antileishmanial potency with respect to ED_{50}s showed ∼6- and ∼2-fold higher efficacy against purified intracellular amastigotes and amastigotes residing in murine macrophages,[12] respectively, in comparison with AG83 promastigotes. Viability of normal murine peritoneal macrophages was only marginally affected even at 213 μM, the ED_{90} of PC–SA liposomes for AG83 promastigotes. Notably, PC–PS liposomes showed no activity against *Leishmania* and murine macrophages.

Evaluation of the toxic effect of SA-bearing liposomes

We used PI uptake assay to test the safety of PC–SA liposomes for mammalian cells. Macrophages treated with 213 μM liposomes remained unstained compared with AG83 promastigotes (Figure 1d), and 50% cytotoxic concentration (CC_{50}) for peritoneal macrophages was 1279 μM (±3.5). The toxicity of PC–SA liposomes for the macrophages and activity against AG83 promastigotes were compared using the selectivity index (SI) ratio (CC_{50} for macrophages/ED_{50} for protozoan).[24] PC–SA liposomes exhibited higher selectivity for the parasites than mammalian cells, with an SI ratio of 10.3. However, PC–PS liposomes showed marginal levels of cytotoxicity against both promastigotes and macrophages.

PC–SA liposomes induce disruption in promastigote cell membrane

Morphological damage inflicted on PC–SA (213 μM)-treated promastigotes was analysed by electron microscopy. Scanning electron micrographs of treated promastigotes revealed shedding of the flagellum, severe distortion and rupture of cell membrane compared with untreated control cells (Figure 2a and b).

Our TEM experiment (Figure 2c and d) revealed a severe disruption of the membrane structure, with strong intracellular vacuolization, accompanied by loss of electron dense intracytoplasmic materials resulting in the formation of 'ghost'-like structures when promastigotes were treated with 213 μM PC–SA liposomes for 1 h.

PC–SA induces disruption of the plasma membrane potential

Previous studies have shown that parasite cell damage with PC–SA liposomes was initiated by binding and accumulation of the vesicles at the cell surface.[10] To determine whether *Leishmania* membrane can be a potential target of PC–SA liposomes, we performed a liposome-induced promastigote membrane depolarization assay using the membrane potential sensitive dye, bisoxonol. PC–SA caused concentration-dependent rapid dissipation of the $\Delta\Psi$ to a critical level (Figure 3a). Such changes were not observed when promastigotes were treated with PC–PS liposomes.

Change in membrane potential causes depletion in cytosolic ATP level and decrease in oxygen consumption rate

Since dissipation of plasma membrane potential leads to strong collapse of bioenergetic metabolism of the parasites,[25,26] we investigated intracellular ATP level and oxygen consumption rate of the treated promastigotes. As shown in Figure 3(b), there was a drastic fall (84%) in ATP levels after 60 min of 213 μM of liposome treatment compared with untreated controls ($P < 0.0001$). O_2 consumption was inhibited by 21% and 97% at 30 and 60 min, post-treatment, respectively (Figure 3c). However, PC–PS liposomes had no effect on the bioenergetics parameters.

Role of electrostatic interactions in PC–SA liposome activity

To better understand the interaction of PC–SA liposomes with the surface of the parasites, we investigated the role of electrostatic interactions through two sets of experiments on promastigotes. First, we investigated the effect of reduction of the salt concentration (Na^+Cl^-) of the incubation medium, preserving its iso-osmolarity by sorbitol. Activity of PC–SA liposomes with ED_{50} decreased nearly 1.5-fold when salt concentration was reduced from 140 to 80 mM. Second, an experiment addressed the possible effect of heparin, a strongly anionic polysaccharide.

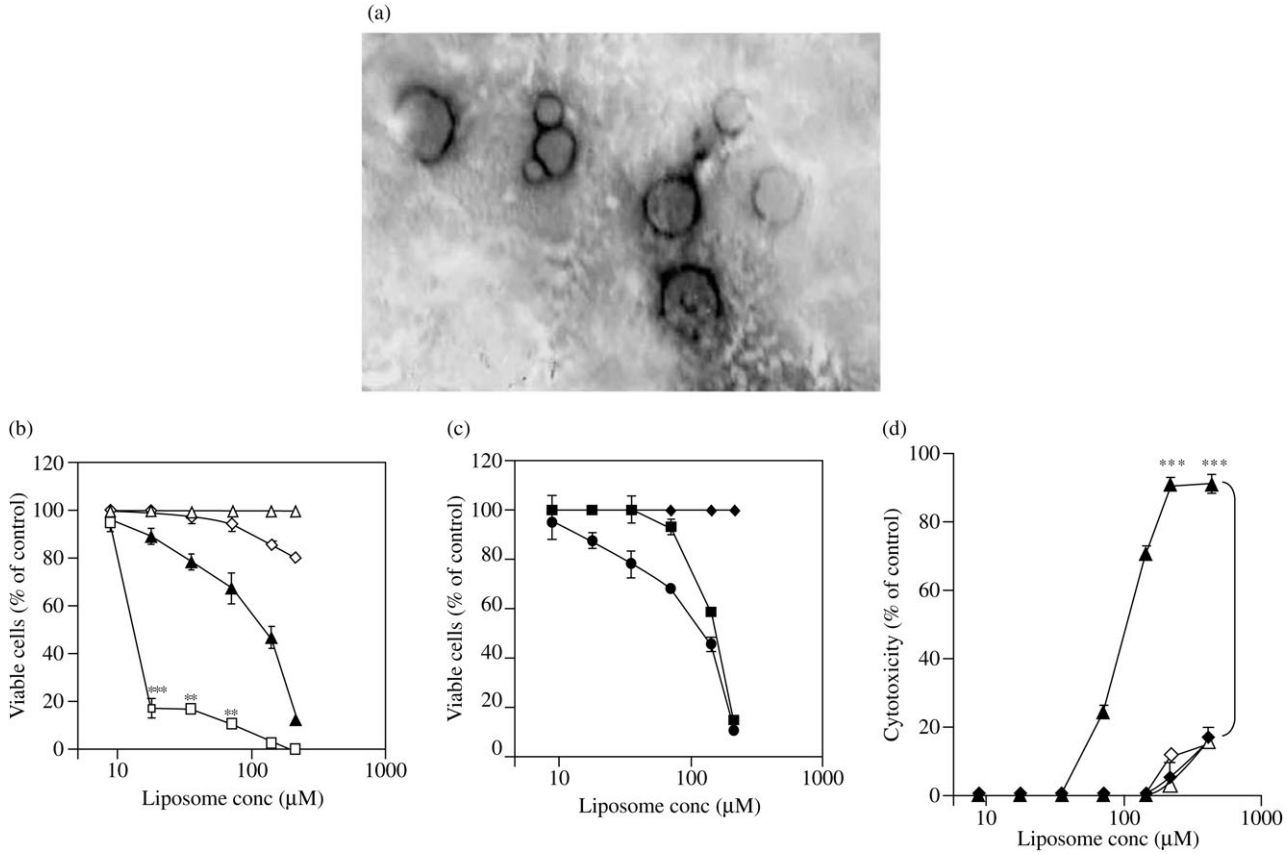

Figure 1. (a) TEM picture of PC–SA liposomes. Magnification 35 000×. (b and c) Activity of SA-bearing liposomes on promastigotes and amastigotes. Different strains of *L. donovani* promastigotes at 22°C and amastigotes and murine macrophages at 37°C were incubated with different concentrations of PC–SA and PC–PS liposomes for 60 min. Viability of cells was evaluated by MTT and calculated as a percentage of control cells incubated in media without liposomes. (b) Promastigotes (filled triangles) and freshly isolated intracellular amastigotes of *L. donovani* AG83 (open squares) and normal murine macrophages (open diamonds) treated with PC–SA liposomes. AG83 promastigotes (open triangles) treated with PC–PS liposomes. (c) Axenic amastigotes of AG83 (filled squares) and promastigotes of R2D2 (filled circles) treated with PC–SA liposomes, and macrophages (filled diamonds) treated with PC–PS liposomes. Data points represent the mean of triplicate samples ± SEM from a single experiment, representative of three different experiments. $^{***}P < 0.0001$, $^{**}P < 0.001$ versus AG83 promastigotes treated with PC–SA liposomes (filled triangles). (d) Cytotoxic activity of PC–SA liposomes through PI uptake. Normal murine peritoneal macrophages and AG83 promastigotes were incubated with graded doses (8.8–426 μM) of PC–SA or PC–PS liposomes for 1 h and subsequently incubated with PI for 5 min. Numbers of PI-stained cells were counted under a confocal microscope. Percentage cytotoxicity of AG83 promastigotes, PC–SA (filled triangles) and PC–PS (open diamonds) treated, and macrophages, PC–SA (filled diamonds) and PC–PS (open triangles) treated, was calculated by the formula: $\%_{cytox} = 100 \times (PI_{exp} - PI_M)$. Data points represent the mean of triplicate samples ± SEM from a single experiment. $^{***}P < 0.0001$.

Preincubation of parasites in the presence of 300 mg/L heparin for 15 min before liposome addition failed to revert the killing activity of the vesicles, whereas at higher concentration (450 mg/L) partial inhibition (35%) was observed. Thus, non-specific charge interaction is not the sole reason for the anti-leishmanial effect. Equivocal activity of PC–SA liposomes against AG83 and R2D2 promastigotes (Figure 1b and c), the latter lacking the repetitive phosphorylated disaccharide region of LPG, proved further that the interaction of PC–SA liposomes with the parasite membrane was possibly through a negatively charged component other than the strongly anionic LPG.

Differential PS exposure by different forms of Leishmania parasite

Since non-specific charge interaction as well as the strongly anionic LPG appeared to have no role in antileishmanial activity of the cationic liposomes, possibly specific interaction with some other membrane component might be the key for the mechanism of action. Recently it was shown that intracellular amastigotes isolated from BALB/c mice have PS enriched surface.[15] The highest susceptibility of intracellular amastigotes isolated from BALB/c mice to PC–SA liposomes (Figure 1b), motivated us to investigate the differential expression of surface PS on *Leishmania* parasites. The relative amount of cell surface PS was determined by binding of fluorescein-labelled annexinV to the cells and subsequent FACS analysis. The shift in fluorescence intensity of annexinV-treated and control cells served as a relative marker of the accessible membrane PS content. Organ-derived amastigotes showed ∼5.3-fold higher annexinV binding compared with AG83 promastigotes. Normal murine peritoneal macrophages were found to express almost no PS on their surface (Figure 4a).

Effect of masking of cell surface PS on the antileishmanial activity of PC–SA liposomes

To investigate the role of PS in interaction with liposomes, PS was blocked by preincubation of promastigotes with annexinV

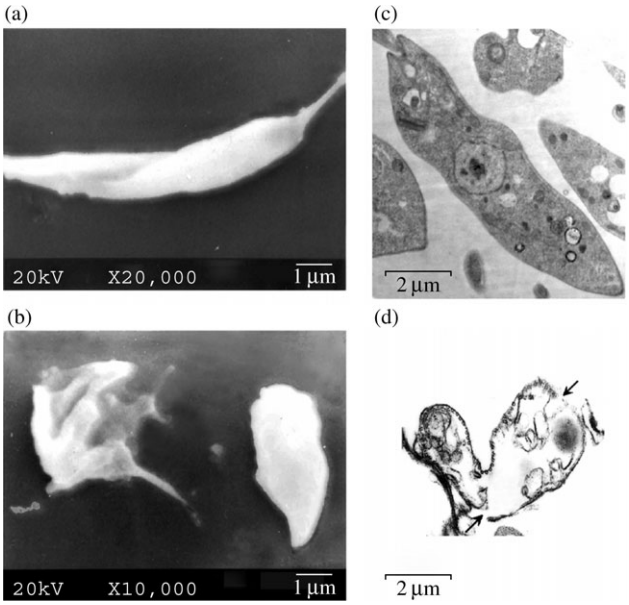

Figure 2. Scanning and transmission electron microscopy of promastigotes treated with PC–SA liposomes. Promastigotes were incubated for 1 h under standard conditions with medium alone (a and c) or 213 μM (b and d) of PC–SA liposomes. Two representative scanning electron micrographs of untreated (a) and treated (b) cells revealed severe distortion and rupture of the cell membrane. Two representative transmission electron micrographs of untreated (c) and treated (d) cells revealed membrane disruption, extensive vacuolization and membrane breakage (arrow) as well as depletion of electron-dense cytoplasmic material. Scale bars: 1 μm (a and b), 2 μm (c and d). Pictures are representative of three similar studies. Contrast and brightness modification was performed in Adobe Photoshop 6.0.

prior to addition of PC–SA liposomes. Killing activity of liposomes (32%, ±2.3) was totally inhibited at 30 min treatment (Figure 4b). Capability of PC–SA liposomes to induce 97% and 100% killing activity at 60 and 120 min, respectively, was drastically reduced to 25% (±4.04), through annexinV blocking of surface PS of promastigotes indicating significant PS-mediated killing. Non-specific negative–positive charge interaction with other membrane components may be responsible for the remaining 25% killing activity.

Effect of PC–PS liposomes on the activity of PC–SA liposomes

To reconfirm the PS-specific affinity of SA-bearing liposomes, PC–SA liposomes were preincubated with either PC–PS or PC–PA or PC–Chol liposomes. Leishmanicidal activities of PC–SA liposomes on promastigotes were inhibited with a gradual increase in the dose of PC–PS liposomes (7:2) in comparison with PC–Chol (7:2) liposomes. Maximum significant inhibition was observed at a concentration of 213 μM PC–PS. More intensive inhibition was observed with PC–PS liposomes composed of 1:1 molar ratio of lipids (Figure 4c), indicating that the inhibition was enhanced with increase in the PS content. Specificity towards PS was further supported by the negligible effect of the anionic PC–PA (7:2) liposomes to inhibit the antileishmanial potency of the PC–SA liposomes.

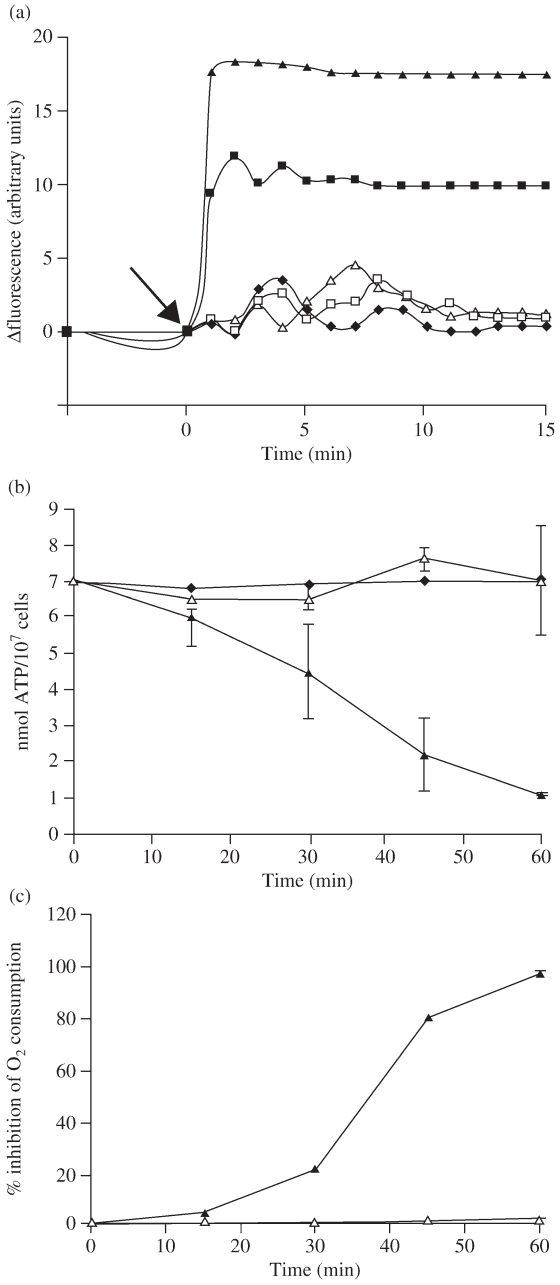

Figure 3. Effect of PC–SA liposomes on plasma membrane potential, intracellular ATP and rate of O_2 consumption of the *L. donovani* AG83 promastigotes. (a) Parasites (2×10^7 cells/mL) were equilibrated with 0.2 μM bisoxonol at 22°C. Changes in fluorescence (arbitrary units) for untreated (filled diamonds) PC–SA liposome treated at 124 (filled squares) and 213 μM (filled triangles) and PC–PS liposome treated at 124 (open squares) and 213 μM (open triangles) concentrations were monitored continuously ($\lambda_{exc} = 540$ nm; $\lambda_{ems} = 580$ nm). The arrow indicates the addition of liposomes at $t = 0$ min. The values represent a single experiment representative of three performed. (b) Relative ATP levels were determined using a Sigma luciferase ATP assay kit at different times after treatment with 213 μM of either SA-bearing (filled triangles) or PS-bearing liposomes (open triangles). Untreated controls (filled diamonds). (c) Oxygen consumption rates of treated and untreated parasite suspensions (1×10^8 cells/mL) were measured in a Clarke-oxygen electrode. Percentage of inhibition of O_2 consumption was determined in the presence of 213 μM PC–SA (filled triangles) and PC–PS liposomes (open triangles) for 15, 30, 45 and 60 min, respectively.

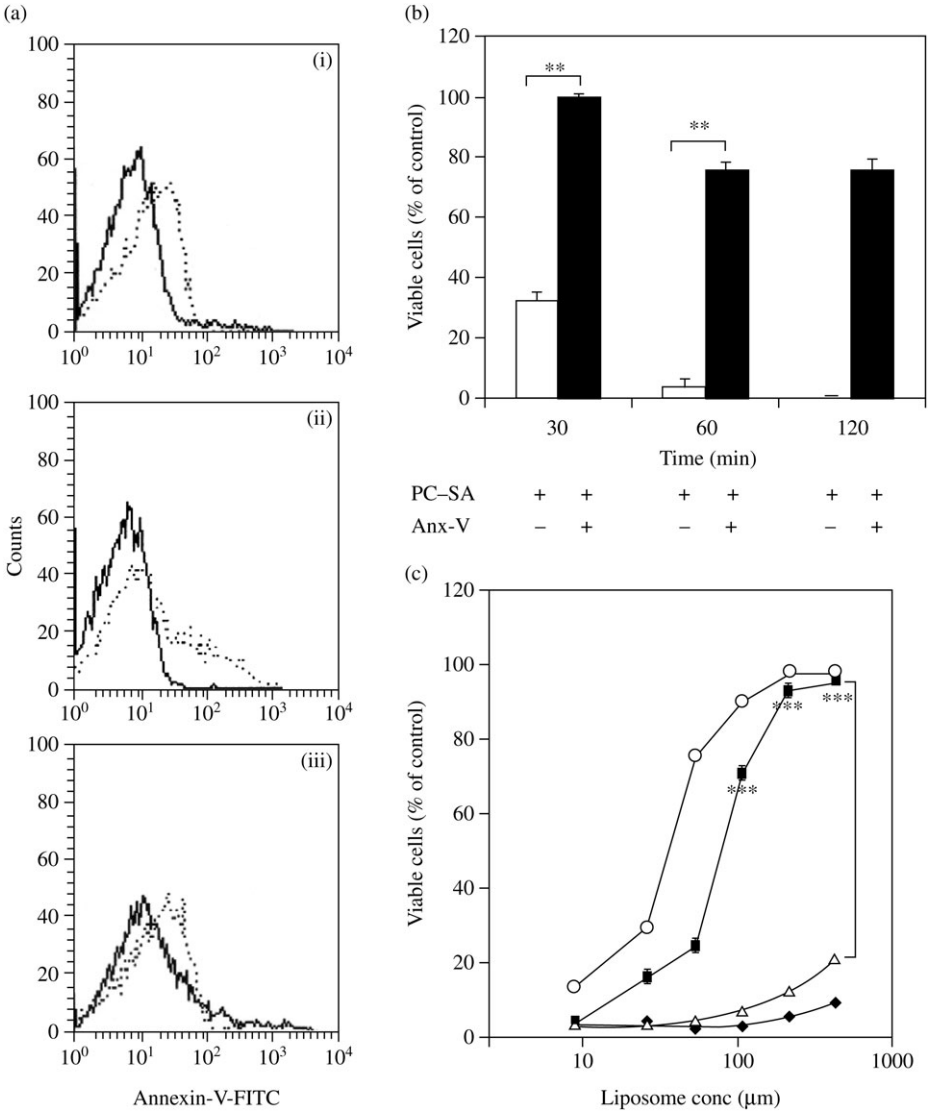

Figure 4. (a) PS is differentially exposed by different forms of *Leishmania*. Cells were incubated with fluorescein-labelled annexinV (broken line) or in buffer alone as control (continuous line) and analysed by flow cytometry. (i) AG83 promastigotes, Δ mean fluorescence intensity (MFI) = 6.92; (ii) freshly isolated AG83 intracellular amastigotes, ΔMFI = 36.56, and (iii) normal murine peritoneal macrophages, ΔMFI = 2.9. Analysis of PS exposure was conducted after monitoring the viability of an aliquot of the parasites by PI incorporation under fluorescence microscope. (b) Blocking of leishmanicidal activity of PC–SA liposomes by annexinV. AG83 promastigotes were incubated with (black bars) or without (white bars) annexinV (1 μg/2 × 10^6) at 26°C for 30 min in binding buffer. PC–SA liposomes were added subsequently and viability of the parasites was determined by MTT assay after 30, 60 and 120 min. (c) Inhibition of leishmanicidal activity of PC–SA liposomes by PC–PS liposomes. PC–SA liposomes (213 μM) were incubated for 30 min with different concentrations of PC–Chol, 7:2 (filled diamonds), PC–PA, 7:2 (open triangles), PC–PS, 7:2 (filled squares) and 1:1 (open circles), liposomes prior to incubation with promastigotes. Data points represent the mean of triplicate samples ± SEM from a single experiment, representative of three different experiments. Significant difference: $^{**}P < 0.001$, $^{***}P < 0.0001$.

Discussion

In the present study, for the first time we report that SA-bearing liposomes kill *Leishmania* parasites through interaction with surface exposed PS. The key for the selectivity towards parasites is due to the abundance of PS on the surface of viable *Leishmania* amastigotes and promastigotes and its lack of expression on normal mammalian cells. Others and we have previously reported on the killing activity of PC–SA liposomes on *Leishmania*[9,11] and other protozoan parasites.[7,8,10] However, why the protozoan parasites are highly susceptible to PC–SA liposomes was not clear. Our present study clearly indicates that the leishmanicidal effect of PC–SA liposomes involves specific interaction with negatively charged PS of parasite membrane, resulting in severe damage of the membrane and ultimate death of the parasite.

Previously, we had shown that these vesicles were cytotoxic against *L. donovani* and were equally effective against other species of *Leishmania*.[9,11] Here, we demonstrated that these vesicles were more effective against intracellular amastigotes. Further, PC–SA liposomes could significantly reduce the organ parasite burden of *L. donovani*-infected BALB/c mice when administered *in vivo* without causing any effect on normal liver function.[11] Herein, a differential susceptibility of different forms

of *Leishmania* to PC–SA liposomes was observed through MTT assay. The rank for susceptibility to the liposomes was freshly isolated intracellular AG83 amastigotes > AG83 promastigotes = R2D2 promastigotes > axenic AG83 amastigotes. The difference in susceptibility could be due to a specific component of the surface membrane that has a higher level of expression on intracellular amastigotes than AG83 and R2D2 promastigotes or axenic AG83 amastigotes. Equal potency of PC–SA liposomes against AG83 promastigotes and LPG mutant R2D2 strain indicated that the component was not the strongly anionic LPG. Almost unaltered viability of macrophages after treatment with PC–SA liposomes indicates that the macrophage is devoid of the specific target component. The ED_{90} of PC–SA liposomes against promastigotes was almost identical to our previous observation with Erythrosine B exclusion.[9] PC–SA liposomes induced marginal haemolysis at a very high dose (data not shown), and were non-toxic towards peritoneal macrophages, demonstrates their specificity for parasites. Our study is the first report showing a cationic vesicle inducing selective membrane disruption in a parasite. A similar mode of killing activity is very common among membrane active leishmanicidal cationic peptides such as temporins and NK-lysin etc.[17,18]

In an attempt to gain insight into the mechanism of how SA-bearing liposomes induce selective membrane disruption of the parasite, effect of charge neutralization of PC–SA liposomes with polyanionic heparin or decrease in electrolyte concentration was investigated and found to play a partial role. Equipotency of PC–SA liposomes for *L. donovani* AG83 and R2D2 indicated involvement of a negatively charged component other than the strongly anionic LPG. Literature survey revealed that surface membrane of *Leishmania* promastigotes contains PC (14.9%), phosphatidylethanolamine (37.7%), phosphatidylinositol (17.9) and PS (9.9%), with traces of cardiolipin and phoshatidylglycerol.[27] Of these, PS was found to be expressed at a higher level on the surface of intracellular amastigotes isolated from BALB/c mice.[15] Surprisingly, we observed a strong correlation of PC–SA-induced parasite killing with the amount of cell surface PS on the different forms of *Leishmania*, and their inactivity toward macrophages correlating with absence of PS on macrophages.[28] This supported our intuition that PS is the component through which PC–SA liposomes interact with the parasite membrane. Recent reports that PS exposure increases the susceptibility of cells to fuse with cationic *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) liposomes,[29] lend support to our view. A similar mode of target selectivity towards cancer cell membrane was reported for NK-lysin derived peptide NK-2.[30] Our results, demonstrating a reduction in killing activity of PC–SA following masking of surface PS with annexinV, confirmed that the small amount of surface exposed PS (10% of total phospholipids) was enough for the observed target selectivity.[27] It is possible that a negative component other than surface PS may affect the sensitivity of parasite to PC–SA liposomes. However, interaction of PC–SA liposomes with biomembrane mimetic model systems (PC–PS, PC–PA or PC–Chol liposomes), prior to parasite membrane exposure, showed that the SA-bearing liposomes had specific affinity for PC–PS liposomes rather than PC–Chol or anionic PC–PA liposomes, further supporting that the specific interaction was mainly through PS.

In conclusion, we propose that SA-bearing cationic liposomes damage *Leishmania* promastigotes and intracellular amastigotes primarily via interaction with surface PS, leading to membrane disruption. The unique mode of selectivity towards PS of *Leishmania* indicates that the SA-bearing liposomes might be valuable as delivery system as well as therapy not only against leishmaniasis but possibly also against other parasitic diseases and cancers which have elevated surface levels of negatively charged phospholipid, i.e. PS.[18,31,32]

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Transparency declarations

None to declare.

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