Author's response to reviews

*Title:* Therapeutic and immunomodulatory activities of short-course treatment of murine visceral leishmaniasis with KALSOMETM10, a new liposomal amphotericin B

*Authors:*

Md Asad (asadseraphic.ni@gmail.com)
Pradyot Bhattacharya (b_pradyot@yahoo.com)
Nahid Ali (nali@iicb.res.in)

*Version:* 3  
*Date:* 15 January 2015

*Author's response to reviews:* see over
Reviewer comments:

Reviewer #1 (Comments for the Author):

Reviewer: Abebe Genetu Bayih

The study has been done to test the efficacy and toxicity of a low dose KALSOME™ 10 for the treatment of visceral leishmaniasis in mice. In addition, the study has investigated the immunomodulatory function of the new drug formulation.

It appears that the study is a continuation of another study done on the same drug formulation (Mishra et al, 2013). Basically, the present study has demonstrated that the drug given ONLY TWICE to BALB/c mice at a dose 7.5mg/kg is sufficient to clear Leishmania donovani infection with no hepatic and renal toxicity. Moreover, it has shown that treatment with the drug shifts the antileishmanial immune response to a predominantly Th-1 type. Thus, the study has shown that KALSOME™ 10 is a promising drug formulation for short-term treatment of kala-azar.

Generally, the experimental data are well presented in the manuscript in a very concise but informative manner. In addition, the manuscript is well-written and the message is easily understandable. However, the design of the experiments missed few fundamental components such as the absence of proper controls. I believe, the authors should address the following issues in order for the manuscript be accepted for publication on BMC Infectious Diseases.

A) Major Compulsory Revisions:

1. The experiments on the efficacy and immunomodulatory of the drug did not involve controls such as the drugs that are currently used for treatment of VL patients such as AmB and AmBisome. Without controls, it is difficult to determine where the efficacy of the new drug falls as compared to the known drugs following the experimental procedures used in this study. Please clarify why a “positive control” was not included in the study.

Response: Experiments added with AmB and AmBisome as “positive controls” in fig. 3.

2. As described in the introductory part of the manuscript, the authors appear to rationalize the usefulness of a liposomal amphotericin B that does not contain cholesterol (KASOME 10) by arguing that the use of cholesterol in formulating liposomal amphotericin B such as AmBisome contributes to exacerbation of the disease. In other words, the authors argue that avoiding the use of the cholesterol “could make this drug more suitable for clearing parasites” (page-6 line-3). Please clarify the justification based on the following points:

a. The scientific background for this argument should be clearly explained and substantiated with published data.

Response: Mechanism of active Leishmania drugs, 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 second one is ergosterol, present in parasite. It is noticeable that affinity of AmB for ergosterol is approximately 8.5 times more than that of cholesterol (Szoka FC mad Tang M. 1993, J Liposome Research; 3: 363-75). For effective treatment, targeted delivery of high doses of AmB is the desired strategy. Encapsulating high AmB content in cholesterol containing liposomes may result in leakage of AmB from the liposomes to the human tissues and organs. To prevent this leakage
from liposomes, 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, the 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 higher affinity of AmB towards ergosterol than cholesterol, greater amount of AmB can be tightly packed inside the liposome and it can be targeted to the desired place (macrophages) more efficiently without leakage of the drug.

Moreover, the fact that cholesterol supports the growth of *Leishmania* is also of relevance in designing VL specific formulation of liposomal AmB. Lipids account for 15% of the dry weight of the leishmanial cells (Meyer and Ilolz, 1966, J. Biol. Chem. 24: 5000-5007). Metabolism of lipids is crucial for several vital physiological processes and affects parasite’s survival (Coppens and Courtoy. 1995, Mol. Biochem. Parasitol. 73,179-188 and Patent No. US 6, 403, 576B1). It is pertinent to note that *Leishmania* require cholesterol for their sustenance (Chandel AKS *et al.*, 2014, World J of Pharmacy and Pharmaceut Sci, 3: 1567-1584) and may fulfill their requirement by salvaging cholesterol from their host macrophage cells. Hence, a prospective antileishmanial drug must affect survival of *Leishmania* by depriving of their cholesterol requirement. Also the delivery of AmB through phagocytic action of reticuloendothelial cells using cholesterol as the encapsulating liposome may negate the benefits of its targeted delivery to the macrophages.

b. The reference cited for the argument (a review by Pucadyil and Chattopadhyay, 2007) does not fully support the statements on the manuscript (page-5 and 6). For example, the review describes that the host cholesterol promotes antigen presentation by infected macrophages.

Response: We have replaced it with more appropriate references by the same group and by others as well to support our statements (Chandel AKS *et al.*, 2014 World J of Pharmacy and Pharmaceut Sci, 3: 1567-1584; Pucadyil TJ *et al.* 2004, Mol Biochem Parasitol, 133: 145-52), which demonstrate experimentally that cholesterol is required for *Leishmania* parasite growth and survival. Depletion of cholesterol from macrophages cell membrane results in significant reduction in the *Leishmania* infection. Therefore, host cholesterol may be involved in sustenance of infection.

c. It appears that the authors do not clearly distinguish the role of the host cholesterol and the one coming from the drugs in promoting or inhibiting VL. How does the cholesterol in the drugs contribute to the internalization of the parasite as the drug is administered well after the parasite establishes itself inside macrophages? Please cite a reference (s), if any, supporting the idea that cholesterol in the liposomal amphotericin B contributes to establishment and/or exacerbation of VL.

Response: There are reports which say that plasma membrane cholesterol of macrophages play a crucial role in leishmanial infection and cholesterol depletion from macrophages results in significant reduction in the *Leishmania* infection (Pucadyil TJ *et al.* 2004, Mol Biochem Parasitol, 133: 145-52). Recent work of Chandel *et al.* demonstrates that exogenous cholesterol, added in the culture, enhances the growth of *Leishmania* promastigotes (Chandel AKS *et al.*, 2014 World J of Pharmacy and Pharmaceutic Sci, 3: 1567-1584). Therefore, after internalization, the cholesterol requirement by *Leishmania* for its sustenance may be fulfilled by salvaging cholesterol from host macrophages. Thus, avoiding cholesterol in liposomal formulation of AmB is a good strategy in drug designing against VL. The manuscript has been modified accordingly.

3. The authors should have shown that the infection was established in all of the groups before treating the mice with the drug. Otherwise, it would be difficult to know if the
near zero liver parasite load on one of the treatment groups is due to failure to establish infection or the result of the antileishmanial effect of the drug at the specified dose. Please respond why you did not show whether the infection worked.

Response: Thirty animals were infected. After two months, six animals were randomly sacrificed to estimate the parasite burden (liver LDA = 10.65±0.92 and spleen LDA = 8.57±0.54). The remaining 24 animals were divided randomly into different groups, keeping a group of animals as infected controls. The manuscript has been modified accordingly.

4. What was the rationale of using Day-14 post-treatment to test the in vivo toxicity? The authors should show that the protocol they used was in accordance with some sort of standard. Or, they should cite a published protocol.

Response: Liposome encapsulation AmB takes few days for slowly releasing the drug and the AmB retention in the blood has been observed even after 7 days (Bekersky I et al., 2002, Antimicrob Agents Chemother; 3: 828–833) and its toxic effect lasts upto 14 days of the drug administration. Hence, we have tested the toxic effect (if any) of the drug on the 15th Day of the drug injection. There are a number of publications where animals where kept for 14 days post liposomal AmB treatment for toxicicity studies (Sharma M et al., 2012, PLoS Negl Trop Dis 6(5): e1629; Banerjee A, et al., 2011, Antimicrob Agents Chemother, 55 (4):1661-70).

B) Minor Essential Revisions:
1. Please describe the drug, KALSOLEME 10, and its formulation in a more detailed manner.

Response: It is sterol enriched mixed lamellarity AmB intercalating liposomes in aqueous suspension where in the aqueous suspension may optionally contain 0.9% saline. Ergosterol constitutes almost 50% of the total liposomal lipid which consists of phosphatidylcholine, ergosterol and AmB in 5:2:1.8 molar ratios. The composition is sonicated during manufacture and before administration for increasing plasma half-life and better bio-distribution. (This portion has been incorporated in the Material and Method Section of the manuscript.

2. Please explain the rationale for selecting two drug doses, 3.5mg/kg and 7.5mg/kg. And, explain why only a double dose of 3.5mg/kg was not tested.

Response: According to WHO, 15-20 mg/kg body weight of Liposomal AmB should be administrated for treatment of VL. Therefore we used 7.5mg/kg double drug doses for our study. Further to check lower dose efficacies, we used 7.5 mg/kg single dose and 3.5 mg/kg single doses. Since 3.5 mg/kg double dose is almost equivalent to 7.5 mg/kg, hence, 3.5 mg/kg double dose was not tested.

3. The time gap between the two injections with 7.5 mg/kg KALSOLEME 10 was not stated in the manuscript.

Response: It was administered on consecutive days.

4. Page-7 line17: please specify how many times the dilution was performed.

Response: It has mentioned in the next sentence that 5 (five) fold serial dilutions were performed.
5. Page-8 line-2: “…..ADULT BALB/c mice…” Please write the actual age of the mice.

Response: 8-10 week old BALB/c mice were taken.

6. Page-9 line-18 to 20: Please write the actual values in the differences b/n each of the treatment groups and the controls. As seen in the figure, there is difference between 3.5 and 7.5mg/kg doses as compared to the control group. Percent reduction could be a better description of the differences.

Response:

Table 1: Percent Reduction in Parasite Burden by KALSOME\textsuperscript{TM}10, AmB and AmBisome therapy, estimated by LDA

<table>
<thead>
<tr>
<th>Drug</th>
<th>LDA Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>3.5 mg/kg SD* KALSOME\textsuperscript{TM}10</td>
<td>53.02</td>
</tr>
<tr>
<td>7.5 mg/kg SD KALSOME\textsuperscript{TM}10</td>
<td>78.1</td>
</tr>
<tr>
<td>7.5 mg/kg DD¶ KALSOME\textsuperscript{TM}10</td>
<td>100</td>
</tr>
<tr>
<td>2.5 mg/kg AmB</td>
<td>40.65</td>
</tr>
<tr>
<td>3.5 mg/kg AmBisome</td>
<td>54.78</td>
</tr>
</tbody>
</table>

* SD: single Dose
¶ DD: Double Dose
This table has been incorporated in the manuscript as well.

7. Page-9 line-21: “…resulted in complete clearance of…” As seen in the figure, the clearance was not COMPLETE in the spleen as one mouse showed some parasites in the spleen.

Response: The sentence in the manuscript has been corrected as almost complete clearance.

8. Please discuss why the difference in the weight of the liver and spleen from treated and untreated mice was non-significant. Why a near complete clearance of the parasite in the spleen and liver did not result in a proportional reduction in the size of these organs?

Response: With treatment liver and spleen weight are decreasing, achieving the weight of normal BALB/c mice. The difference in liver weight of 7.5 mg/kg drug dose treated group was significant compared to normal (p\textless{}0.05, Student t test).
9. Please explain the possible reasons/mechanisms for the high level of immunomodulation by KALSOME 10? As stated in the manuscript, the other lipid formulations are not able to modulate the immune response to L. donovani in infected mice. Again, inclusion of AmBisome control would have shown us the real difference between the two drugs under this experimental condition.

Response: Post infection treatment of AmB (2.5mg/kg) and AmBisome (3.5 mg/kg) has been shown to have immunomodulatory role by AmB only and not by AmBisome (Banerjee et al. J Immunol. 2008). Immunomodulatory role of these drugs and KALSOME™10 have been incorporated in the manuscript.

10. Page-12 line 10 “….Unfortunately these formulations are very costly and REQUIRE SEVERAL DAYS OF HOSPITALIZATION…..” This statement appears to contradict with the current move to the use a SINGLE DOSE AmBisome for the treatment of VL (no need of prolonged hospitalization).

Response: The statement has been modified as “these formulations are imported and costly”.

11. Page-13 line-5 and 6: If the authors knew that the drug at the specified doses was safe with “no hepatic and renal functional impairment”, what is the importance of doing liver and kidney function tests in the current study?

Response: Portion has been re-written.

12. This study was done to test the efficacy of the drug at a lower dose than previously studied (Mishra et al, 2013). However, the authors followed a different protocol from the one used by Mishra et al, 2013. These include the inoculums size and the stage of the parasite used to establish infection. Would it be possible to compare the results of the two studies?

Response: Mishra et al, 2013 measured the efficacy of the drug, KALSOME at 7.5 mg/kg triple dose only and we have used various doses of the drugs and found similar efficacy at 7.5 mg/kg double dose only. We have estimated live, motile Leishmania promastigotes by Limiting Dilution Assay compared to LDU method used by Mishra et al., which may include even dead parasites. We used $2 \times 10^7$ Leishmania amastigites, compared to Mishra et al., used $1 \times 10^7$ Leishmania parasites for the studies.

13. Page-15 line-5 to 13: This was a repeat of what was described in the “results” section. Please delete or re-write it.

Response: This portion has been re-written in the manuscript.

14. There are several typos in the manuscript. Please correct them.
Response: Typos has been corrected in the manuscript.

Response to Reviewer 2
Reviewer: Sukhibir Kaur
Reviewer's report:
Comments
The manuscript by Asad et al., presents protective efficacy of a new formulation of liposomal amphotericin B (KALSOMETM10) in murine model for the treatment of kala-azar. The experimental design, the presentation of the data and the conclusions are scientifically correct.

My major criticism of this paper is that the authors have studied only the cytokine levels for showing the immunomodulatory efficacy of KALSOME 10. Moreover, the efficacy of this new liposomal formulation of amphotericin B has already been reported by Misra et al., 2013 with the same doses. In their study they have used this formulation three times and in the present study the KALSOME10 has been used as a double dose (7.5mg/kg body wt. and 3.5 mg/kg body wt.). In the present study the disease promoting and inhibiting cytokines have been evaluated and no other new data has been generated. If the authors suggest that KALSOME has an immunomodulatory activity then they should have studied other parameters such as immunophenotyping of spleen cells by flow cytometry, to determine the percentage of CD4, CD8 and T reg cells, Delayed type hypersensitivity responses and lymphoproliferative assays.

Response: The suggested experiments have been incorporated.

Another major drawback of the study is that they have not used any positive control such as Ambisome or fungizone for comparison.

Response: The suggested experiments with positive controls have been incorporated.

The authors have also not given detailed protocol followed for treatment and the number of days for which the drug was given.

Response:
The authors should have discussed about the cost effectiveness of KALSOME 10 in comparison with ambisome.
Response: KALSOME<sup>TM</sup> 10 not has been marketed yet. So far it has been tested on animals only. But the price is expected to be 25% less than AmBisome, as 10 times more AmB is associated with the liposome (AmBisome contains 1 mg/ml AmB while Kalsome<sup>TM</sup> 10 contains 10mg/ml AmB).