

Evaluation of toxicity & therapeutic efficacy of a new liposomal formulation of amphotericin B in a mouse model

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Background & objectives: Current therapy for leishmaniasis is limited and unsatisfactory. Amphotericin B, a second-line treatment is gradually replacing antimonials, the first-line treatment and is used as the preferred treatments in some regions. Though, presently it is the only drug with highest cure rate, its use is severely restricted by its acute toxicity. In the present study novel lipid-amphotericin B formulations with lower toxicity than the parent drug were evaluated for the treatment of visceral leishmaniasis (VL) in a mouse model.

Methods: The toxicity and therapeutic efficacy of a new amphiphilic formulation of amphotericin B (Kalsome™10) was compared to that of amphotericin B deoxycholate (Fungizone) in a mouse model of VL using quantitative real-time PCR (qRT-PCR).

Results: The toxicity of amphotericin B was significantly less with liposomal formulation as compared to the deoxycholate form, evidenced by reduced nephrotoxicity and higher tolerated dose in BALB/c mice. The therapeutic efficacy was evaluated by quantitative real time (RT) PCR using primers highly specific for the ITS region of *Leishmania donovani*. There was reduction in parasite load by 2 log unit after 7 days of treatment and finally resulting in complete clearance of parasite from infected mice after 30 days of treatment with Kalsome™10.

Interpretation & conclusions: This new formulation showed a favourable safety profile and better efficacy when compared to conventional amphotericin B. If production cost is kept low, it may prove to be a feasible alternative to conventional amphotericin B.

Key words Amphotericin B - efficacy - leishmania - liposomal formulations - toxicity

Visceral leishmaniasis (VL) or kala-azar is a chronic parasitic disease and is considered one among the ten main tropical illnesses by the World Health Organization¹. It is usually a fatal disease if not treated

and is characterized by irregular fever, malaise, loss of weight, hepatosplenomegaly, and anaemia with or without lymphadenopathy. Unfortunately, as yet no effective vaccine is available against leishmaniasis

and control of the disease relies only on chemotherapy. Presently there are about 25 compounds and formulations showing anti-leishmanial effects but only a few have been proven worthy². Most of the drugs have one or more limitations like unaffordable cost, difficulty in administration, toxicity or more importantly the development of resistance in the parasite. Pentavalent antimony, sodium antimony gluconate (SAG) has long been the cornerstone of anti-leishmania chemotherapy for more than 60 years. However, for the last few years outbreaks of SAG resistant kala-azar in various endemic regions of India have been reported³. As a result, the erstwhile second line treatment, amphotericin B (AmB) has now moved to the forefront for the treatment of kala-azar in India.

Amphotericin B, a polyene macrolide antibiotic, remained the gold standard for the treatment of fungal infection and was strongly recommended as an alternative first-line treatment for leishmaniasis. Despite its high efficacy, its inherent nephrotoxicity significantly limits its successful chemotherapeutic use. Amphotericin B (deoxycholate) induced toxicity is explained by binding of amphotericin B to lipoproteins and internalization of amphotericin B-lipoprotein complex, which is mediated by low density lipoprotein (LDL) receptors⁴. Amphotericin B associated with LDL exhibits toxicity for renal cells. Thus, to increase therapeutic index of the drug and to reduce toxicity, several new lipid formulations of amphotericin B have been developed by replacing deoxycholate with other lipids in which toxicity is partially ameliorated. These formulations include liposomal amphotericin B [L-AmB (Ambisome)], amphotericin B colloidal dispersion [ABCD (Amphocil)] and amphotericin B lipid complex [ABLC (abelcet)]⁵. However, all presently available liposomal amphotericin B formulations are expensive, costing ₹21,855 per patient (per treatment as much a US \$600) for complete kala-azar treatment⁶. This cost is far beyond the reach of poor patients and for government organizations engaged in kala-azar control programmes. Further, these formulations use sterols, such as cholesterol, cholesterol succinate and cholesterol sulphate. Cholesterol is required for the endurance of *Leishmania* and the parasite meets its requirement by salvaging cholesterol from these molecules. Thus, cholesterol containing liposomes nullify the advantage of carrier molecule in targeted delivery of drug. Therefore, suitable lipid constituents must be used as a carrier molecule which should

adversely affect the survival of *Leishmania* by depriving the parasite of its cholesterol requirement. Keeping this in mind, new ionic amphiphilic formulation of amphotericin B (Kalsome™10) has been developed⁷. It is basically, a sterol enriched with mixed lamellarity amphotericin B intercalating liposomes comprising phosphatidylcholine and ergosterol in a ratio where ergosterol constitutes up to 50 molar % of the total lipids of the liposomal formulations. If production cost is kept low, the drug is expected to reduce the cost of kala-azar treatment drastically.

The aim of the present work was to evaluate, *in vivo* anti-leishmanial efficacy and related side effects of Kalsome™10 in the BALB/c mice. Conventional methods of detection and quantitation of the parasite burden in different mouse tissues and for evaluation of anti-leishmanial efficacy of drugs remains microscopic enumeration of amastigotes against host cell nuclei⁸. This method is time-consuming and subjective and may not be reliable if the parasites are not equally dispersed on the slides. Therefore, culture microtitration was developed⁹. This technique is more sensitive than the imprint method, but is labour intensive and time consuming. Other limitations include very low number of the parasites¹⁰. Since recurrences of leishmaniasis are associated with tissue loads of residual and latent parasites after treatment, non-quantitative PCR tests are of little value in indicating a positive or negative result¹¹. Therefore, in present study, therapeutic efficacy of a new anti-leishmanial formulation was evaluated by quantitative real time PCR (qRT-PCR) using primers from the conserved sequences of internal transcriber spacer (*ITS*) region of *L. donovani* nuclear DNA (nDNA), as a proof of evidence of its anti-leishmanial activity.

Material & Methods

The study was carried out between 2005 and 2007 at the All India Institute of Medical Sciences (AIIMS), New Delhi. Amphotericin B deoxycholate (Fungizone) was purchased from Ambalal Sarabhai Enterprises Ltd, Vadodara, India. New formulation of the liposomal amphotericin B, Kalsome™10, was a kind gift from Lifecare Innovations Pvt. Ltd. Gurgaon, India. For use, the commercially available AmB deoxycholate was reconstituted in sterile, chilled, triple-distilled water to obtain a stock solution of 5 mg/ml. All other reagents used were of analytical grade (Sigma-Aldrich, St Louis, USA).

Experimental animals and strain of Leishmania donovani: Experiments were conducted on BALB/c male mice (5-6 wk old, 25±30 g) purchased from Laboratory Animal Facility of National Institute of Nutrition, Hyderabad, India. The animals were maintained by giving a standard pellet diet and water *ad libitum*. Mice were checked daily for their mortality and waning prior to commencement of the study and only healthy mice were included. The study protocol was approved by the Institute's Animal Ethics Committee.

HM/IN/KE16/1998 strain of *L. donovani* isolate from an untreated child from Muzaffarpur district of Bihar (eastern India) was used for infection establishment¹². The parasite culture is being maintained in Medium 199 with 25mM HEPES, pH 7.4 (Hi-Media, Mumbai) supplemented with 10 per cent heat inactivated foetal bovine serum (Sigma-Aldrich, USA) at 22° C in a Biological oxygen demand (BOD) incubator.

Electron microscopic analysis of Kalsome™10: To increase the plasma half-life of the liposomal drug Kalsoma™10 (10 mg/ml of amphotericin B) for facilitating better bio-distribution in the body bath sonication was carried out for 45 min on ice. Sonicated and non-sonicated preparations of Kalsome™10 were observed under the scanning electron microscopy. Analysis was carried out on LEO scanning electron microscope available at electron microscopy facility of AIIMS.

In vivo single dose drug toxicity study: Groups of six mice each were injected with various concentrations of conventional Fungizone (ranging from 1-3 mg/kg of body weight) or new liposomal amphotericin B formulation: Kalsome™10 (range, 15-20 mg/kg) and empty liposomes *i.e.* liposomes without AmB (range, 20-100 µl) as control, intravenously. The toxicity of the drug was evaluated *in vivo* by determining the 50 per cent lethal dose (LD₅₀). For this, the mortality rate and activity levels of animals were monitored for 24 h.

Chronic toxicity study: The chronic toxicity was performed according to the Organization for Economic Co-operation and Development (OECD) test guidelines with minor modifications, as the purpose was not regulatory clearance but to provide an evidence of its efficacy¹³. Healthy mice were randomly assigned into four groups: A, B, C and D (6 per group). Daily administration of the LD₅₀ as estimated above for Fungizone and Kalsome™10 through the known route was given for 1 wk to group A and B, respectively.

In group C normal saline was given while in group D empty Kalsome (liposomes without AmB) was given using the same route. Animals were sacrificed on day 28 after the last dose of the drug. Blood, liver, kidney and spleen tissues were collected aseptically for histopathological evaluation. Biochemistry analysis of blood urea nitrogen (BUN), creatinine and glucose level, was done using the commercially available kits in a semi-automated chemistry analyzer (Techno-168) (I.S.E Sr.t. group, Italy s.r.l.).

Establishment of infection and therapeutic protocols: To determine, the duration required to establish the *L. donovani* infection, group of six BALB/c mice, were infected with *L. donovani* (MHOM/IN/KE16/1998) strain¹². For infection, the log-phase promastigotes of the parasite were washed twice using PBS buffer (pH 7.4) at 2300 g at 4 °C. Finally, the pellets were re-suspended in PBS buffer for intravenous infusion and approximately 1×10⁷ promastigotes/ml were injected into the tail vein using 26 gauge needle. The day of infection was termed as day 0 (D0). The animals were daily observed for gross physiological and behavioural changes, if any. These included loss of hair, other signs of being unwell such as fever, loss of weight and appetite and enlargement of spleen and liver were observed for 3 wk post-infection.

After visible splenic enlargement, and other symptoms, two mice were sacrificed on every alternate day. Blood was collected and tested for the presence of antibodies against visceralising species of *Leishmania* using the commercially available Lc-rK39 (recombinant antigen prepared from *L. chagasi*) dipstick test kits (Insure™, In-Bios Int, USA) and Ld-rKE16 (recombinant antigen prepared from *L. donovani*) spot test (Signal KA™, Span Diagnostic Ltd., Surat, India). Liver and spleen samples were taken out under sterile conditions, homogenized in mortar and pestle and were subjected to PCR analysis for confirmation of infection. For DNA isolation 300 µl of homogenized sample was taken and re-suspended in two volumes of lysis buffer [50mM NaCl, 50mM Tris-HCl, 10mM EDTA (pH 7.4), 1 per cent Triton X-100, 200 µg/ml of proteinase K]. The lysed samples were then incubated in water bath for 2 h at 60°C. Thereafter the genomic DNA of *Leishmania* was extracted using phenol-chloroform extraction and finally suspended in Tris-EDTA (TE) buffer or sterile water. A 1020 bp region of genomic DNA was amplified, using the following primer set¹⁴. Forward, ITSF→5'CTGGATCATTTTCCGATG-3', and Reverse, ITSr→5'ACACTCAGGTCTGTAAAC-3'.

Since amplicon size of our product was 1000 bp (ideal 100-500 bp real time PCR), the RT-PCR was standardized with low salt concentration and adjusted reaction time. PCR was performed in 50 μ l reaction volumes containing 100 ng of genomic DNA, 10 pmol each of the gene-specific forward and reverse primers, 10 μ M of each dNTP, 2 mM MgCl₂ and 5 units of Pfu Taq DNA polymerase (Bangalore Genei, India). The conditions for PCR were as follows: 95°C for 2 min, then 34 cycles at 95°C for 20 sec, 53°C for 30 sec and 72°C for 1 min. Final extension was carried out at 72°C for 1 min.

Leishmania infected mice were randomly assigned to three groups; groups A, B and C (6 mice per groups) and treatment was started one week after establishment of infection. Groups A and B mice were treated on each alternate day with Fungizone (2 mg/kg of body weight) and 0.75X dose of Kalsome™ (7.5 mg/kg of body weight, half of the LD₅₀), respectively. Group C including six healthy mice was used as negative control

group (Fig. 1). Drugs were injected by the intravenous route in 0.1 ml volume. Three mice were sacrificed by cervical dislocation at day 7, and the remaining three at the termination of the study *i.e.* day 28 post-treatment.

Assessment of efficacy

Determination of parasite burden: Detection of parasitic load in liver was carried out by blinded microscopic enumeration with Giemsa-stained liver impression smear in three groups. Leishman-Donovan units (LDU) were calculated per organ for the liver by using the formula [LDU= number of amastigotes per 1,000 nucleated cells x organ weight (in g) x 2x10⁵], according to Stauber's formula¹⁵.

(ii) *Quantitative real-time PCR for quantitative analysis of parasite load:* Both liver and spleen are well accepted system for assessment of *L. donovani* infection, however, due to larger volume of the tissue liver was used for quantitation of parasite load. A real-time hot-start PCR was performed with the Smart

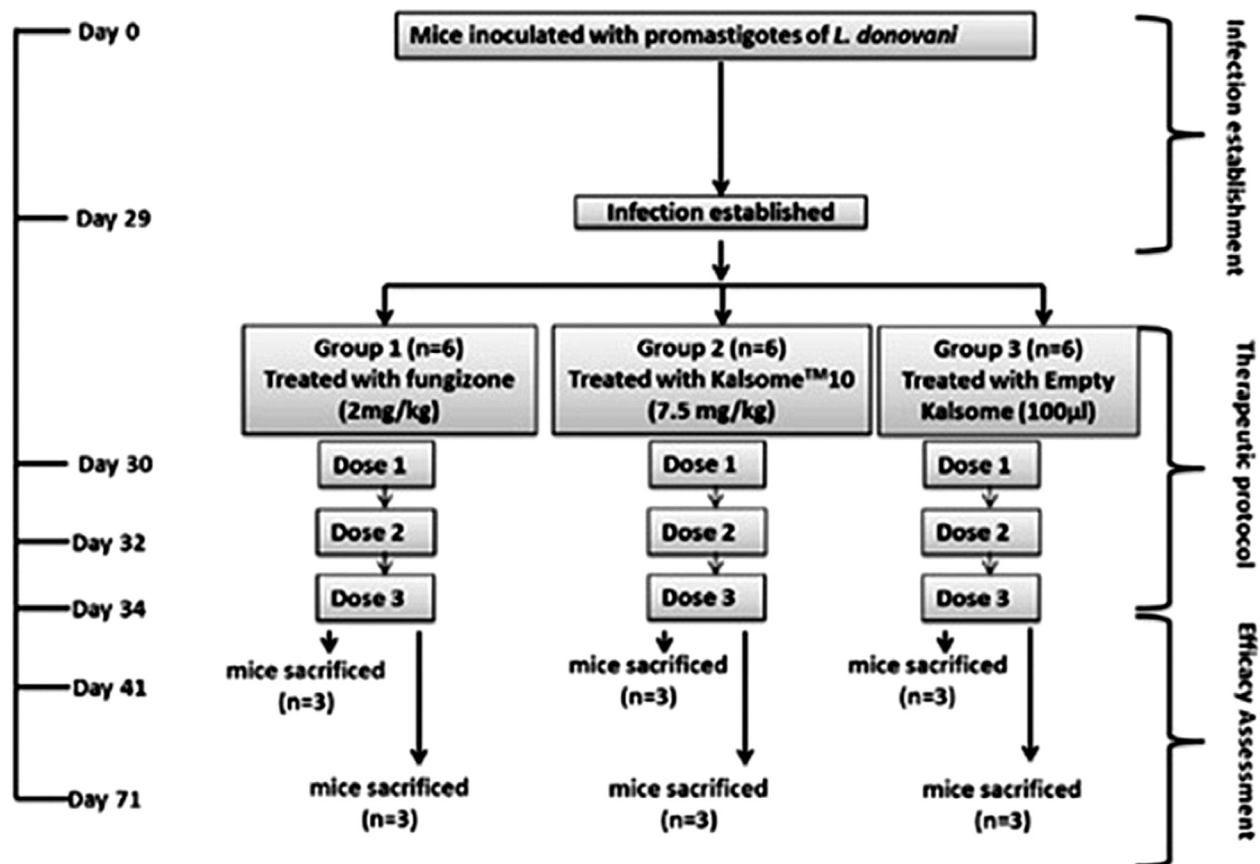


Fig. 1. Flowchart summarizing the experimental details of the study.

Cycler DNA Master SYBR Green Kit (Cepheid, USA) in a Smart Cycler (Cepheid, USA) to determine the parasitic load in liver tissues of control group as well as in Fungizone and Kalsome -treated mice. The 25 μ l reaction mixture contained 12.5 μ l DNA Master SYBR Green, 0.5 μ M each primer and 1 μ l of template. Time and temperatures were same as used in normal PCR reaction. For fluorescence signal acquisition, channel F1 was used and standard threshold of 30 fluorescence was set. The fractional cycle number reflecting a positive PCR result is called the cycle threshold (C_T).

Statistical analysis: To determine the variability of the assays, intra- and inter-assay (repeatability) precision was measured. Three replicates of eight different concentrations of *L. donovani* DNA were tested simultaneously in the same run. Variability is shown as the mean \pm standard deviation (SD) and reported as the coefficient of variation (CV). Statistical procedures were performed using Microsoft Excel and STATA Statistics Package 9 (Stata Corp. College Station, Tx, USA). Statistical analyses were performed by using an unpaired t-test (two-tailed) followed by Mann-Whitney test using, GraphPad Prism5 version (GraphPad Software Inc., La Jolla, CA).

Results

Electron microscopic features of the new formulation: The sonicated and non-sonicated drug samples revealed typical micro sphere structure of approximately 1.0 μ m in diameter. These micro spheres tend to aggregate in non-sonicated samples, while after sonication these got separated (Fig. 2A & B). This finding is crucial for the clinical use of this formulation.

LD₅₀ evaluation: The LD₅₀ for standard drug Fungizone was estimated to be 2 mg/kg of body weight of the mice. The LD₅₀ for KalsomeTM10 (10 mg/ml) was 15 mg/kg of body weight of the mice. Hence, KalsomeTM10 (10 mg/ml) was markedly less toxic to mice. Study on empty Kalsome revealed that the carrier molecule used was non-lethal to the animals (Fig. 3).

Toxicity in animal models: Treatment with new liposomal formulation was found to be associated with less nephrotoxicity compared with treatment with fungizone. The level of serum creatinine and blood urea nitrogen increased significantly in mice treated with Fungizone. Creatinine value increased >2-fold over base line values (0.7 \pm 0.04 and 0.28 \pm 0.02 mg/dl; P >0.001) and blood urea nitrogen level increased by > 2.5 fold (64.8 \pm 1.04 and 22.4 \pm 1.74 P <0.001).

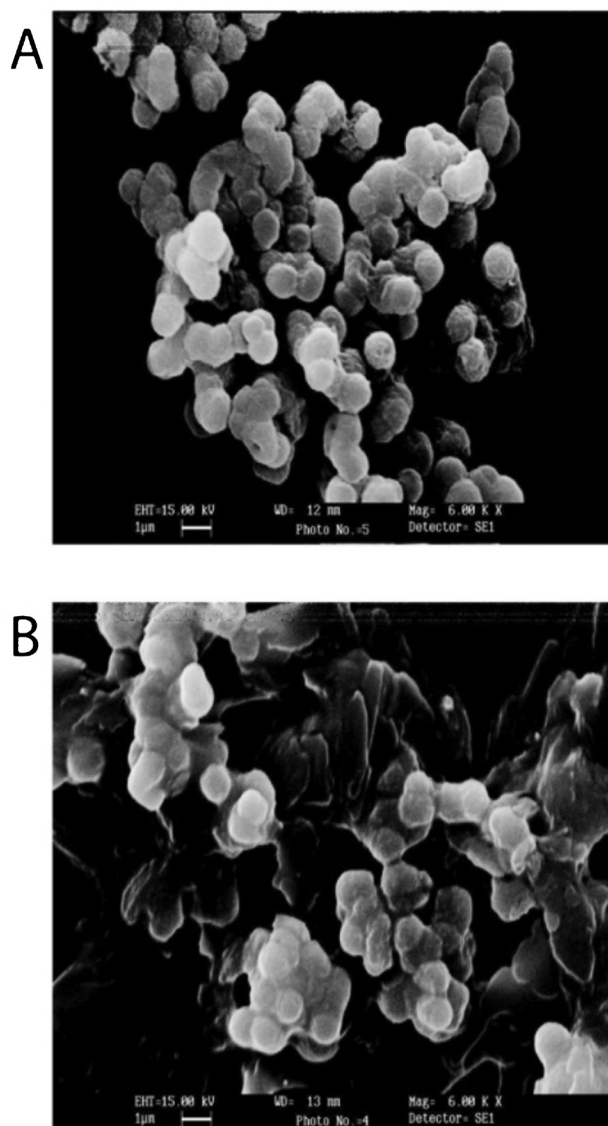


Fig. 2. Amphotericin B loaded liposomal microspheres (KalsomeTM10) analyzed by scanning electron microscopy. (A) sonicated (A) nonsonicated preparation of KalsomeTM10.

Mice treated with Kalsome showed minimal renal function impairment (creatinine value 0.40 \pm 0.04 mg/dl; and blood urea nitrogen level 24.1 \pm 0.9 mg/dl) (Table I). Serum glucose levels were almost similar in all experimental groups. The analysis of kidney tissue revealed normal histopathology in all treatment groups except the 2 mg/kg Fungizone group, which showed focal lesions consisting of inflammatory cells. Some dilation and engorgement were seen in blood vessels. Mild hemosiderosis and a few megakaryocytes were also seen showing degenerative features. The histology assessment in the spleen and liver did not reveal any vascular changes (data not shown). Empty

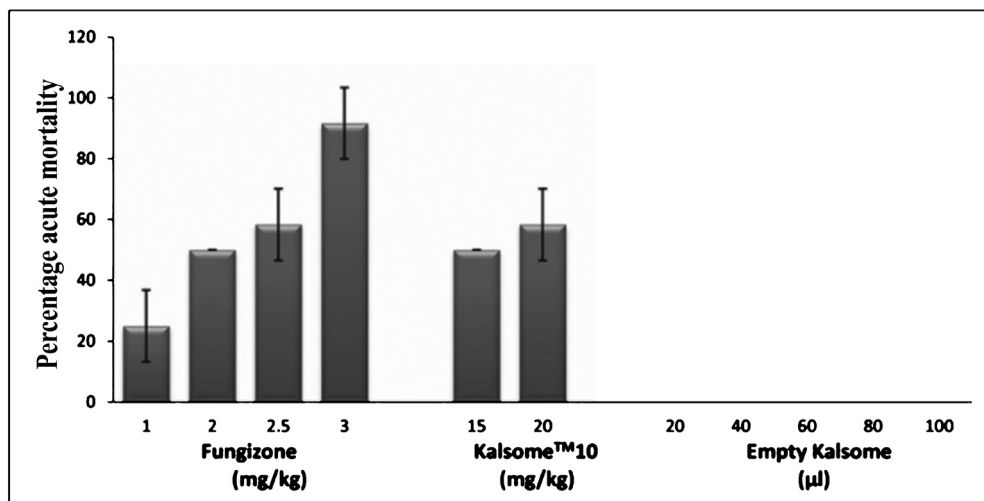


Fig. 3. Single dose drug toxicity study. Values are expressed as mean of three separate experiments \pm SEM.

liposome did not impart any untoward characteristic to amphotericin B liposomes. Mice injected with new liposomal formulation showed more tolerance to the drug as shown by the high tolerated dose of AmB.

Assessment of infection establishment: Serological tests (both rK39 and rKE16) were found positive for antibodies against *Leishmania* in all the six mice in group I on day 29. Further establishment of infection was confirmed by conventional PCR using *ITS* region primers of *L. donovani* (Fig. 4).

qRT-PCR for detection of visceral leishmaniasis from mouse tissue: Quantitative real-time PCR was standardized using the same *ITS* region primers of *L. donovani* and a standard curve having C_T value on X-axis and number of parasitic genomes on Y axis were plotted (Fig. 5). To determine the detection limit of assay and establish a standard curve that could be used for quantitation, serial dilutions of *L. donovani* DNA with final concentrations ranging from 10,000 parasites

to about 1 parasite per reaction were used to a real-time PCR analysis. As little as 0.6 parasite could be detected, corresponding to approximately 600 fg, per reaction in a 20- μ l reaction volume. The mean standard curve, calculated from three independent experiments, was linear over 8-log range of DNA concentrations, with a correlation coefficient of 0.756 (Fig. 5). A negative control consisting of the reaction mixture and water instead of template DNA was added in each run. A melting curve analysis of PCR products showed that the melting temperature of the DNA amplicons was ca. 88.5°C. Real-time PCR efficiency was calculated from the slope. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ as described by Pfaffl¹⁶ (Fig. 5). Investigated transcripts showed 112 per cent real-time PCR efficiency.

Three replicates of eight numbers of 4-fold DNA concentrations, from 10^4 parasites per reaction to 0.6 parasite per reaction, were assessed in a single run. Three

Table I. Changes in serum glucose, serum creatinine and blood urea nitrogen (BUN) level in BALB/c mice treated with Fungizone, Kalsome™10, normal saline and empty kalsome

Treatment group	Dose	Serum glucose level (mg/dl)	Serum creatinine level (mg/dl)	Blood urea nitrogen level (mg/dl)
Normal saline	100 μ l/day	79.03 \pm 0.167	0.28 \pm 0.02	22.4 \pm 1.74
Empty Kalsome	100 μ l/day	78.9 \pm 0.163	0.29 \pm 0.016	21.23 \pm 0.74
Fungizone	2 mg/kg/day	79 \pm 0.297	0.7 \pm 0.04*	64.8 \pm 1.04*
Kalsome	15 mg/kg/day	79.18 \pm 0.319	0.4 \pm 0.04	24.1 \pm 0.9

Values are expressed as mean of three separate experiments \pm standard errors of mean (S.E.M)

* $P < 0.001$ compared with control (normal saline injected mice)

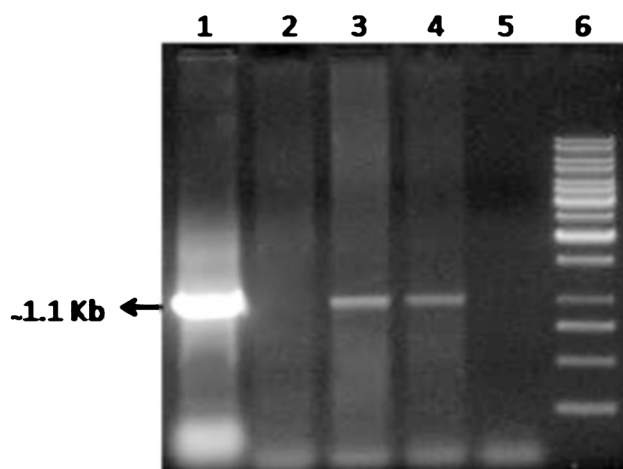


Fig. 4. PCR detection of *Leishmania* infection in mice. BALB/c mice were infected with $\sim 1 \times 10^7$ promastigotes/ml of *L. donovani*. After 3 wk post-infection liver and spleen samples were taken out under sterile conditions, DNA was isolated and subjected to PCR analysis for confirmation of infection. Mice injected with normal saline were served as negative control. Lane 1 - Positive control (KE-16 strain DNA), lane 2 - PCR product from liver tissue sample of saline infected mice (healthy mice), lane 3 - PCR product from liver tissue sample of *Leishmania* infected mice (challenged mice), lane 4 - PCR product from spleen tissue sample of *Leishmania* infected mice (challenged mice), lane 5 - Blank (distilled water), and lane 6 - 1 Kb molecular weight marker.

replicates of 4-fold *L. donovani*_DNA dilutions were performed on different days. The inter-assay variations of C_T values for the number of parasitic genome ranging from 10^4 to 0.6 were 2.89, 0.82, 0.84, 2.94, 2.62, 2.48, 1.26 and 1.24 per cent, respectively (Fig. 5).

Efficacy of KalsomeTM10 in experimental VL: Microscopic enumeration for the assessment of the amastigote burden one week after the completion of therapy showed that in untreated animals the amastigote burden (expressed in millions of LDU) was 200 ± 18

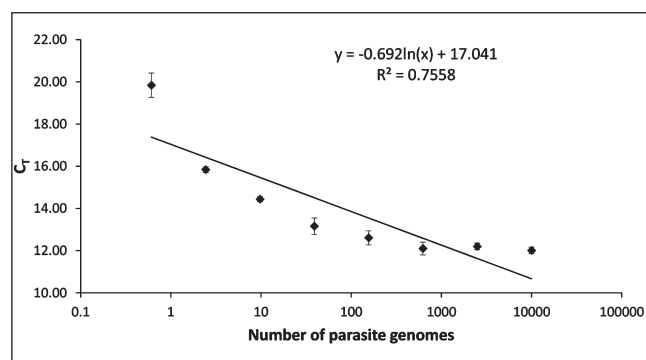


Fig. 5. Sensitivity of qRT-PCR. A plot of mean cycle threshold (C_T) value against the logarithmic concentration of parasite DNA (10-1000 parasites per reaction).

LDU but in Fungizone treated mice it decreased to 2.3 ± 2 LDU (Table II). However, the decrease in parasite burden was more in KalsomeTM10 (7.5 mg/kg)-treated group (1.4 ± 1.4 LDU). The amastigote suppression efficacy of KalsomeTM10 was 98.85 per cent as compared to 99.3 per cent for Fungizone which is a standard drug. After one month, no LDU was detected in any of the treated groups resulting in complete clearance of parasite.

Results were also confirmed by qRT-PCR. Of total DNA eluted from 100 mg of tissue, ~ 100 ng of DNA was used for qPCR experiment. The number of *Leishmania* genomes found in 100 ng is further calculated in per gram of liver. The mean \pm SD C_T value for the control group was 12.23 ± 0.127 . At day 7, the mean C_T value decreased to 14.15 ± 0.22 and 14.58 ± 0.21 in the Fungizone and Kalsome treated groups, respectively. The amastigote suppression efficacy of fungizone was 98.79 per cent and that of Kalsome was 99.55 per cent in these experimental conditions. The C_T value >35 was observed at day 30 for both the treated groups (Table III). In qRT-PCR evaluation the mice treated with KalsomeTM10 reduced parasite burden by about 2.34 log units within one week and finally resulted in complete clearance of parasites. In all of the assays, the C_T values of negative controls were always >35 .

Discussion

Efficient and cost effective short course treatment of leishmaniasis remains a much desired research question¹⁷. The major limitations of the existing treatment options for VL are the emergence of unresponsive strains^{18,19} and cost of the treatment^{20,21}. Amphotericin B is the current second line drug of choice with 97 per cent cure rate and no reported resistance^{22,23}. The limitation is infusion related toxicities, especially the renal toxicity^{24,25} and other adverse effects restrict its use. However, the systemic side effects are reported to be considerably reduced by the particulate forms of liposomes which are highly effective as well as require a shorter course of 3-5 days therapy and facilitate targeted delivery of liposomal amphotericin B but, the stability and cost of these formulations seriously limit their widespread use.

Choice of sterols is a critical factor in designing of liposomal formulation of a drug specifically to prevent binding of liposomal amphotericin B to mammalian host cells. The therapeutic antimicrobial action as well as toxicity of amphotericin B to the host cells result from its binding with sterols of the cell membranes, resulting

Table II. Efficacy of new amphiphilic formulation of amphotericin B (Kalsome™10) against established *L. donovani* infection in BALB/c mice

Treatment group	Body wt (g) ^a	Relative liver wt (%)	Amastigote load [x10 ⁶ (% efficacy) ^b]	
			One week post- treatment	One month post- treatment
Fungizone (2 mg/kg)	23 ± 0.7	2.8 ± 0.1	2.3 ± 2 (98.85%)*	0
Kalsome (7.5 mg/kg)	24 ± 0.8	2.5 ± 0.5	1.4 ± .14 (99.3%)* ^s	0
Positive control	22 ± 0.5	3.5 ± 0.6	>200 ± 20	>230 ± 5

Data are means ± SEM for 6 mice in each group

^aOrgan weight/body weight; ^bAmastigote load or LDU= number of amastigotes per 1,000 nucleated cells x organ weight (in g) x 2x10⁵.
Per cent efficacy= [1- (mean amastigote load in drug treated mice/mean amastigote load in control mice)] x 100

*P<0.001 compared with control (normal saline injected mice); ^sP<0.001 (compared with Fungizone injected mice)

Table III. Results of real-time and conventional PCR assays for *Leishmania* DNA in mouse tissue samples

Treatment group	Results after one week of treatment		Results after one month of treatment	
	Conventional PCR	qRT-PCR (C _T)	Conventional PCR	qRT-PCR (C _T)
Fungizone (2 mg/kg)	+	14.15 ± 0.22	-	>35
Kalsome (7.5 mg/kg)	-	14.58 ± 0.21	-	>35
Control group	+	12.23 ± 0.12	+	12.15 ± 0.13

Values are mean ± SEM of n=3

in pore formation and finally leading to cytolytic action of the drug. Ergosterol in combination with certain lipids in liposomes appears to be a better choice since the drug has stronger affinity for the ergosterol or their precursor in *Leishmania*²⁶ than to cholesterol, present in most mammalian cell membranes. Also, cholesterol is an essential requirement for the endurance of *Leishmania*, and cholesterol containing liposomes may serve as a source of cholesterol for *Leishmania*.

In the present study the toxicity as well as efficacy of the new liposomal formulation of amphotericin B were evaluated against established *L. donovani* infection in BALB/c mice. The study demonstrated a major reduction in the toxicity of amphotericin B upon incorporation in liposomes compared with amphotericin B deoxycholate. The evaluation of the toxicity of liposomal amphotericin B (Kalsome™10) in mouse model involved the study of LD₅₀ profile of new drug. Further, the safety study of lipid-based carrier *i.e.* empty Kalsome (liposome without amphotericin B) showed it to be safe for application. *In vivo* single dose acute toxicity study showed that amphotericin B upon encapsulation in liposomes induced less toxicity in comparison with amphotericin B deoxycholate which would allow accommodation of much higher doses of the drug for *in vivo* use. Mice treated with new liposomal

formulations of amphotericin B showed a maximum tolerated dose that was 7-fold greater than amphotericin B deoxycholate which would be advantageous to less susceptible or drug-resistant isolates.

In vivo renal toxicity was assessed by estimating serum creatinine, blood urea nitrogen and glucose level in mice. Though the levels of serum creatinine and blood urea nitrogen were elevated in mice treated with amphotericin B deoxycholate, no renal or hepatic alterations occurred, as evidenced by normal levels of creatinine and blood urea nitrogen levels in liposomal amphotericin B treated mice.

The present study showed *in vivo* efficacy of this new liposomal formulation of amphotericin B using the *L. donovani* infected BALB/c mouse model by real time PCR. The conventional PCR and gel based visualization of amplified products has several limitations such as time, low sensitivity, short dynamic range < 2 logs, low resolution, non-automated, only size-based discrimination and non-quantitative results²⁷. To overcome these limitations, highly sensitive and specific quantitative real time PCR method was used for anti-leishmanial efficacy. Though, 100-500 bp is optimal size for amplicons of real time PCR, but up to 1000 bp was possible with low salt concentration

and adjustment in reaction time. This method was used not only to detect the *L. donovani* infection in the experimentally infected animals but also to quantify the parasite load accurately up to a minimum detection level of 1 parasite per reaction. Treatment of infected mice with KalsomeTM10 revealed that it reduced parasite burdens more efficiently than amphotericin B deoxycholate. Though, potential limitation of this study included the fact that daily dose- sub acute toxicity tests and subchronic toxicological evaluations could not be done as per OECD guidelines. Hence, these results cannot be used for regulatory purposes, as the purpose of this study was only collecting the evidence of efficacy and diagnostic modalities to assess the efficacy. Single dose acute toxicity experiments and chronic toxicity experiments were performed according to the OECD test guidelines¹³ and the results showed that the new formulation was efficacious and displayed no major toxic effects in the animals.

Conflicts of interest: Authors declare no conflicts of interests.

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