KALSOME™10 AS A POTENTIAL THERAPEUTIC AGENT AGAINST VL
Introduction

Visceral leishmaniasis (VL) or kala-azar, a disseminated infection of the lymphoreticular system, is caused by the protozoan parasite(s) Leishmania donovani and/or L. infantum/chagasi. An estimate of 0.2-0.4 million global VL cases are reported each year with more than 90% of them occurring in India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia (Alvar, Velez et al. 2012). Human infections can be asymptomatic or oligosymptomatic with manifestations including persistent low-grade fever, hepatosplenomegaly, cachexia, pancytopenia and hypergammaglobulinemia. During active VL the parasites multiply within the macrophages of spleen, liver and bone marrow resulting in fatal outcome if untreated (Goto and Prianti 2009). The course of disease progression signifies cell-mediated immune response (CMI) to play an important role in protection or development of VL (Asad and Ali, 2014). Active VL is characterized by suppression of CMI, which is apparent from the unresponsiveness of patients to different delayed type hypersensitivity (DTH) tests (Leishmanin skin test or Montenegro test) as well as the defective lymphoproliferative response of the peripheral blood mononuclear cells (Bhattacharya and Ali 2013). Recovery from infection following an effective chemotherapy, on the other hand, is associated with a strong cell-mediated DTH response (Saha et al. 2006). Thus a favorable CMI in response to appropriate treatment marks the successful cure of VL.

In the absence of a vaccine, chemotherapy is the mainstay to combat the disease. Until the year 2000, pentavalent antimonials (Sbv) were the frontline treatment for VL. However, with the emergence of antimony-resistant parasites in parts of northern Bihar, India, amphotericin B (AmB) was introduced which renders high cure rates (>95%) in VL patients (Mondal et al. 2010). However, AmB which requires intravenous administration of long duration is highly toxic and has frequent adverse effects, including infusion related fever and chills, nephrotoxicity, hepatotoxicity and hypokalemia (Saha et al. 2007). To overcome its drawbacks lipid formulations of AmB (L-AmB) have been developed to reduce organ toxicity as well as treatment duration. Most of these formulations are, however, exorbitantly costly. Although WHO has recently reduced the price of one such formulation, AmBisome, the cost of
these L-AmBs still precludes their widespread use in the developing countries (Ejazi and Ali 2013). In this context an Indian formulation of L-AmB, Fungisome has been introduced. A clinical trial with this drug at doses of 2 mg/kg for 10 days, 3 mg/kg for 5 days and 3 mg/kg for 7 days yielded cure rates of 100%, 90.9% and 100% respectively (Bodhe et al. 1999). These encouraging results paved the way for a short-course therapy to determine whether a relatively larger amount of Fungisome could be safely and effectively administered at single (5 and 7.5 mg/kg) or double (5 mg/kg) doses. The treatment regimens manifested a successful cure rate of 50-90% at 6 months posttreatment. A close look at the immunological profile of the VL patients at one week after treatment revealed a significant fall in plasma IL-10 levels in all successfully cured patients. Investigations on the antigen-specific cytokine production by PBMCs showed an enhanced Th1 type response with upregulated IFNγ, IL-12 and TNFα and reduced IL-10 and TGFβ production one week posttreatment in patients who were successfully cured at 6 months irrespective of the drug-dose (Mondal et al. 2010). These results with Fungisome could match the immunological profile obtained with AmB as early as one week posttreatment demonstrating the importance of immunomodulatory effects exerted by the disease inhibiting cytokines for successful cure (Saha et al. 2007; Mondal et al. 2010).

Most of the L-AmBs in clinical use contain cholesterol as one of the constituents and is useful for stability and targeted delivery of the drug (Bodhe et al. 1999; Timmers et al. 2000; Sundar and Rai 2005). Cholesterol, on the other hand, plays a crucial role in active VL facilitating the internalization of the parasites (Pucadyil et al. 2004). Recent work of Chandel et al. demonstrates that exogenous cholesterol, if added in the culture, can enhance the growth of Leishmania promastigotes (Chandel, 2014). Therefore, after internalization, the cholesterol requirement by Leishmania for its sustenance may be fulfilled by salvaging cholesterol from host macrophages. Hence, formulation of liposomal AmB devoid of cholesterol is a good strategy in designing antileishmanial drugs. Thus, new amphiphilic L-AmB, KALSOMETM10 has been developed where AmB is intercalated with sterol and phosphatidyl choline. This is a sterol rich drug where ergosterol constitutes 50% molarity of total lipid in the liposome (Asad et al. 2015). The absence of cholesterol could make this drug more
suitable for clearing parasites. A recent study with KALSOMETM10, at 7.5 mg/kg triple dose, reported successful therapy (98.85% amastigote suppression) of BALB/c mice with established L. donovani infection (Mishra et al. 2013). Here we have evaluated lower doses of KALSOMETM10 (3.5 mg/kg single dose, 7.5 mg/kg single dose and 7.5 mg/kg double dose) for their efficacy in curing murine VL. In addition, we were interested to investigate whether a similar modulation of cytokine profile as observed with AmB treatment could be obtained with KALSOMETM10 to identify the possible mechanism of cure. Along with efficacy, tolerability of these doses was also evaluated through the different liver (SGOT, SGPT and alkaline phosphatase) and kidney (urea and creatinine) functioning parameters.
**Results**

**Effective clearance of *Leishmania* parasite by KALSO**

To estimate their efficacies, low doses of KALSO (3.5 mg/kg single dose, 7.5 mg/kg single dose and 7.5 mg/kg double dose) were injected into 2 month infected BALB/c mice. One month after treatment LDA was performed to evaluate parasite burden at the sites of infection *i.e.* liver and spleen. Treatment with 3.5 mg/kg and 7.5 mg/kg single doses of KALSO showed a substantial fall (~3-fold) in parasite levels in these organs compared to infected controls. Administration of 7.5 mg/kg double dose, on the other hand, resulted in almost complete clearance of parasite from both liver and spleen (Fig. 5.1 A and C). Although all the drug doses were significantly effective in clearing the parasites from liver and spleen (*P* < 0.001), 7.5 mg/kg double dose appeared to be most effective with parasite clearance efficiency significantly higher (*P* < 0.001) than even 3.5 mg/kg single dose. The weights of infected liver and spleen also gradually decreased with increase in drug doses (Fig. 5.1 B and D)) when compared with infected controls, although the difference was not significant. Moreover, the efficacy of KALSO 10 was comparable to that of AmB and AmBisome (Fig. 5.1 A and C). Treatment of infected mice with 3.5 mg/kg single dose of KALSO 10 generated similar efficacy as that with a single dose of 3.5 mg/kg AmBisome and 2.5 mg/kg AmB when compared in both liver and spleen.
Figure 5.1: Efficacy of KALSOME™10 in murine VL. Two months infected animals were treated with 3.5 mg/kg single dose (SD) and 7.5 mg/kg SD and double doses (DD) of KALSOME™10 in 200 μl of 0.02 M PBS through the tail vein. One month post treatment, LDA was performed for evaluating their efficacy. Parasite burdens (A and C) and weights (B and D) of liver and spleen show the changes at different doses with normal and infected mice. Data expressed as means ± SE for six mice per group and are representative of two independent experiments with similar results. ***P < 0.0001.
In vivo toxicity study of effective dose of KALSOME™ 10 in non-challenged BALB/c mice

To detect any functional abnormality of liver due to KALSOMETM 10 treatment, the drug was administered into normal healthy BALB/c mice at three different doses (3.5 mg/kg single dose, 7.5 mg/kg single dose and 7.5 mg/kg double dose) and SGPT, SGOT and alkaline phosphatase levels measured at 14 days posttreatment. Treatment with single drug doses of 3.5 mg/kg and 7.5 mg/kg resulted in comparable levels of SGPT and alkaline phosphatase whereas administration of 7.5 mg/kg double dose exhibited a minor increase in these parameters compared to untreated controls (Fig. 5.2 A and C). Levels of SGOT in mice treated with 3.5 mg/kg single dose remained comparable to normal values (Figure 4B). However, 7.5 mg/kg single dose and double dose treated mice exhibited some rise in SGOT levels compared to controls. These differences, however, were insignificant indicating no aberration in liver function due to drug administration.

Along with hepatotoxicity, nephrotoxicity is also a major challenge for a prospective drug candidate against leishmaniasis. The mean level of creatinine for all the drug doses was found to be similar (Fig. 5.2E) with a small but insignificant increase in urea levels at 7.5 mg/kg single and double doses when compared to untreated controls (Fig. 5.2 D).
Figure 5.2: Toxicity study of KALSOME™10 in normal mice. Healthy BALB/c mice were treated with 3.5 mg/kg SD and 7.5 mg/kg SD and DDs of KALSOME™10 in 200 µl of 0.02 M PBS through the tail vein. Fifteen days post treatment, liver [SGPT (A), SGOT (B) and Alkaline Phosphatase (C)] and kidney [urea (D) and creatinine (E)] functioning tests were performed on mice serum and compared with that of healthy controls. Data represented as means ± SE for six mice per group and are representative of two independent experiments with similar results.
**KALSOME™ 10 Against VL**

**KALSOME™ 10 mounts a protective response against VL by regulating different disease promoting/inhibiting cytokines**

VL is associated with impaired cell mediated immunity marked by the inability of T cells to proliferate or to produce IFNγ in response to leishmanial antigens. Recent investigations have reported the ability of these cells to respond to leishmanial antigens with the production IL-10 and TGFβ, the key disease promoting factors in VL (Gregoriadis 1995).

The development of resistance and control over parasites require the production of IL-12 from antigen presenting cells and IFNγ from T cells (Gregoriadis 1995). To investigate whether KALSOME™ 10 therapy can modulate the disease promoting immune response to effective immunity we carried out a detailed immunoprofiling of leishmanial antigen stimulated splenocyte culture supernatants of normal, infected and treated mice. Three month infected BALB/c mice demonstrated significantly higher levels of IL-10 (P <0.0001) and TGFβ (P < 0.001) in comparison to normal mice, whereas there was no change in the levels of IL-12 and IFNγ when compared to normal controls (Fig. 5.3 A & B). Treatment with KALSOME™ 10 at 3.5 mg/kg single, 7.5 mg/kg single and double doses exhibited varying curing efficacies ranging from 53-100% (Table 5.1). Interestingly, however, significant fall in the levels of IL-10 (P < 0.01) and TGFβ (P < 0.01) was observed in all the groups in comparison to infected mice. Nevertheless, 7.5 mg/kg double dose, which showed the most effective cure, resulted in almost complete inhibition of both of IL-10 and TGFβ (Figure 5C and 5D). Again, KALSOME™ 10 induced significantly (P < 0.001) higher levels of IL-12 and IFNγ at 7.5 mg/kg double dose (Figure 5A and 5B) with 7.5 mg/kg single dose also promoting significant (P <0.0001) elevation of IFNγ (Fig. 5.3 B) emphasizing immunomodulation from disease promoting cytokine milieu to a strong IL-12 and IFNγ secretion for effective cure against VL.
Figure 5.3: Immunomodulatory role of KALSOMETM10. Two month infected BALB/c mice were treated with 3.5 mg/kg SD and 7.5 mg/kg SD and DDs of KALSOMETM10. Leishmanial antigen stimulated culture supernatants of splenocytes obtained from normal, infected and treated mice were used for cytokine profiling [IL-12 (A), IFN-γ (B), IL-10 (C) and TGFβ (D)]. Data represented as means ± SE for six mice per group and are representative of two independent experiments with similar results. *P < 0.005, **P < 0.001, ***P < 0.0001.
Table 5.1 Percent reduction in parasite burden by KALSOME™10, AmB and AmBisome therapy, estimated by LDA

<table>
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<tr>
<th>Drug</th>
<th>percent reduction in LDA Value</th>
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<tr>
<td></td>
<td>Liver</td>
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<tr>
<td>3.5 mg/kg SD* KALSOME™10</td>
<td>53.02</td>
</tr>
<tr>
<td>7.5 mg/kg SD KALSOME™10</td>
<td>78.1</td>
</tr>
<tr>
<td>7.5 mg/kg DD¹ KALSOME™10</td>
<td>100</td>
</tr>
<tr>
<td>2.5 mg/kg AmB</td>
<td>40.65</td>
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<tr>
<td>3.5 mg/kg AmBisome</td>
<td>54.78</td>
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*SD: single Dose; †DD: Double Dose.
KALSOME™10 treatment induced protective immunity correlates with a protective cytokine milieu

To compare the immunomodulatory effects of KALSOME™10 with AmB and AmBisome, we administered 3.5 and 7.5 mg/kg (single and double) doses of KALSOME™10 and AmBisome in groups of normal mice. Another group was injected with 2.5 mg/kg dose of AmB, the maximum tolerable dose of this drug, keeping an untreated group as control. After 10 days the animals were sacrificed and ConA stimulated splenocytes were cultured for cytokine ELISA and FACS analysis. In cytokine ELISA study, we found that IFNγ secretion was significantly high from 7.5 mg/kg double dose of KALSOME™10 treated mice compared to all other groups (Fig. 5.4 A). This elevated secretion of IFNγ correlated well with the suppression of IL-10 production by this dose. Similarly, significantly high frequencies of CD4+ and CD8+ cells producing IFNγ were observed by 7.5 mg/kg double dose of KALSOME™10 compared to 2.5 mg/kg AmB and all administered doses of AmBisome (Fig. 5.4 C & D). Although IFNγ production from CD8+ cells was significantly high in AmB treated group, the highest production of IFNγ was still observed by 7.5 mg/kg double dose KALSOME™10 treatment. Significant suppression of IL-10 production was observed in 2.5 mg/kg dose of AmB and 7.5 mg/kg double dose of AmBisome (Fig. 5.4B), suppression of IL-10 production by CD4+ and CD8+ T cells in 7.5 mg/kg double dose KALSOME™10 treated group was most prominent compared to normal (Fig. 5.4 E & F). Moreover, the lowest level of IL-10 was detected from CD4+ cells of the same group, reaching almost baseline.
Figure 5.4: Immunomodulatory role of KALSOME™10 on the prophylactic model. Normal healthy mice were treated with 3.5 mg/kg SD and 7.5 mg/kg SD and DDs of KALSOME™10 and 2.5 mg/kg AmB and 3.5 mg/kg SD and 7.5 mg/kg SD and DDs of Ambisome in seven different groups with four mice in each group. One group was kept as normal healthy control. After 10 days mice were sacrificed and ConA (2.5 µg/ml) stimulated splenocytes were used for flow cytometry (After 12 hrs incubation) and for cytokine ELISA after 72 hours of incubation. Data represented as means ± SE for four mice per group and are representative of two independent experiments with similar results. * $P < 0.005$, ** $P < 0.001$, *** $P < 0.0001$. 
Discussion

AmB, a polyene macrolide, has emerged as the main antileishmanial drug following the prevalence of SAG resistant *Leishmania* strains in India. This drug has proved to be very effective against VL with a cure rate of almost 100% (Sundar and Chakravarty 2008). However, the toxic effects associated with AmB administration limits its utility (Davidson et al. 1991; Sundar et al. 2011). Several lipid formulations of AmB especially AmBisome have been exploited very effectively for reducing its toxic effects and targeting delivery of AmB against *Leishmania* infection (Gregoriadis 1995; Croft and Olliaro 2011). Unfortunately, these formulations are costly and imported, thereby further increasing the treatment cost. Furthermore, recent reports of VL relapses and development of PKDL after apparent cure with AmBisome (Kumar et al. 2011; Pandey et al. 2012) demonstrate the need to develop new L-AmBs. In addition to its antileishmanial activities, AmB has been found to possess immunomodulatory function. Therapy with AmB induced elevated production of TNFα, IFNγ and IL-12 in splenocytes of treated mice, and PBMC of kala-azar patients with reduced IL-4, IL-10 and TGFβ production (Saha et al. 2007; Banerjee et al. 2008). Interestingly AmB could upregulate IFNγ as well as suppress IL-10 and TGFβ in normal mice splenocytes and human PBMCs suggesting its inherent immunomodulatory activity (Saha et al. 2007). AmBisome, on the contrary, could neither promote Th1 response nor downregulate Th2 cytokines (Banerjee et al. 2008) suggesting a lack in immunomodulatory function in this L-AmB, whereas essential for parasite clearance and prevention of relapse following drug treatment (Saha, et al. 2007). Therefore the need for an efficient and cost effective liposomal AmB, for short course treatment of leishmaniasis, having a long-lasting protective effect remains. Herein we investigated the curative efficacy and immunomodulatory activity of a new liposomal formulation of AmB, KALSOME™10 at single (3.5 mg/kg and 7.5 mg/kg) and double dose (7.5mg/kg) therapy. While all the doses led to significant reduction in the parasite burden in the two months infected BALB/c mice, treatment with 7.5 mg/kg double dose led to an almost complete cure in both liver and spleen. These doses were found to be safe with no hepatic and renal impairment. Further, as observed in the therapy with free AmB (Saha et al. 2007), KALSOME™10
maintained the inherent immunomodulatory efficacy of the AmB and augmented Th1 immune response by suppressing the disease promoting cytokines, IL-10 and TGFβ.

*Leishmania* is unique in having the ability to survive and multiply within host neutrophils and macrophages. AmB promotes leishmanicidal activity by virtue of its high intercalation affinity for ergosterol or its precursor present on the parasite (Meyerhoff 1999). It, however, can also interact with the cholesterol present in host cell membrane inflicting toxicity and functional impairment of the reticuloendothelial system (RES) (Berman et al. 1986). In KALSOME™10, AmB is intercalated with ergosterol which constitutes 50% molarity of the total lipid of the liposome (Asad et al. 2015). Mechanism of antileishmanial activity of AmB is based on its interaction with the sterols present on parasite cell membrane and the subsequent parasiticidal activity. There are two sterols of relevance while designing a drug to treat *Leishmania* infection. One of them is cholesterol, present mostly in human kidney cell membranes and the other is ergosterol, present in the parasite. It is noticeable that affinity of AmB for ergosterol is approximately 8.5 times more than that of cholesterol (Szoka, 1993). For effective treatment, targeted delivery of high doses of AmB is the desired strategy. Encapsulating higher amount of AmB in cholesterol containing liposomes may result in leakage of AmB from the liposomes to the human tissues and organs. To prevent this cholesterol has been replaced by ergosterol. This strategy ensures that AmB will not be released from liposomes until it reaches the target. The breaking up of the liposomes occurs inside the macrophages, residence of *Leishmania*. Thus ergosterol encapsulated AmB has a definite edge over cholesterol encapsulated AmB due to its slow release and greater target specificity. Also due to the higher affinity of AmB towards ergosterol than cholesterol, a greater amount of AmB can be tightly packed inside the liposome and it can be targeted to the macrophages more efficiently (Asad et al. 2015). In our study, we re-evaluated the tolerability of KALSOME™10 at 3.5 mg/kg, 7.5 mg/kg single and double doses by assessing different nephro- and hepatotoxic parameters and found it to be non-toxic at all of these doses. Since L-AmBs are not entirely free of infusion related toxicities (Sundar and Chakravarty 2008; Mishra et al. 2013; Asad et al. 2015) and AmBisome has been reported to show
nephrotoxicity at 5 mg/kg triple dose (Afrin and Ali 1997; Sundar and Chakravarty 2008; Sundar et al. 2008; Asad et al. 2015) these results were very encouraging and could provide a new antileishmanial treatment option.

The parasiticidal efficacy of KALSOME™10, at 7.5 mg/kg triple dose, has already been established in L. donovani infected BALB/c mice (Asad et al. 2015). Our interest was to determine whether it could successfully maintain its leishmanicidal activity at lower doses (3.5 mg/kg single dose, 7.5 mg/kg single and double doses) lower than the reported one. Herein we showed that therapy of established murine VL with increasing doses of KALSOME™10 led to a progressive reduction in the parasite load in the visceral organs with 7.5 mg/kg double dose exhibiting complete parasite clearance in both liver and spleen. Additionally a reduction in parasite load with increasing drug doses correlated with the decrease in the organ weight synchronizing with a progressive cure. The parasite killing efficiency achieved by KALSOME™10 at such low doses could be matched only by very few antileishmanial drugs (Sachdeva et al. 2014). While 5 mg/kg triple dose of AmBisome showed similar efficacy in murine VL, it required longer treatment duration (Larabi et al. 2003). Even in kala-azar patients a similar dose (5 mg/kg/day for 3 days in a span of 10 days for a total dose of 15 mg/kg) of AmBisome was reported to cure only 90% of the patients with few relapses (Davidson et al. 1996; Lanternier and Lortholary 2008). Thus KALSOME™10 has the potential to become a successful treatment alternative to the presently used antileishmanial chemotherapies at doses that are safe and tolerable.

It is well established that the course of disease progression following L. donovani infection is modulated by a range of T cell responses and cytokine network. Extensive studies have identified IL-10 as the major player in the disease pathology of VL (Asad and Ali, 2014). It can render macrophages unresponsive to activation signals and inhibit killing of amastigotes by downregulating the production of NO (Sundar and Chakravarty 2008). TGFβ has also been reported to have inhibitory effects on the action of macrophages and its blockade has been found to limit parasite replication in host cells (Kumar and Nylen 2012). Effective elimination of IL-10 and TGFβ synchronizing with the upregulation of IFNγ and IL-12 could be key factors for therapeutic success against VL (Saha et al. 2007; Croft and Olliaro 2011; Sundar et al.
KALSOME™10 Against VL

2011; Bogdan 2012; Kushawaha et al. 2012). It has been reported that chemotherapy with SAG and AmB cause downregulation of disease promoting cytokines IL-10 and TGFβ, leading to enhanced IL-12 and IFNγ levels correlating with cure (Ghalib et al. 1995; Saha et al. 2006; Saha et al. 2007). Herein we also found that treatment of infected mice with increasing doses of KALSOME™10 (3.5 mg/kg single dose and 7.5 mg/kg single and double doses) correlated with the reduction of both the disease promoting cytokines, IL-10 and TGFβ. Moreover, IL-10 was completely absent and secretion of TGFβ was almost negligible at 7.5 mg/kg double dose of KALSOME™10. Subsequently, we observed an elevation in both IL-12 and IFNγ levels in infected mice treated with 7.5 mg/kg double dose. Treatment with 7.5 mg/kg double dose effectively suppresses disease promoting cytokines IL-10 and TGFβ, thereby boosting IL-12 and IFNγ levels. This immune modulation by KALSOME™10 may be responsible for the almost complete parasite clearance observed by 7.5 mg/kg double dose.

Notably, KALSOME™10 showed a marked immunomodulatory effect at single and double doses of 7.5 mg/kg by significantly increasing IFNγ level leading to suppression IL-10. Elevated production of IFNγ from CD4+ and CD8+ T cells in 7.5 mg/kg KALSOME™10 treated groups led to the maximum suppression of IL-10 production from these cells. Such an elevation of IFNγ was almost absent in AmBisome at all comparative doses. Although some immunomodulatory activity was detected by AmB treatment, the best results were observed only with KALSOME™10. Therefore, encapsulation of AmB in our formulation not only maintains the inherent immunomodulatory effect of AmB but also enhances it, which is a prerequisite for long-term protection.