Immunoadjuvant chemotherapy of visceral leishmaniasis in hamsters using Amphotericin B encapsulated nano-emulsion template based chitosan nanocapsules

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ABSTRACT

The accessible treatment options for life-threatening neglected visceral leishmaniasis (VL) disease have problems regarding efficacy, stability, adverse effects and cost, making treatment a complex issue. Herein, we formulated nanometric amphotericin B (AmB) encapsulated chitosan-nanocapsules (CNC-AmB) using polymer deposition technique mediated by nano-emulsion template fabrication. CNC-AmB exhibited good steric stability in vitro where chitosan content was found to be efficient in preventing their destabilization in the presence of protein and Ca$^{2+}$. Toxicity study on J774A model cell line and erythrocytes revealed less toxicity of CNC-AmB compared to commercialized AmB formulations such as Fungizone® and AmBisome®. Experimental results of in vitro (macrophage amastigote system, IC$_{50}$ = 0.19±0.04 µg AmB/ml) and in vivo (Leishmania donovani infected hamsters, 86.1±2.08% parasite inhibition) in conjunction with effective internalization by macrophages illustrated the efficacy of CNC-AmB to augment antileishmanial property. Quantitative mRNA analysis by RT-PCR showed that improved effect was synergized with up regulated Tumor Necrosis Factor-α (TNF-α), Interleukin-12 (IL-12) and inducible nitric oxide synthase, and down regulated Transforming Growth Factor-β (TGF-β), IL-10 and IL-4. These research findings suggest that developed cost-effective CNC-AmB immunoadjuvant chemotherapeutic delivery system could be a viable alternative to the current high-cost commercial lipid based formulations.

Key words: Nanocapsules, Nano-emulsion, Chitosan, Macrophages, Cytokines
INTRODUCTION

Visceral leishmaniasis (VL), a vector-borne disease is caused by intra-macrophage trypanosomatid protozoa of the genus *Leishmania* (1,2) and is manifested by hematological and hepatosplenic abnormalities with defective T-cell-dependant immune responses and is usually fatal if not treated properly (3,4). Human VL causes an estimated 50,000 deaths annually, a rate surpassed among parasitic diseases only by malaria, and 2,357,000 disability-adjusted life years lost, placing leishmaniasis ninth position in a global analysis of infectious diseases (5).

Despite the availability of several chemotherapeutic drugs, successful management of VL has not yet been attained. Indeed, amphotericin B (AmB) is used as a prototype leishmanicidal drug due to its excellent efficacy with commercially great success. It possesses selective killing activity against *Leishmania* mediated through its higher binding affinity for predominant 24-substituted sterols (ergosterol and episterol) in the plasma membrane of parasite as these sterols are not found in mammalian cells (6). However, toxic side effects, in particular hematological intolerance and nephrotoxicity produced by AmB at therapeutic doses have often limited its clinical application (7).

In the past few years, the capability of the drug delivery systems have been critically tested to enhance the accessibility of the drug to reticuloendothelial system (RES) organs (liver and spleen), ensuring delivery of less amount to the kidney and lungs, thus able to decrease AmB mediated toxicity (8,9). The investigations for such newer less toxic formulations of AmB have led to the development of commercial preparations for therapeutic use such as liposomal AmBisome®, Abelcet® a AmB-lipid complex, and micellar Amphotec® (10,11). Though liposomes and lipid complexes have succeeded in reducing the adverse-effects of AmB, but lower stability and prohibitive higher cost restrict their clinical utility. On the contrary, mixed
micellar formulation is cost-effective but cannot improve the tolerability of the AmB. Accordingly, there is a need for the development of stable and tolerable low cost formulations. Alternatively, we developed a novel stable nano-emulsion template (NET) based polymeric chitosan nanocapsule (CNC) formulations of AmB having an oil-based central cavity, which represents hydrophobic lipid particles and surrounding chitosan has hydrophilic properties. The amphiphilic properties are obtained by the inclusion of an oil phase in an oppositely charged chitosan polymer (see Fig. S1 in the supplementary material). The resulting nanocapsule carrier loads easily and can be used to stabilize a higher magnitude of insoluble hydrophobic or amphiphilic drugs. The rationale behind the choice of chitosan biopolymer includes its excellent biocompatibility, biodegradability (12), “generally regarded as safe” (GRAS) approval (13) and its cell mediated immune-enhancing effects favoring elevated macrophages (MPφ) uptake of carrier system (14). Stimulation of Th1 and suppression of Th2 immune responses is considered a promising therapeutic strategy for leishmaniasis (15) and chitosan has been accounted to stimulate MPφ to produce various pro-inflammatory cytokines including IL-1, IL-6, TNF-α, nitric oxide (NO) and granulocyte MPφ colony stimulating factor (GM-CSF) (14, 16, 17). Chitosan also induces immunologic adjuvant effects by active binding to the specific receptors on MPφ (18-20). Furthermore, acid resistive property of chitosan in overcoming immediate lysosomal digestion within MPφ for short duration is another advantage favorable to provide sustained AmB release at the particular target site (RES organs, host for the intramacrophage Leishmania parasite). Additionally, surface adsorbing ability of chitosan on lipid droplets (21), that results in long-term physically stable polymeric CNC in both liquid and dry form, which makes them unique and applicable as a substitute for much less stable liposomes.
In the present paper, we report design and antileishmanial evaluation of developed AmB carrier system (CNC-AmB) against *L. donovani* amastigotes, tested in an experimental model of visceral leishmaniasis in hamsters as well as in murine macrophage cell lines (J774A) and the activity was compared with commercially available Fungizone and Ambisome formulations. Moreover, immunomodulatory role of nanocarriers was also assessed in hamsters and cytotoxicity study was carried out against J774A cells and erythrocytes.

**METHODS AND METHODS**

**Materials.** AmB was a kind gift from Intas Pharmaceuticals (Ahmadabad, India). Soya lecithin, soyabean oil, Tween 80, low molecular weight chitosan (75-85% deacetylation and 20-200 cps. viscosity) and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) were supplied by Sigma Aldrich (MO, USA). Dialysis bag (cut-off mol. wt. 12 KD) was purchased from HiMedia (Mumbai, India). HPLC grade acetonitrile was supplied from SD Fine Chem Ltd. (Mumbai, India). All other chemicals and solvents were of analytical grade procured from local suppliers unless mentioned. Ultrahigh pure water produced by three-stage Millipore Milli-Q plus 185 purification system (Bedford, US) was utilized throughout all experiments.

**Parasites.** The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) was used for both *in vitro* and *in vivo* experiments. These parasites and the macrophage cell line J774A were maintained in RPMI-1640 medium (Sigma, USA) supplemented with 10% heat inactivated fetal bovine serum (HIFBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in humidified atmosphere of 5% (v/v) CO²/air mixture (22).

**Animals.** Syrian golden male hamsters, *Mesocricetus auratus* (45–50 g) reared in institute facilities were used to study the antileishmanial effects of the AmB nanocarriers because the hamster is the most appropriate experimental model as it largely reflects the clinic-
immunopathological features of progressive human VL, including a relentless increase in visceral parasitic burden, cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia and ultimately death (23,24). In vivo studies were carried out with prior approval of the Animal Ethics Committee of Central Drug Research Institute and according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Fabrication of chitosan nanocapsule from the nano-emulsion template. Polymeric chitosan nanocapsule (CNC) was generated with preformed chitosan polymer in two steps. Firstly, oil-in-water (o/w) nano-emulsion-template (NET) was formulated through modified spontaneous emulsification solvent evaporation (25). Probe sonicator (SONICS, USA) was used to supply high energy to prepare formulation, however prior to sonication the oil phase and the water phase were prepared separately. Briefly, the organic phase consisted of absolute ethanol, 10% (w/w) soyabean oil, 3% (w/w) nonionic lipophilic emulsifier (soya lecithin) was dispersed by means of a magnetic stirrer until complete dissolution of SL was obtained. The aqueous phase composed of 4% (w/w) hydrophilic emulsifier (Tween 80), 2.25% (w/w) osmotic agent (glycerol) and water, was obtained by dissolving the stabilizer in the water. Finally AmB solution in acidic methanol was added in the oil phase. The NET was obtained by adding the oil phase to the aqueous phase both being adjusted to the same temperature. Afterwards sonifier high energy was supplied at 40% amplitude for 4 min and the excess of solvents mixture (ethanol/water) was subsequently removed under reduced pressure at 50 °C until the desired final volume (10 ml).

Secondly, CNC nanocarrier was generated by coating droplets of NET with the chitosan deposition on the water/oil surface (26). In order to prepare CNC-AmB, the chitosan solution using 1% (v/v) glacial acetic acid with varying compositions (0.1, 0.2, 0.4, 0.6, 0.8 and 1% w/v
coded by NC-1, NC-2, NC-4, NC-6, NC-8 and NC-10, respectively) and ethanol in equimolar ratio was gradually added in the continuous aqueous phase of the NET in 1:10 ratio and was stirred uncovered at room temperature for complete evaporation of ethanol (IKA, Germany). The polymer precipitated onto the droplets, forming chitosan-coated oil nanodroplets (CNC). This CNC dispersion was sterilized by filtration through a disposable syringe filter (0.45 μm Millipore filter) with not more than 5 ml of dispersion was filtered through each disposable syringe filter. Developed CNC were recovered by ultracentrifugation at 50000 × g for 20 min at 4 °C (Beckman Coulter, Fullerton, CA, US) and thereafter the sediment was subjected to lyophilization. To examine the in vitro cellular internalization, fluorescent FITC-loaded nanocarriers were prepared in the same way incorporating 0.5 mg/ml FITC instead of AmB.

Physicochemical characterization. Average hyrodianamic diameter (D_{H}) and polydispersity index (PDI) of prepared nanocarriers were determined using a photon correlation spectroscopy (PCS) and the zeta potential was determined using electrophoretic mobility with laser-based multiple-angle particle electrophoresis analyzer at 25°C (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) just after dispersion in deionized water. All of the measurements were performed in triplicates.

The morphologic characteristic was ascertained using high resolution transmission electron microscopy (HR-TEM). For the HR-TEM measurement, samples (1mg/20 ml) were prepared as a thin aqueous film supported on a 300-mesh copper grid. Negative staining was performed using a droplet of 2% (w/v) phosphotungstic acid. Direct imaging of dried samples was executed at a 200 kV acceleration voltage using HR-TEM (Tecnai™ G² F20, Eindhoven, The Netherlands).
In order to quantify the amount of AmB loaded into nanocarrier, a certain amount of formulation was dispersed in dimethyl sulfoxide in triplicate, vortexed for 20 min and centrifuged for 20 min at 2767×g. Afterwards, 1 part of the supernatant was mixed with 9 parts of methanol and the AmB in the resulting solution was quantified by high performance liquid chromatography (LC-10ATvp HPLC instrument, Shimadzu, Tokyo, Japan) using a Lichrosphere reverse-phase C₁₈ column (250 × 4 mm, 5 µm; Merck, Darmstadt, Germany) having acetonitrile with potassium dihydrogen phosphate buffer (pH 3.5, adjusted with orthophosphoric acid), (60:40, v/v) as mobile phase at 1.0 ml/min flow rate and column effluent was detected with a UV detector at 405 nm. Results are expressed as AmB actual loading (DL, mg of AmB encapsulated per 100 mg of formulation) and encapsulation efficiency (EE, ratio of actual and theoretical AmB loading×100) ± SD of values collected from five different batches.

Dialysis membrane diffusion technique was utilized to investigate release profile of AmB from developed nanocarriers (27). The dialysis bags was filled with formulation sample of a volume equivalent to 2 mg of AmB, hermetically sealed and suspended in dissolution apparatus (DISSO 2000, Labindia, India) containing 250 ml of phosphate buffered saline (PBS) (pH 7.4) along with 0.5% Tween 80, thermostated at 37±1º C with moderate shaking at 100 rpm. At predetermined time intervals, aliquots of the external release medium (1 ml) were withdrawn, replenished with equal volume of fresh PBS and analyzed for the amount of released AmB using HPLC method as described previously.

Developed nanocarriers were incubated with 10 wt% BSA solution and 0.2 M CaCl₂ solution, respectively at RT at a concentration of 1 mg/ml. At each time point, an aliquot of sample solution was collected to measure the size using PCS as described previously.
In vitro biological evaluation. (a) Macrophage uptake study. Qualitative in vitro uptake experiment was performed in order to examine the effect of the surface modification of NET with chitosan on the endocytic internalization mechanism that was responsible for enhanced internalization of the CNC. Non-infected J774A cells (10^5 cells/well) were seeded into 96-well plates and incubated with NET and chitosan coated NET (i.e., CNC) formulations incorporating FITC as fluorescent marker (0.1 mol%), for 24 h and cell-associated fluorescence was analyzed using fluorescence activated cell sorter (FACS) instrument (Beckman Coulter MPL FC 500, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

(b) Toxicity determination assay: (i) In vitro cytotoxicity against non-infected J774A MPφ cells. The cytotoxicity of AmB loaded formulations was assessed by the MTT proliferation assay (28). Non-infected J774A macrophage cells (5 × 10^4 cells/well) were aliquotted into 96-well plates and incubated in triplicate with NET-AmB, CNC-AmB and commercial Fungizone (AmB formulated in 41 mg sodium desoxycholate with 20.2 mg sodium phosphate buffer), and Ambisome (AmB formulated with 213 mg hydrogenated soy phosphatidylcholine, 52 mg cholesterol, 84 mg distearoylphosphatidylglycerol, 0.64 mg alpha tocopherol, 900 mg sucrose, and 27 mg disodium succinate hexahydrate as buffer to form liposome) at 0.25, 2.5, 12.5 and 25 μg/ml equivalent AmB concentrations at 37±1 °C for 24 h. At determined time, the formulations were replaced with DMEM containing MTT (500 μg/ml) and cells were then incubated for additional 4 h, facilitating MTT to be reduced by viable cells with the formation of purple formazan crystals. MTT was aspirated off and dimethylsulfoxide (200 μl) was added to ensure solubilization of the formazan crystals. The optical density (OD) was measured at 570 nm using a multiwell microplate reader (BIO-TEK, Model- Power wave XS, Crailsheim, Germany). The experiment was repeated for reproducibility and the AmB concentration required to kill 50% of
the cells (CC<sub>50</sub>) was calculated using sigmoidal regression analysis by plotting a graph of the 
ODs against AmB concentration taking the OD of the control well as 100% viability.

(ii) **In vitro erythrocyte toxicity:** The erythrocyte toxicity assay was performed following the 
previously reported procedure with minor modifications (29) to assess the hemolytic potential of 
the developed injectable nanocarriers. Erythrocytes were collected from blood of Wistar rat by 
centrifugation (1420×g for 5 min) and resuspended in PBS. Erythrocytes suspension in PBS 
served as negative control and that which was dispersed in deionized water considered to be 
producing 100% hemolysis provided positive control. To study haemolysis, erythrocytes 
suspension was incubated in triplicate with NET-AmB, CNC-AmB and commercial Fungizone 
and Ambisome at 0.25, 2.5, 12.5 and 25 μg/ml equivalent AmB concentrations at 37±1 °C for 30 
min. After centrifugation at 9300×g for 20 min released haemoglobin in the supernatant was 
analyzed by measuring the absorbance at 576 nm using a multiwell microplate reader.

(c) **In vitro inhibitory effect on L. donovani intra-MPφ amastigotes.** The activity of NET-
AmB and CNC-AmB against intracellular amastigotes was evaluated as per protocol described 
earlier (30). Briefly, MPφ (10<sup>5</sup> cells/well) in 24-well plates (Nunc, IL, USA) were infected with 
promastigotes expressing green fluorescent protein (GFP) at multiplicity of 10 parasites per 
MPφ. After 12 h incubation 24-well plates were washed thrice with PBS (pH 7.2) to remove 
non-phagocytosed promastigotes and re-supplemented with complete medium RPMI-1640 
(Sigma, USA). The cells were incubated with AmB loaded NET-AmB, CNC-AmB and 
commercial Fungizone as well as Ambisome at different drug concentrations, and drug-free NET 
and CNC with the same amount of formulations in triplicate for 48 h. The untreated infected 
MPφ served as control. After 48 h treatment, cells were removed, washed in PBS, and 
quantitated by flow cytometry equipped with a 20 mW argon laser with excitation at 488 nm and
emission at 515 nm. Multiparametric data were analyzed by Kaluza analysis software (Becton Dickinson). The inhibition of parasite growth was determined by comparing the fluorescence levels of drug-treated parasites with that of untreated control parasites and the 50% inhibitory concentrations (IC50) of each compound was calculated by linear regression analysis.

**In vivo biological evaluation (a) In vivo AmB assay in *L. donovani* infected hamsters.** The effect of NET-AmB, CNC–AmB, Fungizone and Ambisome on the *in vivo* efficacy was studied at 1 mg/kg body weight drug equivalent dose against *L. donovani* amastigotes in a hamster model (31). After 30 days of established infection hamsters (n = 5 in each group) were dosed 1 mg/kg for 5 consecutive days with AmB loaded formulations by intraperitoneal route, and infected untreated hamster group was used as control. Treated hamster groups were sacrificed one week after treatment and compared with the infected untreated control. The splenic dab smear of all performed animals, was monitored microscopically using Giemsa-stained imprints, in which parasite burdens were measured by counting the number of amastigotes per 100 MPφ nuclei. The percentage of inhibition (PI) was calculated using the formula (32):

\[
PI = \left( \frac{PP - PT}{PP} \right) \times 100
\]

where PP is the number of amastigotes per 100 MPφ nuclei in spleen before treatment whereas PT is the number of amastigotes per 100 MPφ nuclei after treatment.

**(b) In vivo immuno-modulatory responses in *L. donovani* infected hamsters.** Quantitative real time-PCR (qRT-PCR) was performed in triplicate to assess the expression of mRNAs for various cytokines (TNF-α, IL-12, IL-4, IL-10 and TGF-β) and inducible NO synthase (iNOS) in splenic cell smear of differently treated infected hamsters as recommended by the manufacturer. mRNA from splenocytes of different groups of experimental hamsters was isolated using Tri reagent (Sigma, USA) as directed by manufacturer’s protocol. cDNA was synthesized using first strand...
cDNA synthesis kit (Fermentas, USA) as per manufacturer’s protocol. qRT-PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 40 cycles, each consisting of denaturation at 95°C for 30s, annealing at 55°C for 40 s, and extension at 72°C for 40 s per cycle using the iQ5 multicolor real-time PCR system (Bio-Rad, USA). cDNAs from infected hamsters were used as comparator samples. Quantification of the PCR signals was performed by comparing the cycle threshold (CT) value of the gene of interest with the cycle threshold value of the reference gene hypoxanthine phosphoribosyltransferase (HPRT). Values are expressed as fold increase (fold change, FC) of mRNA relative to those in unstimulated cells.

Statistical analysis. Values are presented as mean±standard deviation (SD) of three to five independent measurements. Statistical significance of differences was analyzed by one-way analysis of variance (ANOVA) and p values less than 0.05 were considered to be statistically significant.

RESULTS

Fabrication and characterization of nanocapsules. CNC were produced by applying polymer deposition and adsorption approach where chitosan was allowed to become electrostatically anchored and deposited on negatively charged water/oil surface of NET droplets that was produced by application of ultrasound energy using sonifiers employing Tween 80 and SL as surfactants, and soyabean oil as the central oily core. The excipients which we have used in formulation are generally considered safe for use in parenteral formulations. The combination of surfactants with oils to form NET offers an advantage over commercial micellar or co-solvent system in terms of drug solubilization capacities for amphiphilic compounds (33). Chitosan concentration was optimized by measuring the change in zeta potential, size and stability of nanocapsule dispersions. The CNC prepared using 0.6% w/v chitosan (NC-6) had the electric
charge of (+) 29±0.82 mV with 145.8±8.91 nm size (n=3) and observed as the most stable formulation, while NC-4 (0.4% w/v chitosan) showed aggregation characteristics while NC-8 exhibited larger size (Fig. S2) and therefore, 0.6% w/v chitosan concentration was selected for optimized CNC. The characteristics of the optimized formulations (NET and CNC i.e., NC-6 using 0.6% chitosan) are showed in Table S1. The mean size (PDI) of the NET and CNC was around 101.4±7.08 nm (0.105±0.03) and 145.8±8.91 nm (0.122±0.04), respectively (n=3) as determined by PCS. HRTEM explorations of the optimized delivery system confirmed the nano sizes of the carriers (100-150 nm). The important relevant results from the TEM study (Fig. S3) illustrates that the prepared CNC has spherical morphology and development of CNC is signified by the continuous chitosan shielding layer of about 16 nm thickness over plain NET droplets.

Developed formulations were found to be highly efficient for AmB entrapment (Table S1). Fig. S4 shows the cumulative percentages of AmB released as a function of time. These curves showed the greater release of AmB from NET compared to CNC in initial 24 h period, after that release from swelled CNC was exceeded. Results of stability study (Fig. S5) demonstrate higher stability of CNC-AmB compared to NET-AmB and commercial formulations.

Assessment of AmB formulation toxicities. (a) Cytotoxicity to J774A MPφ cells.

Metabolism of treated cells was measured by reduction of dimethylthiazole diphenyltetrazolium bromide (MTT) by mitochondria. The cytotoxicity induced by various concentrations of AmB formulations is shown in Fig. 1. The rank order of cytotoxicity of the four formulations in terms of CC_{50} against the J774A cell line was Fungi zone (0.41±0.05 µg/ml)<NET-AmB (9.61±0.16 µg/ml)<Ambisome (9.84±0.16 µg/ml)<CNC-AmB (10.96±0.11 µg/ml). Results demonstrated that CNC-AmB formulation is least cytotoxic (higher CC_{50}).
(b) Hemotoxicity to erythrocytes. Formulated CNC-AmB exhibited hemotoxicity up to 3.291±0.82%, NET-AmB illustrated up to 7.832±1.67 whereas commercial formulations Ambisome and Fungizone showed hemotoxicity up to 6.321±1.02 and 100% respectively (Table 1). All tested formulations composed of AmB concentration in the range of 0.25-25µg/ml.

In vitro uptake studies. Fig. S6 in the supplementary material depicts the macrophagic uptake of developed nanocarrier formulations in J774A by flow cytometry analysis. This study represents comparative uptake among NET and CNC. Herein, we revealed almost two times (>1.87) enhanced uptake of CNC over NET in J774A cell lines.

Antileishmanial efficacy assessment of AmB formulation. (a) In vitro assessment in L. donovani intra-MPφ amastigotes. Concentration of AmB at which nearly 50% (IC_{50}) and 90% (IC_{90}) of clinical isolates of L. donovani amastigotes were killed, was calculated using log phase transgenic GFP-expressing intracellular amastigote by flow cytometry. The percent cell death was measured in terms of decrease in Mean Fluorescence Intensity (MFI) values on treatment with different concentrations of formulations. Results are presented in form of dose response curve (Fig. 2) and also reported in terms of IC_{50} as well as IC_{90} (Fig. 3). It has been observed that IC_{50} value of CNC-AmB and NET-AmB was 0.19±0.04 and 0.31±0.05 µg/ml while Ambisome and Fungizone exhibited 0.29±0.03 and 0.48±0.05 µg/ml, respectively. It is evident that the activity of CNC-AmB formulation is 1.5 times, 1.4 times and 2.4 times higher compared to NET-AmB (p<0.05), Ambisome (p<0.05) and Fungizone (p<0.05), respectively.

(b) In vivo assessment in L. donovani infected hamsters. From in vivo experiment results (Table 2) it was clearly manifested that CNC-AmB was significantly more active (86.1±2.08% inhibition) compared to NET-AmB (64.4±6.91% inhibition), whereas 69.8±3.13 and 55.5±4.34% parasite inhibition was observed with Ambisome and Fungizone, respectively. The rank order of
Quantitative mRNA RT-PCR studies in *L. donovani* infected hamsters. The antileishmanial efficacy of CNC-AmB was supported by a surge in iNOS, TNF-α and IL-12 mRNA levels. The levels of Th-2 cytokines IL-4, IL-10 and TGF-β mRNA, on the other hand, were observed to be down-regulated in the treated hamsters. Study showed significant differences between the treated and control groups (Fig. 4).

**DISCUSSION**

A variety of intracellular parasites including *Leishmania* can survive and multiply within cells of the MPS particularly MPφ which serve as reservoir for them owing to the development of potential ability to resist phagocytosis activity. Nanocarrier systems can be advantageous in treating these types of infection because they themselves are concentrated within phagocyte cells and provide direct contact of drug bearing carrier with parasites.

In the present study, we developed and evaluated a novel NE-template (NET) based stable lipid formulation, the CNC-AmB, in treatment of *L. donovani* infection *in vitro* and *in vivo* and its toxicity profile. Additionally, we investigated the changes on phagocytosis activity and cytokine production of MPφ after stimulation by CNC-AmB. To assess the efficacy of CNC-AmB, we compared its study results with that of NET-AmB and, commercial Fungizone and Ambisome.

Biocompatible excipients were chosen for the primary NET formulation, mainly soybean oil as a hydrophobe to generate core/shell of CNC, Tween 80 nonionic surfactant and MilliQ water as the aqueous phase. An additional, somewhat neutral component, SL, was introduced in the formulation which increases the nano-emulsion stability significantly, creating a ‘framework’
in the shell (34-36). Indeed, the addition of surfactants has been shown an important parameter to
efficiently reduce droplet size (37). The combination of surfactants with oils to form NET offers
an advantage over a micellar or co-solvent system in terms of drug solubilization capacities for
lipophilic compounds, because of the extra locus for solubilization provided by the oil phase
(33). The chitosan polymer was added in the continuous phase (even after nano-emulsion is
complete) and their deposition onto the emulsion droplets was induced by solvent evaporation,
resulted in CNC shell formation.

The zeta potential value and nanometric size are important particle characteristics as it
can influence both nanocarrier stability as well as cell adhesion (38). High positive zeta potential
of CNC due to continuous opaque shielding layer (thickness around 15.4 nm) of chitosan and
particulate form (150–200 nm) as confirmed by HR-TEM study, provides stability to the
formulation. Moreover, it is preferentially engulfed by the MPφ due to easy interaction of
cationic particles at MPφ negative surface by ionic adsorption and promote subsequent cellular
uptake leading to passive targeted drug delivery (39).

AmB molecule entrapped (97.8±2.11% efficiency) in CNC formulation by intercalation
between hydrophilic chitosan polymer and hydrophobic cavitation bubbles i.e., oil core,
generated by a succession of mechanical depressions and compressions in liquid dispersion of
NET due to sonifier energy (40). Minor decrease in the entrapment efficiency of CNC compared
to NET may be partially due to a longer exposure of NET droplets to the solvent during the
modification process, leading to a loss of the drug (Table S1). Fig. S4 showed that release ratio
of drug from NET-AmB significantly exceeded with the CNC in 24 h, which indicated
restriction of drug release due to chitosan surface layer. Thereafter, release from CNC-AmB
increased comparatively because of the hydrophilic chitosan which allows greater ease for the
release medium to penetrate, facilitating release of AmB from the swelled CNC. However, it has been reported that the incorporation of AmB to fat emulsions resulted in precipitation of AmB (41), whereas in the developed CNC it is highly stable.

Because nanocarrier stability is greatly influenced by surface charge (zeta potential), as shown in Fig. S5, negatively charged (mainly attributed to the presence of the natural surfactant lecithin in composition) NET droplets exhibited higher increase in size due to less stability compared to positive CNC. Negative charge of NET endorses the adsorption of cationic proteins and, sodium and calcium ions that are present in the biological fluids (serum) leading thereby to the neutralization of the surface charge, the breakdown of the system and the leakage of the entrapped agents. On the other hand, positive chitosan-surfactant layers around NET droplets in CNC carrier provided long term stability.

Model drug AmB and its commercial formulations are renowned for its hemotoxicity, arises due to eryptosis trigger, i.e. cell membrane scrambling and cell shrinkage, through stimulation of increased Ca$^{2+}$ entry into erythrocytes (42). In this aspect AmB-induced hemolysis was studied \textit{in vitro} which is a reliable measure for estimating the membrane damage caused \textit{in vivo}. Evaluation of toxicity measures of CNC-AmB against the J774A cell line clearly demonstrated its comparative non-toxicity (higher CC$_{50}$ 10.96 µg/ml than that of commercial formulations) towards the MP$\phi$ cells which confers to the hemo-compatibility behavior of chitosan (43).

When evaluated during \textit{in vitro} MP$\phi$ uptake studies, CNC-AmB showed elevated internalization in contrast to NET-AmB (Fig. S6). It has been shown that the expression of activation markers such as major histocompatibility complex (MHC) class I and II, Fc receptors, and mannose receptor are induced after chitosan treatment (18, 44). Possibility is that the binding
of N-acetyl-glucosamine unit of low molecular weight chitosan content of CNC favors the recognition by mentioned receptors, mediate the internalization of the drug carrier which is thought to be a prerequisite for enhancing activation of inflammatory cells including MPφ, the favorable site of *L. donovani* replication (14, 45). Increased delivery of CNC to the MPφ population would be expected to result in increased efficacy of CNC-AmB in *Leishmania* infected MPφ and hamsters.

When evaluated under *in vitro* studies, AmB in formulations retained its antileishmanial activity against intra-MPφ amastigotes. The 50% parasite growth inhibitory effectiveness of CNC-AmB formulation for the amastigotes, the stage responsible for VL, was 1.5-fold, 1.4-fold and 2.4-fold better compared to NET-AmB, Ambisome and Fungizone, respectively. These results supported the output from *in vivo* studies in golden hamsters in which the inhibition of *L. donovani* infection was significantly greater with CNC-AmB. Commercial formulation Ambisome, a liposomal drug carrier resulted in 1.65 times efficacious compared to Fungizone (p<0.05). It is interesting to note that primarily developed NET-AmB formulation is more effective compared to Fungizone and almost similar to that of Ambisome, while CNC-AmB is better than Ambisome (Table 2), suggesting that difference in activity of the CNC-AmB is attributable to uptake and interaction with host cells rather than intracellular distribution. The results demonstrated that at 1 mg/kg AmB, under the treatment regimen employed, CNC-AmB elicited a marked improvement over Ambisome in terms of parasite burden and survival. Substantial increased efficacy and decreased toxicity results are concordant with previous lipid-complexed AmB formulation studies (46, 47).

Accumulating evidences have shown that, in hamster, protection against *Leishmania* is associated with Th2-to-Th1 switching for complete parasite clearance. For this reason, we
assessed several Th1 (TNF-α, IL-12) and Th2 (TGF-β, IL-4 and IL-10) cytokines to investigate the possible role of the cytokine immune response in drug free CNC and CNC-AmB treatment of VL. Furthermore, iNOS chemokine in MPφ can be induced by many cytokines (48). After activation MPφ respond to the particulate CNC-AmB carrier via phagocytosis and secrete pro-inflammatory cytokines. Consecutively, this may lead to a cascade of adverse reactions which, in some occasions, cause severe damages to the host. Therefore, production of pro-inflammatory cytokines and internalization response were utilized to evaluate the interaction between the nanocarrier and the MPφ. The result in this study demonstrated that chitosan induced significantly elevated release of the TNF-α and IL-12 cytokines (Fig. 4 and S7) confirmed the reactivity of the J774 cells with splenic MPφ and ensured Th1-mediated protection (49). Results presented here show that LMW chitosan activated moderately the iNOS pathway in resident MPφ as reported earlier (50). Additionally, probable cumulative effect of IL-12 along with iNOS mediated the parasite killing (51, 52). Conversely, result showed favorable decrease in infection susceptible IL-10, IL-4 and TGF-β gene expression which are potent inhibitors of MPφ activation and killing of *Leishmania* organisms (53, 54). Decisively, our scientific finding suggest that CNC-AmB treatment may function equally well in immunocompromised patients, thereby increasing its potential efficacy in human immunodeficiency virus-leishmaniasis coinfection therapy.

**Conclusion.** Developed CNC-AmB represents a novel colloidal carrier, remarkably effective for experimental visceral *L. donovani* infection in hamster. At an equivalent dosage and treatment frequency it far surpasses commercial AmB formulations and results in effective clearance of parasites, using a limited biologically safe and widely spaced therapeutic regimen. The presence of discernible changes in the cytokine responses indicate that this immunochemotherapeutic
strategy is dependent on host cytokine-based immunity and CNC-AmB can be considered as ‘potential protective AmB delivery system’. In addition, the composition and manufacturing procedures of the CNC make feasible the production of a stable AmB delivery system that could be an economically interesting alternative to the current high-cost commercial lipid based formulations. The present work clearly supports the application of nano-emulsion template based chitosan nanocapsules (CNC) as a novel therapeutic AmB delivery approach for safer and cost-effective treatment of VL.

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REFERENCES


Figure legends

FIG. 1. Mitochondrial toxicity (MTT test) of AmB loaded formulations (NET-AmB and CNC-AmB) and comparison with that of commercial Fungizone and Ambisome against macrophages J774A after 24 h incubation at 37°C (5% CO₂) at 1 × 10⁵ cells/well in RPMI supplemented with 5% HIFBS.

FIG. 2. In vitro dose-response curve of AmB loaded NET-AmB, CNC-AmB, Fungizone and Ambisome, and AmB-free NET and CNC with the same amount of formulations against L. donovani amastigote infected macrophages observed after 48 h of incubation.

FIG. 3. In vitro antileishmanial activity (IC₅₀ and IC₉₀) of NET-AmB, CNC-AmB compared to Fungizone and Ambisome in L. donovani amastigote infected macrophages observed after 48 h of incubation.

FIG. 4. In vivo immuno-modulatory responses regarding splenic iNOS and cytokines (TNF-α, IL-12, IL-4, IL-10 and TGF-β) mRNA expression in infected control and treated (NET-AmB, CNC-AmB and compared to Fungizone and Ambisome) Syrian golden hamsters by quantitative RT-PCR. Analysis showing the relative fold changes of iNOSs and cytokine expression level±S.D. (n= 5 hamsters/time point) in comparison to reference gene (HPRT).
FIG. 1. Mitochondrial toxicity (MTT test) of AmB-loaded formulations (NET-AmB and CNC-AmB) and comparison with that of commercial Fungizone and Ambisome against macrophages J774A.1 after 24 h incubation at 37°C (5% CO₂) at 1 × 10⁶ cells/well in RPMI supplemented with 5% FBS.
TABLE 1 Hemolytic potential of various AmB-loaded nanocarriers and commercial formulations at different drug concentrations. *Data are reported as mean of three (n=3) independent experiments±SD.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Percent hemolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NET-AmB</td>
</tr>
<tr>
<td>0.25</td>
<td>0.167±0.34</td>
</tr>
<tr>
<td>5</td>
<td>0.639±0.24</td>
</tr>
<tr>
<td>10</td>
<td>2.439±0.54</td>
</tr>
<tr>
<td>25</td>
<td>7.832±1.67</td>
</tr>
</tbody>
</table>
**TABLE 2** *In vivo* antileishmanial activity of NET-AmB, CNC-AmB and compared to Fungizone and Ambisome in the established Syrian golden hamster model infected with *L. donovani* amastigotes. A shortened treatment course was given; 5 intraperitoneal injections over a 5 day period on days 31, 32, 33, 34 and 35 post-infection making a total dose of 5 mg of AmB/kg body weight of hamster.

<table>
<thead>
<tr>
<th>Group (n=5)</th>
<th>Before treatment</th>
<th>NET-AmB</th>
<th>CNC-AmB</th>
<th>Fungizone</th>
<th>Ambisome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amastigotes/100 MPϕ (Nuclei)³</td>
<td>402.2±42.31</td>
<td>143.3±25.42</td>
<td>55.8±10.31</td>
<td>178.9±28.44</td>
<td>121.6±4.83</td>
</tr>
<tr>
<td>Percentage inhibition of splenic amastigote (Load)²</td>
<td>-</td>
<td>64.4±6.91</td>
<td>86.1±2.08</td>
<td>55.5±4.34</td>
<td>69.8±3.13</td>
</tr>
</tbody>
</table>

³ The NET-AmB group is statistically significantly different from the Fungizone and Ambisome groups (P <0.01), and the CNC-AmB group is statistically significantly different from the Fungizone and Ambisome group (P<0.05) (Tukey test followed by Dunn’s multiple comparisons test).

² The NET-AmB group is statistically significantly different from the Fungizone and Ambisome groups (P <0.01), and the CNC-AmB group is statistically significantly different from the Fungizone and Ambisome group (P<0.01) (Tukey test followed by Dunn’s multiple comparisons test).