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Reference:
Riaz Amina, Hendrickx Sarah, Elbrink Kimberley, Caljon Guy, Maes Louis, Ahmed Naveed, Kiekens Filip, Khan Gul Majid.- Preparation and characterization of nanostructured lipid carriers for improved topical drug delivery : evaluation in Cutaneous leishmaniasis and vaginal candidiasis animal models
AAPS PharmSciTech / American Association of Pharmaceutical Scientists - ISSN 1530-9932 - 21:5(2020), 185
Full text (Publisher's DOI): https://doi.org/10.1208/S12249-020-01717-W
Full text (Publisher's DOI): https://doi.org/10.1208/S12249-020-01819-5
To cite this reference: https://hdl.handle.net/10067/1700940151162165141

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Nanostructured lipid carriers as topical drug delivery vehicle: *in vitro* and *in vivo* evaluation in a cutaneous *Leishmania* and vaginal *Candida* laboratory model.

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Abstract:

The use of nanocarriers in drug delivery has provided new opportunities in topical treatment of diseases affecting the skin and mucosa by improving drug permeation and accumulation in the various skin strata, reducing systemic side effects and enhancing the solubility of poorly soluble drugs. The present study aimed to evaluate the application of amphotericin-B loaded nanostructured lipid carriers (AmB-NLCs) for topical treatment of cutaneous leishmaniasis and vulvovaginal candidiasis. AmB-NLCs were characterized for particle size, zeta potential, polydispersity index and encapsulation efficiency. The surface morphology of AmB-NLCs was evaluated using scanning electron microscopy and Fourier transform infrared spectroscopy was done to check chemical interactions between excipients. Thermal properties of prepared AmB-NLCs were evaluated using differential scanning calorimetry. The AmB-NLCs were further characterized for *in vitro* drug release, *ex vivo* skin permeation and deposition before evaluating their *in vitro* and *in vivo* antileishmanial and antifungal efficacy. Significant accumulation in the skin (22.7 ± 2.6%) supported the topical application potential. Encapsulation of AmB in NLCs resulted in enhanced *in vitro* potency against various *Leishmania* and fungal strains as compared to the plain AmB solution. Topical application of AmB-NLCs on *L. major* infected BALB/c mice resulted in a significant reduction in parasite load compared to the control group. Topical AmB-NLCs gel demonstrated superior efficacy in the vaginal *Candida albicans* rat model as compared to plain AmB gel. In conclusion, NLCs may have promising potential as carrier for topical treatment of various conditions of skin and mucosa.

Key Words: Nanostructured lipid carriers, Amphotericin B, Topical treatment, Cutaneous Leishmaniasis, Vulvovaginal candidiasis,

INTRODUCTION

Effective drug delivery requires the active ingredient to attain therapeutic concentration at site of action for a given time period. Advances in drug delivery research have enabled the formulation scientists to explore routes of administration other than the conventional oral or parenteral routes. Topical drug delivery generally refers to the application of drugs on skin or mucous membranes for a localized effect. Some of the major advantages accompanying topical delivery include improved drug bioavailability, reduced systemic toxicity, improved patient compliance, avoidance of hepatic first-pass elimination and more convenient application (Tan, Feldman et al. 2012). The development of an effective topical drug delivery vehicle requires overcoming several barriers and limitations requiring cautious selection of active ingredient as well as vehicle ingredients to achieve therapeutic drug concentrations at the target site (Singh Malik, Mital et al. 2016). The present study specifically involved two infectious diseases with topical manifestations.

Cutaneous leishmaniasis (CL) is the most prevalent of all forms of leishmaniasis and is caused by one of several *Leishmania* parasite species that are transmitted by the bite of sand flies (de Vries, Reedijk et al. 2015). Almost 90% of all CL cases arise in only seven countries including Iran, Afghanistan, Brazil, Algeria, Saudi Arabia, Syria and Peru (Hepburn 2003). Selecting the most suited therapy for CL depends on the *Leishmania* species as well as the clinical stage. Local therapy alone is reserved for the cases with smaller and limited lesions, whereas those with larger and widespread lesions should benefit from systemic or oral therapy along with local treatment (Arana 2018). Topical application of a gel or cream is advantageous over systemic therapy or intralesional injections to avoid systemic side effects, improve patient compliance and less need for rigorous follow-up. However, conventional gels and creams have proven to be far from effective due to various problems including the inability to cross the dermal *stratum corneum* (SC) and reaching the target site at therapeutic adequate concentrations (Van Bocxlaer, Yardley et al. 2016).

Mycoses, especially skin and mucosal fungal infections represent a common disease rising worldwide (Havlickova, Czaika et al. 2008, Dorgan, Denning et al. 2015). Vaginal candidiasis or vulvovaginal candidiasis (VVC) is the most common and affects 75% of all women of child bearing age (Song, Wang et al. 2004). Although the main yeast species responsible for VVC is *Candida albicans,* other species including *C parapsilosis, C. glabrata* and *C. krusei* are also emerging as cause of candidiasis. For less problematic fungal infections,

topical application of antifungal drugs directly on the skin or mucosa is favorable as this will result in a more localized effect and reduced systemic toxicity potential.

Amphotericin B (AmB) belongs to the class of the polyene antibiotics and is a broad-spectrum antifungal and antileishmanial agent typically administered intravenously (Lemke, Kiderlen et al. 2005). Its mechanism-of-action involves the formation of complexes with ergosterol present in cell membranes leading to the formation of pores in the membrane and subsequent cell death (Brajtburg, Powderly et al. 1990). AmB is not very soluble in water (<1mg/L) at physiological pH, which is the main reason for its low oral bioavailability. AmB is currently used as a second-line drug for CL and is generally reserved for the more severe cases because of its relative high toxicity when administered intravenously (Croft, Seifert et al. 2006). A topical formulation of AmB would therefore be desirable as it would reduce systemic side effects and improve patient compliance. Topical AmB already demonstrated some efficacy in treating CL lesions (López, Vélez et al. 2018). Although the first-line treatment for VVC is still fluconazole or another azole, AmB becomes increasingly important in fluconazole-resistant strains (Sobel 2016). Also in pregnant women with VVC, AmB is the drug of choice owing to its low teratogenic potential (King, Rogers et al. 1998). Conventional topical AmB-formulations, including gels, creams or ointments, often only show limited efficacy and local skin irritation. As AmB is highly lipophilic with a high molecular weight (924.079 Da), its absorption through skin and mucosal tissues is low (Sheikh, Ahmad et al. 2014).

The use of nanocarriers (NCs) in drug delivery has provided new opportunities in topical treatment of various diseases, including CL and VVC. Different types of NCs have been evaluated in treating CL lesions by both systemic (Heidari-Kharaji, Taheri et al. 2016) and topical (Ammar, Nasereddin et al. 2019) routes and demonstrated significant advantages over the conventional dosage forms. The NCs display improved efficacy owing to many factors, including an improved drug permeation across the SC barrier, an enhanced drug accumulation in the various skin layers, a sustained drug release over a longer period of time and enhanced solubility of poorly soluble drugs (Espuelas, Schwartz et al. 2016). The application of NCs in VVC can also result in controlled drug absorption from the vaginal epithelium as compared to conventional gels while simultaneously retaining other aforementioned advantages (Ensign, Cone et al. 2014, Trombino, Mellace et al. 2016). Encapsulation of drugs into NCs has already shown to improve solubility, therapeutic activity, stability and bioavailability over the free drug (Singh, Singh et al. 2016). Nanostructured lipid carriers (NLCs) are the second generation of lipid nanoparticles and have various advantages over the first generation solid lipid nanoparticles (SLNs) and other types (polymeric, metallic) of nanoparticles (Das, Ng et al.

2012). Our research group has already prepared and optimized drug-loaded NLCs using curcumin (Cur-NLCs) as model drug, owing to its common availability, non-toxicity and cost effectiveness (Riaz, Ahmed et al. 2019). NLCs based on glyceryl monostearate (GMS) and isopropyl myristate (ISM) had average particle size (PS) of 312 ± 1.89 nm, zeta potential (ZP) of -38.4 ± 0.93 mV and encapsulation efficiency (%EE) of 88 ± 2.45%. Prepared NLCs showed a significant targeting potential to macrophages and accumulation in deeper skin layers. The *in vitro* antileishmanial assays demonstrated a superior efficacy for drug-loaded NLCs as compared to the plain drug solution.

The aim of the present study was to incorporate AmB in the previously optimized NLCs and to characterize the novel AmB-loaded NLCs (AmB-NLCs) for their PS, ZP, %EE, PDI and *in vitro* drug release. The potential of AmB-NLCs as drug vehicle to improve the topical delivery was evaluated in CL and VVC laboratory models. The *in vitro* antileishmanial and antifungal efficacy of the AmB-NLCs was evaluated and compared with that of the plain AmB solution against various *Leishmania* and fungal species. Finally, the efficacy of the optimized AmB-NLCs was confirmed *in vivo* against *L. major* infection in the BALB/c mouse model and the *C. albicans* VVC rat model.

MATERIALS AND METHODS

Chemicals

Isopropyl myristate (IPM), methanol, amphotericin B (AmB) and Giemsa stain were purchased from Sigma Aldrich, glyceryl monostearate (GMS) from Sasol (Witten, Germany), Phospholipon® 90G from Lipoid AG (Switzerland), Carbopol®934 from Avonchem and $Tween_{80}$ from BDH Lab England.

Microbial strains and cell culture

Promastigotes of *L. major* JISH 118 and *L. tropica* AO-21 were kindly provided by Prof. Croft (London School of Hygiene and Tropical Medicine). Cultures were grown at 26°C in RPMI-1640 medium (Gibco, Essonne, France) supplemented with 1% 2 mM L-glutamine (Gibco), 5% heat inactivated fetal bovine serum (iFBS) (Gibco) and 2% penicillin-streptomycin (Gibco) and were sub-cultured twice weekly. To maintain parasite virulence, the number of passages *in vitro* was kept as low as possible. MRC-5 cells were cultured in minimum essential medium (MEM) supplemented with 5% iFBS, 16.5 mM $NaHCO_3$ and 20 mM L-glutamine at 37°C under 5% CO_2. The fungal species *Trichophyton rubrum*,

C. albicans and *Aspergillus fumigatus* were cultured in RPMI respectively at 37°C, 27°C and 37°C.

Animals

Female Swiss mice (15-20 g), female BALB/c mice (15-20 g) and female rats (175-200 g) were purchased from Janvier (France) and were provided with a standard rodent diet and water *ad libitum*. The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its latest version) and was approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD 2017–80 (06-06-2019).

Preparation of AmB-loaded NLCs (AmB-NLCs):

Drug-loaded NLCs were prepared based on our previous experiences using curcumin as model drug and optimized for concentration of lipids, surfactants and drug using Design Expert software (Trial Version 11, State-Ease Inc., MN). It was demonstrated that the PS of Cur-NLCs augmented by increasing the lipid and drug concentrations, whereas the concentration of surfactant had an insignificant effect. On the other hand, the %EE improved by increasing the concentration of lipids, drug and surfactants. An optimized formulation was proposed by the software, containing 150 mg surfactant, 8 mg drug and 808.93 mg of solid and liquid lipid blend (for 20 ml formulation). The observed values for PS (312 ± 1.89 nm) and %EE (88 ± 2.45) correlated well with the expected values of PS (317.8 nm) and %EE (89) for drug-loaded NLCs. AmB was loaded in the previously optimized NLCs using a similar technique with little modification. Briefly, required quantities of GMS (3.43%), IPM (0.6%) and Phospholipon® 90G (0.1%) were melted and mixed at 65°C. AmB (0.04%) was dissolved in 2 ml basic methanol and added in the lipid melt. An aqueous phase consisting of water and $Tween_{80}$ (0.75%) was maintained at the same temperature as that of the lipid melt. The aqueous phase was then added onto the lipid phase at once and subjected to high speed homogenization for 10 minutes at 26,000 rpm using a Heidolph silent crusher M. Temperature was maintained at 65°C throughout the process. The resulting nanodispersion was allowed to cool at room temperature before the volume was adjusted using distilled water. Finally, to further reduce the particle size, the liquid dispersion containing NLCs was subjected to probe sonication for 1 minute (cycles of 20 seconds sonication and 20 seconds rest) using Misonix-XL-2000 series.

Preparation of AmB-NLCs based Carbopol gel

To improve topical applicability, AmB-NLCs were incorporated into a gel based on Carbopol 934 (0.5%). The measured quantity of Carbopol was introduced into the AmB-NLCs dispersion and was allowed to stir till complete hydration (1 hour), after which a drop of triethanolamine was added to neutralize and form the AmB-NLCs gel. Glycerol (2% w/w) was added as humectant and the gel was stored at 2-8°C prior to use. For comparison of efficacy, a reference gel (RG) of AmB was prepared by incorporating the solution of AmB in already prepared carbopol 934 gel (0.5%) by stirring.

HPLC conditions for quantification of AmB

Quantification of AmB was done using High performance liquid chromatography (HPLC) (Varian ProStar, Model 210) equipped with UV-visible detector (Varian, Model 340), as described previously (Javed, Hussain et al. 2015). The mobile phase used was mixture of acetonitrile and sodium phosphate buffer (pH 7.4) in a ratio of 40:60 that was supplied isocratically at a rate of 1 ml/min. One hundred microliter of the sample was injected into the HPLC column (Agilent, Zorbax C8, 5um, 4.6×150 mm) and the absorbance of the eluent was measured at 406 nm.

Characterization of AmB-NLCs

AmB-NLCs were characterized for PS, PDI and ZP using the Zetasizer ZS90 (Malvern Instruments, UK). AmB-NLCs were diluted (1:10) with double distilled water before running each experiment. Results are presented as mean value of three measurements. The %EE was determined by measuring the amount of un-entrapped drug remaining in the supernatant after separating the AmB-NLCs pellet by centrifugation. Briefly, the AmB-NLCs dispersion was centrifuged (HERMLE Labortechnik GmbH Z326 K) at 13,000 rpm at 4°C for 40 minutes. The amount of un-entrapped drug was determined by separating the supernatant and measuring the absorbance of AmB at 406 nm using UV-visible spectrophotometer (HALO DB-20 UV-VIS double beam). The experiment was conducted in triplicate and the Eq. (1) was used to determine the % EE (Butani, Yewale et al. 2016).

$$\%EE = Mi - Mf/Mi \times 1000 \quad \text{Eq. (1)}$$

Where M_i and M_f are the amounts of drug, loaded initially and found in supernatant at the end, respectively.

Surface morphology of AmB-NLCs was determined using Scanning electron microscopy (SEM). Sample images were taken by using a field emission gun SEM (MIRA3 TESCON) in secondary electron imaging mode and at voltage of 5KV.

FTIR analysis was carried out to study any interaction between AmB and lipids used in NLCs formulation with FTIR spectrophotometer (100 L160000A, Perkin Elmer, USA). Infra-red spectra were obtained for GMS, IPM, AmB and AmB-NLCs over a wavelength range of 4000-600 nm^{-1} (Phatak and Chaudhari 2013).

Thermal properties of dried GMS, IPM, AmB, blank NLCs and AmB-NLCs were evaluated using DSC equipment (DSC25, TA instruments, New Castle, DE, USA). Blank NLCs and AmB-NLCs dispersions were dried at room temperature. Samples (8-10 mg) were evaluated using aluminum pans (Tzero) on calibrated equipment (using indium and sapphire standards) under nitrogen gas purge (50 ml/min). Samples were heated from -50°C to 200°C at a heating rate of 10°C/min. Data obtained was examined and quantified using TA instruments TRIOS software.

Characterization of AmB-NLCs gel

The AmB-NLCs gel was evaluated for its physicochemical properties including colour, odour, pH, stability and consistency (Hajjar, Zier et al. 2018). The chemical formula of AmB is $C_{47}H_{73}NO_{17}$ and AmB has a molecular weight of 924.091 $g.mol^{-1}$ and a melting point of 170°C respectively.

A Brookfield viscometer was used to study the rheological properties of the AmB-NLCs gel. Spindle no s63 was used for measurements and different shear speeds were used varying from 2 to 120 rpm at 25°C. The apparent viscosity of AmB-NLCs gel was measured at each speed and a viscosity curve was constructed between apparent viscosities and shear speeds (Zhu, Chen et al. 2018).

The spreadibility, which can be defined as the ease with which a formulation spreads on the skin, was determined by using a wooden block and a glass slide apparatus. Briefly, 0.1 g of AmB-NLCs gel was placed on the glass slide and the time for the upper moveable slide to get separated from the fixed slide after addition of 50 g weight to the pan was noted. Spreadibility was calculated by applying the following formula.

$$S = \frac{M \times L}{t} \quad \text{Eq. (2)}$$

Where S denotes spreadibility coefficient and M, L and t are weight on the pan, the length of glass slide and the time taken by the upper slide to get separated from the fixed slide respectively (Maitra, Goyal et al. 2017).

In vitro drug release

An *in vitro* drug release study from the AmB solution, AmB-NLCs, RG of AmB and AmB-NLCs gel was conducted using a shaker water bath (Memmert SV 1422). Briefly, measured quantities of sample (containing 0.5 mg of AmB) were placed in a dialysis bag (MWCO 12000 Da, Sigma Aldrich) and the dialysis bag was then placed in 60 ml of release medium (phosphate buffer, pH 7.4) containing 1% $Tween_{80}$. The drug release study was conducted by placing this assembly into the shaker water bath for 24 hours during which a fixed volume of samples was withdrawn at predetermined time intervals (0.5, 1, 2, 4, 6, 8, 12 and 24 hours) and replaced with fresh buffer (pH 7.4). Samples were analysed for AmB quantification by using UV-Visible spectrophotometer (HALO DB-20 UV-VIS double beam) at 406 nm. For this purpose a standard curve of AmB was prepared in the same medium (phosphate buffer, pH 7.4 having 1% $Tween_{80}$) and the AmB concentrations in the samples were calculated via the standard curve (y=0.0155x+0.0426) (Santiago, e Silva et al. 2018).

Ex vivo skin permeation and deposition

AmB-NLCs and AmB solution were subjected to an *ex vivo* skin permeation and skin deposition study using fresh skin excised from healthy rats (Joshi and Patravale 2008). Hair and subcutaneous fat tissues were removed from the skin before mounting it between the donor and receptor compartments of a Franz diffusion cell (custom made) with the SC side facing the donor compartment. The applied Franz diffusion cell had an effective area of 0.77 cm^2 available for permeation and a capacity of 5.2 ml. The receptor compartment was filled with phosphate buffer pH 7.4 having 1% $Tween_{80}$ and was continuously stirred at 300 rpm throughout the experiment. A weighed quantity of gel corresponding to 0.25 mg of AmB was placed in the donor compartment and samples were taken from the receptor compartment at predetermined time intervals up to 24 hours. After each time a sample was taken, the medium in the receptor compartment was replaced with fresh buffer to maintain sink conditions. The amount of AmB in each sample was determined using HPLC as described above. The amount of AmB permeated per unit area (ug/cm^2) was plotted against time and the flux (J_{max}) was calculated using Eq. (3) (Butani, Yewale et al. 2016).

$$Jmax = \frac{(amount\ of\ drug\ permeated)}{(time)\times(area\ of\ membrane)}$$ Eq. (3)

At the end of the experiment, the excess formulation remaining on the skin surface was wiped off and the amount of AmB retained inside the skin was quantified. For this purpose, the skin was cut into smaller pieces and soaked in basic methanol overnight, followed by 5 sonication cycles (15 minutes) in an ultrasound bath. AmB was recovered in the supernatant after centrifugation at 600 rpm for 10 minutes, passed through a 22 µm membrane filter and quantified using HPLC.

MRC-5 cytotoxicity assay

Cytotoxicity of the prepared AmB-NLCs and AmB solutions was assessed on MRC-5 cells. Briefly, MRC-5 cells were seeded in 96-well microtiter plates in MEM medium (having 16.5 mM $NaHCO_3$, 5% iFBS and 20 mM L-glutamine) at a concentration of 10^4 cells/well. Sample dilutions of AmB-NLCs and AmB solution were incubated at 37°C in presence of 5% CO_2 for 72 hours. After incubation, 50 µl of resazurin solution (7-hydroxy-3H-phenoxazin-3-one 10-oxide) was added and the fluorescence was determined spectrophotometrically (TECAN® GENios, excitation 550 nm – emission 590 nm) after 4 hours. Results are expressed as the % reduction in cell viability of each well containing a sample dilution as compared to the untreated positive control wells and CC_{50} values (concentration that is cytotoxic to 50% of cells) were determined (Mothana, Al-Musayeib et al. 2012).

Skin irritation study

The skin irritation potential of AmB-NLCs gel was evaluated in rats (3 animals in each group *i.e.,* control and treatment) according to Organization for Economic Co-operation and Development (OECD) test guidelines 404: acute dermal irritation/corrosion (James and Sunday 2014). Hairs on the back of animals were removed using an electric razor 24 hours before sample application. Rats were anesthetized using 0.2 ml ketamine HCl injection to avoid any movement after gel application for at least 2 hours. Weighed quantity of gel (0.5 g) was applied topically on shaved back of the animals in treatment group whereas the control group received topical application of distilled water (0.5 ml). Gel was kept in contact with skin by the aid of adhesive plaster tape and bandage dressing for 24 hours. After completion of 24 h time period, dressing and any material were removed and skin was washed with distilled water. Exposed areas were examined for signs of irritation at 1 h, 24 h, 48 h, 72 h, 7 days and 14 days. Scoring for erythema and edema was done according to the grading of skin reactions table as presented

in OECD guidelines on a scale of 0-4 with "0" depicting no reaction and "4" demonstrating severe erythema and edema.

Antileishmanial activity of AmB-NLCs

Against promastigotes: To evaluate the efficacy of the AmB-NLCs and AmB solution against *Leishmania* promastigotes, 10 µl of drug dilutions were added in each well of a 96-well microtiter plate containing 200 µl of parasite inoculum (100,000/well) except for the positive (untreated parasite inoculum) and negative control (only growth medium) wells. After 72 hours of incubation at 25°C, 50 µl of resazurin solution was added in each well and the fluorescence (excitation wavelength 550 nm, emission wavelength 590 nm) was measured after 24 hours. The resulting IC_{50} values are the results of 6 independent assays run in duplicate and were determined based on the percentage reduction in parasite burden as compared to parasite growth in the positive control wells. IC_{50} (concentration that inhibits 50% growth) values for both AmB-NLCs and AmB solution were calculated (Vermeersch, da Luz et al. 2009).

Against intracellular amastigotes: Primary mouse peritoneal macrophages (PMM) were used to assess the sensitivity of amastigotes towards test compounds. PMM were obtained 24 hours after intraperitoneal stimulation of Swiss mice with a 2% starch solution in PBS. PMM were seeded in RPMI-1640 medium in 96-well microtiter plates at a final concentration of 3×10^5 cells/well and were allowed to differentiate and adhere for 24 hours. After 24 hours, macrophages were infected with metacyclic promastigotes at an infection rate of 10 promastigotes per macrophage. After 24 hours of infection, non-internalized promastigotes were removed by discarding the medium from the wells and the infected PMM were replenished with fresh medium containing 2-fold drug dilutions. Then, the plates were incubated at 37°C under 5%CO_2 for 4 days. Positive controls were infected macrophages without any treatment and negative controls were non-infected macrophages. After incubation, macrophages were fixated with methanol and stained with a Giemsa staining solution. Parasite burdens were assessed microscopically and compared with untreated positive controls. Infection was considered adequate if 70 to 80% of macrophages were infected in the positive control wells. Results are expressed as percentage reduction in parasite burden compared to untreated infected macrophages and IC_{50} values were calculated for both AmB-NLCs and the AmB solution. Results are expressed as average values of six independent replicates each run in duplicate (da Luz, Vermeersch et al. 2009).

***In vitro* antifungal activity of AmB-NLCs**

Antifungal assays were performed on three fungal species, as described previously (Van de Ven, Paulussen et al. 2012). Briefly, RPMI medium was used for culturing of fungal species and the susceptibility assay was performed in 96-well microtiter plates using 5×10^3 colony forming units (CFU) per well. The plates were incubated for 48 h at 37°C in case of *A. fumigatus*, 24 h at 37°C for *C. albicans* and 7 days at 27°C for *T. rubrum* respectively. After completion of incubation period, 10 µl of resazurin was added in each well and cell viability was checked fluorimetrically after incubating for 17 h, 4 h and 24 h respectively.

Efficacy of topical AmB-NLCs in infected BALB/c mice *in vivo*

Female BALB/c mice were infected with early passage promastigotes of *L. major* JISH 118 by subcutaneous injection of 4 x 10^7 promastigotes/200 µL in RPMI-1640 at the base of the tail. The area around the tail base was shaved using an electrical razor 24 h before infection. At 14 days post-infection, the animals were regrouped into 5 groups with comparable mean lesion sizes (Table 1). Treatment was started 14 days post-infection and continued for 10 days. During treatment, animals were observed daily and the lesion size was measured using a digital caliper. The percentage of reduction in mean lesion size as compared to control group could be followed during treatment. After completion of treatment, the animals were euthanized and the skin around the infection site was collected, weighed and subjected to parasite extraction. Briefly, excised skin was incubated in 1.5 ml of collagenase medium for 2 hours at 37°C under 5% CO_2. Next, the skin piece was homogenized using the gentleMACS Dissociator™ (Miltenyi). The cell suspension was strained (70 µm mesh) and washed twice with 5ml PBS. After straining, the suspension was centrifuged at 3000g for 8 minutes and the cell pellet was suspended in 1 ml RPMI. Starting from 100µL of sample, 10-fold serial dilutions were prepared in duplicate to determine parasite burdens by limiting dilution assay. Results are expressed as parasite burden per mg of lesion for each group.

Efficacy of topical AmB-NLCs gel in VVC rat model *in vivo*:

Efficacy of topical AmB-NLCs gel was evaluated and compared with AmB RG in an established rat model of VVC (de Wit, Paulussen et al. 2010). The ovaries of female rats were removed 3 weeks before start of experiment to allow sufficient time for recovery. The pseudo-estrus was induced by injecting subcutaneous estradiol benzoate (1 mg) and progesterone (200 µg) 3 days before experiment and then on day 2 and 7. Rats were infected with intravaginal administration of 10^7 CFU of *C. albicans* and treatment was started next day after

infection. Carbopol gel was used to improve retention of drug-loaded nanocarriers in the vagina. Animals were divided into three groups: control group (n=4) and three treatment groups (n=2) receiving topical AmB-NLCs gel or AmB RG or 1% miconazole (MIC) gel as reference. Topical gel (200 µl) was applied intravaginally twice daily and treatment was continued for 10 days. Fungal burden was estimated by taking vaginal swabs at days 4, 7 and 11 after start of treatment.

RESULTS AND DISCUSSION

Preparation of AmB-NLCs and AmB-NLCs based Carbopol gel

AmB-NLCs were successfully prepared, similarly to the Cur-NLCs previously made by our group (hot homogenization followed by probe sonication). Actually, AmB could be encapsulated into the NLCs in exactly the same way. Prepared AmB-NLCs were also successfully converted into a gel made up of Carbopol 934 to increase their ease of application.

Characterization of AmB-NLCs

The PS, PDI, ZP and %EE of AmB-NLCs were 288 ± 1.12 nm, 0.286 ± 0.08, -38.8 ± 1.98 and 90 ± 2.78, respectively. The PS of AmB-NLCs was smaller as compared to Cur-NLCs (312 ± 1.89 nm) which may be attributed to the lower molecular weight of curcumin (368.38 Da) compared to AmB (924.07 Da), since it has been reported that low molecular weight polymers produce bigger sized nanoparticles compared to high molecular weight polymers (Palacio, Orozco et al. 2011).

There was no significant difference in PDI, ZP and %EE between AmB-NLCs and Cur-NLCs. The high negative value of ZP (-38.8 ± 1.98) confirms the physical stability of the nanodispersion by conferring sufficient repulsion between negatively charged colloidal particles (Chetoni, Burgalassi et al. 2016). The PDI value of 0.286 ± 0.08 denotes a relatively narrow size distribution. Although the ideal PDI value for a supreme quality colloidal dispersion is claimed to be ≤1, many researchers also reported PDI values of ≤3 as ideal for a uniform and stable system (Shah, Eldridge et al. 2014).

The low drug concentration in combination with the high concentration of lipids in the NLCs formulation resulted in a higher %EE (90 ± 2.78) since a higher amount of lipid blend was available to encapsulate drug molecules. In case of topical application, this high %EE can help in reducing skin irritation by avoiding direct contact of the drug with the skin surface

(Butani, Yewale et al. 2016). SEM images of AmB-NLCs show that AmB-NLCs are spherical in shape with a smooth surface (Figure 1).

The FTIR spectra of GMS, IPM, AmB and AmB-NLCs are presented in Figure 2. The FTIR spectrum of GMS shows characteristic peaks at 1729.77 cm^{-1}, 2913.94 cm^{-1} and 3306.85 cm^{-1} due to C=O stretching, $–CH_2–$ stretching and OH stretching vibrations respectively, while the FTIR spectrum of IPM shows peaks at 2922.83 cm^{-1}, 2853.53 cm^{-1}, 1732.99 cm^{-1}, 1466.90 cm^{-1} and 1248.51 cm^{-1} due to CH_2 stretching (asymmetric), CH_2 stretching (symmetric), C=O stretching, C-O-H in plane band and C-O stretching respectively. The FTIR spectrum of AmB shows peaks at 1691.55 cm^{-1}, 1552.18 cm^{-1} and 1007.25 cm^{-1} that represent C=O stretching, in plane NH_2 bend and out of plane C-H bend respectively (Bhosale, Bhandwalkar et al. 2016). Absence of peaks of AmB in the FTIR spectrum of AmB-NLCs demonstrates successful encapsulation of the drug within the solid lipid NLCs-matrix (Teng, Yu et al. 2019).

DSC is an important technique in determining the state of drug encapsulated in NLCs (whether crystalline or amorphous) by observing the temperature changes at phase transition (Madane and Mahajan 2016). Thermograms obtained for GMS, IPM, AmB, Blank NLCs and AmB-NLCs are presented in Figure 3**.** The melting peak for AmB appeared at 137°C whereas melting peaks for GMS and IPM appeared at 57°C and 9°C respectively. Blank NLCs demonstrated melting temperature of 54°C due to combined effect of solid and liquid lipid that resulted in lowering of melting temperature. The melting peak for AmB is absent in the thermogram of AmB-NLCs that depicts drug dispersion in the lipid matrix up to molecular level and conversion of drug state from crystalline to amorphous.

Characterization of AmB-NLCs gel

The AmB-NLCs gel had a yellowish colour and was odourless, smooth, stable, homogeneous and translucent. The pH of the gel was 6.4 ± 0.13, which shows compatibility with the pH of the skin. Figure 4a demonstrates the effect of varying shear rate on the apparent viscosity of the AmB-NLCs gel and reveals that the apparent viscosity was inversely related to the shear rate. At 6 rpm, the viscosity of the AmB-NLCs gel was high (19996 cp) but decreased with increasing shear rate. By looking at the viscosity curve, it can be concluded that the AmB-NLCs gel showed shear thinning behaviour that is appropriate for pharmaceutical topical gels. Viscosity at a high shear rate of 100 rpm (1200 cp) was much lower than the original viscosity, which points to deformation of gel structures at high shear rates. Storage and transport of the prepared gel can, however, happen without any problem as long as the AmB-NLCs gel is not subjected to changes in shear which may possibly alter viscosity and stability of the gel

(Chawla and Saraf 2012). Spreadibility of a topical formulation is of key importance in the application of a formulation on skin and its overall performance. In general, the application of a gel on ulcerated skin is simpler if the gel would easily spread (Sachan, Gupta et al. 2016). The spreadibility of AmB-NLCs gel was calculated to be 52 ± 5.126 g.cm/sec, which is acceptable for a skin formulation (Hafeez and Kazmi 2017).

In vitro drug release

The *in vitro* drug release demonstrates that almost 50% of the encapsulated drug was released within the first 6 hours, followed by a more sustained release. The cumulative percentage drug release from AmB-NLCs and AmB-NLCs gel was 75% and 68% respectively after 24 hours (figure 4b). The drug absorbed on the nanoparticle surface and/or encapsulated in the outer shell became available for release soon after start of experiment and explains the higher initial release, whereas sustained release can be explained by drug release from the inner core of NLCs (Fang, A Al-Suwayeh et al. 2013). Similar results were reported in literature where AmB-loaded solid lipid nanoparticles (SLNs) demonstrated a burst release during the initial 8 hours followed by a sustained release over the next 72 hours (Jain, Gupta et al. 2014). This slower and sustained drug release from the nanoparticles will help in reducing the cytotoxicity of AmB to normal cells, which indeed can be observed upon the abrupt release of large amounts of the drug when using other formulations (Jansook, Pichayakorn et al. 2018).

The dissolution data analysis program (DDSolver) was used to fit the drug release data to various models including zero-order, first-order, Korsmeyer-peppas and Higuchi models. The resulting R^2 values demonstrated that drug release followed the Korsmeyer-Peppas model (Table S1). The diffusion exponent (n) was calculated and its values (≤ 0.4) revealed a fickian release for both AmB-NLCs and AmB-NLCs gel that was diffusion controlled (Dash, Murthy et al. 2010).

Ex vivo skin permeation and deposition

In case of topical application of an active ingredient, a high retention of the drug in the skin is desirable to improve efficacy. The drug permeation study (Figure 4c) demonstrated that AmB-NLCs showed higher AmB permeation (231.86 ± 2.48 µg/cm^2) across the skin compared to the AmB solution (72.65 ± 3.14 µg/cm^2). The data presented in Table 2 clearly indicate that NLCs were able to enhance skin permeation of AmB compared to the conventional gel. Similar results were reported in the past, where an AmB-loaded SLN gel formulation was able to enhance the AmB permeation compared to conventional gel (Butani, Yewale et al. 2016). In

another study, capsaicin-loaded NLCs showed higher skin permeation compared to SLNs and plain drug solution (Agrawal, Gupta et al. 2015). In case of NLCs, the higher drug permeation across the skin can be attributed to the structural composition of the lipid nanoparticles.

The amount of AmB accumulated in the skin (Table 2) was higher for AmB-NLCs (22.71 ± 2.58%) compared to AmB solution (12.82±1.08%) and can be attributed to the occlusive effect, smaller particle size and the penetration enhancing effect of the surfactants (Fang, Fang et al. 2008). Compared to plain drug solutions, NLCs can cause localized delivery of AmB into specific skin layers because of their lipidic composition resembling lipids of the SC layer (Schäfer-Korting, Mehnert et al. 2007). Comparable results were reported previously, where a higher amount of fluconazole was accumulated in the skin for NLCs (22.56 ± 3.27%) compared to SLNs (18.34 ± 4.24%) and plain drug solution (10.62 ± 2.45%) (Gupta and Vyas 2012). These higher drug concentrations in the skin for NLCs and SLNs were attributed to the lipid composition of the nanoparticles, their smaller particle sizes and their interaction with the SC lipids.

MRC-5 cytotoxicity assay

The *in vitro* cytotoxicity of both the AmB solution and AmB-NLCs was examined on MRC-5 cells and CC_{50} values were >11.33 µM for both. The CC_{50} value of AmB-NLCs was not reached in the assay demonstrating that even the highest in-test concentration of the AmB-NLCs dispersion was non-cytotoxic. Lipid-based nanocarriers are generally well tolerated due to the presence of physiological lipids and have proven to be biocompatible by many researchers (Silva, González-Mira et al. 2011, Severino, Andreani et al. 2012, Dolatabadi, Azami et al. 2018). SLNs have been proposed to be suitable carriers for drug delivery owing to their very low *in vitro* cytotoxicity (Müller, Rühl et al. 1997). In another study, drug-loaded NLCs were proposed to be the superior drug delivery system for dermal application (Gokce, Korkmaz et al. 2012).

Skin irritation study

Skin irritation potential of AmB-NLCs gel was evaluated using rats and skin condition of representative animals from both control and treatment groups are presented in Figure S1**.** No erythema or edema was observed at any observation time point (score of "0"). The AmB-NLCs gel is shown to be safe for topical application based on the visual observations of this study.

In vitro activity of AmB-NLCs against promastigotes and intracellular amastigotes

Antileishmanial assays were performed against promastigotes and intracellular amastigotes and the IC_{50}-values for both the AmB-solution and the AmB-NLCs are presented in Table 3. On both promastigotes and intracellular amastigotes, AmB-NLCs showed higher growth inhibition with IC_{50}-values for AmB-NLCs about 7-fold and 4-fold higher in comparison to free AmB. These results can be explained on the basis of some intrinsic characteristics of lipid nanoparticles, including protection of labile encapsulated drugs, provision of controlled drug release, enhanced bioavailability of incorporated drugs by improved transportation to target sites and enhanced macrophage targeting potential (Müller, MaÈder et al. 2000, Üner 2006, Song, Lin et al. 2015). The sustained drug release of AmB-NLCs in addition to the plausible protection they offer to the labile drug through encapsulation most likely resulted in prolonged drug exposure causing more effective parasite killing compared to the plain solution. In the past, many researchers have demonstrated enhanced activity of antimicrobial drugs when encapsulated in lipid nanoparticles (Gandomi, Atyabi et al. 2012, Nasseri, Golmohammadzadeh et al. 2016, Fazly Bazzaz, Khameneh et al. 2018, Zhu, Cao et al. 2018). In a recent study, paromomycin-sulphate (PM) loaded SLNs were shown to inhibit parasite growth more effectively than non-formulated PM (Kharaji, Doroud et al. 2016).

The higher activity of AmB-NLCs against intracellular amastigotes may be linked to an enhanced uptake of the nano-sized NLCs by macrophages resulting in higher intracellular drug accumulation. Nanoparticles are readily phagocytosed by macrophages, which can result in passive targeting of encapsulated drugs to the infected macrophages (Kunjachan, Gupta et al. 2011, Jain and Jain 2013). The nanoparticle size has a significant effect on macrophage uptake with 70-85 nm being the minimum size required for phagocytosis and an enhanced phagocytosis for particles between 100-1000 nm before becoming constant at 1000 nm (Xie, Tao et al. 2014). In a recent study proposing enrofloxacin-loaded SLNs as promising carriers for therapy of intracellular bacterial infections, the SLNs demonstrated a 27.06 to 37.71 times higher intracellular drug accumulation compared to free drug at similar extracellular concentrations. Moreover, intracellular uptake of SLNs was influenced more profoundly by size as compared to surface charge (Xie, Yang et al. 2017).

Antifungal activity of AmB-NLCs *in vitro*

The *in vitro* IC_{50} values for AmB solution, AmB-NLCs and blank NLCs against various fungal species are presented in Table 4. Encapsulation of AmB into NLCs resulted in significant reduction in IC_{50} values as compared to free AmB. AmB-NLCs were about 8, 7 and

16 times more effective than free AmB against *C. albicans*, *A. fumigatus* and *T. rubrum* respectively. These findings are consistent with the findings against *Leishmania*. Encapsulation of AmB in to nanoparticles demonstrated a significant reduction in IC_{50} values where PLGA nanoparticles containing AmB were up to 30 times more effective than AmB (Van de Ven, Paulussen et al. 2012). This enhanced activity of encapsulated active ingredient could be due to improved contact of nanocarriers with the microorganisms and more efficient delivery of drug in a sustained manner (Nasseri, Golmohammadzadeh et al. 2016).

Efficacy of topical AmB-NLCs in BALB/c mouse model for CL

At the start of the treatment, there was no significant difference in mean lesion size between the different groups and almost all lesions were in nodule phase (Table 5). At the end of the 10-day treatment period, the mean lesion size among the different groups showed no significant difference compared to control group, although the AmB-NLCs group showed the lowest percentage increase in mean lesion size compared to other groups (Figure 4d). As can be observed in Figure 5, almost all lesions progressed to the ulcerative form in the untreated, AmB-solution treated and blank NLCs-treated groups, whereas in the AmB-NLCs treated and lAmB-treated groups lesions remained in nodule stage and did not aggravate. Limiting dilution assays reveal a significant reduction in parasite burden for both the AmB-NLCs and the lAmB-treated group compared to the control group (Table 5). The AmB-solution failed to significantly reduce the parasite load as compared to untreated control group, whereas blank NLCs showed some minimal activity as well.

One of the most important criteria for the evaluation of drug efficacy in CL is the reduction in average lesion size upon treatment. Although no significant decrease in lesion size could be observed for the AmB-NCLs, previous research already demonstrated that a significant decrease in relative lesion area might only appear later (Bavarsad, Bazzaz et al. 2012, Mayelifar, Taheri et al. 2015). Hence, the comparison of the remaining parasite burdens after treatment might be a more appropriate evaluation parameter for short time periods. In this study, both the intravenous lAmB and topical AmB-NLCs were effective and inhibited parasite growth to almost similar extent. Nanoparticulate systems are especially useful as macrophages immediately engulf such carriers by endocytosis, resulting in localized drug delivery at the infected site. Such macrophages may also act as secondary drug reservoir resulting in prolonged drug effects. Drug-loaded NLCs were basically explored for the topical treatment of CL owing to their potential to target deeper skin layers and macrophages with minimal systemic exposure (Pople and Singh 2010). In the past, our group demonstrated a significant

accumulation of fluorescent labeled NLCs in deeper skin layers (Riaz, Ahmed et al. 2019). None of the treatment groups showed a complete eradication of parasites, which might be attributed to the fact that BALB/c mice are highly susceptible to infection with this particular virulent *L. major* strain and that they are not able to immunologically clear the infection as opposed to humans who are more resistant to leishmaniasis. Interestingly, blank NLCs also demonstrated some activity that could be triggered by some immunological responses. Whether or not any formulation component other than active drug ingredient has some activity against *Leishmania* is not known at this time. It was reported that paromomycin (PM) showed an improved *in vivo* efficacy in the BALB/c mice model infected with *L. tropica* and significantly reduced parasite burden as compared to free PM (Heidari-Kharaji, Taheri et al. 2016). However, the route of administration in the aforementioned study was either intramuscular or intralesional. Many researchers have proposed topical use of lipid nanoparticles for treatment of CL, whether alone or in combination to standard treatment (Ghadiri, Vatanara et al. 2011, Monteiro, Löbenberg et al. 2017) but to our knowledge so far no *in vivo* study showing efficacy for lipid nanoparticles (SLN or NLCs) using the topical route of application has ever been performed.

Efficacy of topical AmB-NLCs gel in VVC rat model *in vivo*

AmB-NLCs demonstrated 8-fold enhanced *in vitro* activity against *C. albicans* as compared to free AmB. It was decided to evaluate the efficacy of AmB-NLCs gel in rat model of vulvovaginal candidiasis (VVC). Animal models for VVC could demonstrate a high variation in fungal burden between animals, one of the reasons for which may be a varied level of muco-adhesion to the vaginal epithelium that can be achieved (Fernandes Costa, Evangelista Araujo et al. 2018). *Candida albicans* is naturally present in vagina of human females whereas it is not the part of vaginal microbiota of female rats (Cassone and Sobel 2016). However, despite these points, animal models still provide a good insight in to the clinical efficacy potential. Treatment was continued for 10 days and average fungal burdens (log CFU/swab ± SEM) taken at days 4, 7 and 11 days for animals of different groups (Figure 6). AmB-NLCs demonstrated improved efficacy in lowering the fungal burden as compared to control group and AmB RG, which is in line with the *in vitro* results. NLCs were reported to enhance the antimicrobial activity of *Astronium* species against *C. albicans* both *in vitro* and *in vivo*. The aforementioned plant extract demonstrated MIC of 125 µg/ml which was reduced to 15 µg/ml after encapsulation in to NLCs. Results of the *in vivo* study demonstrated that NLCs had

improved antimicrobial activity as compared to non-incorporated extract (Bonifácio, dos Santos Ramos et al. 2015).

CONCLUSION

In conclusion, AmB-NLCs seem to be an attractive and cost effective formulation for topical application, which might be applicable in the treatment of CL and VVC. Drug-loaded NLCs showed a superior *in vitro* activity against different *Leishmania* and fungal species and higher skin retention as compared to the free drug. The superior *in vivo* activity of the drug-loaded NLCs over the plain drug solution supports the targeting potential of NLCs to infected macrophages and deeper skin layers in topical treatment of CL. Moreover, topical application does not require trained personal or hospital admission as required for intravenous or intralesional injections which could definitely improve patient compliance. Drug-loaded NLCs-based topical gel also demonstrated a superior activity in the elimination of fungal burdens in the VVC model as compared to plain drug gel which suggests the possibility of using NLCs as an improved drug delivery carrier in topical treatment of mucosal diseases.

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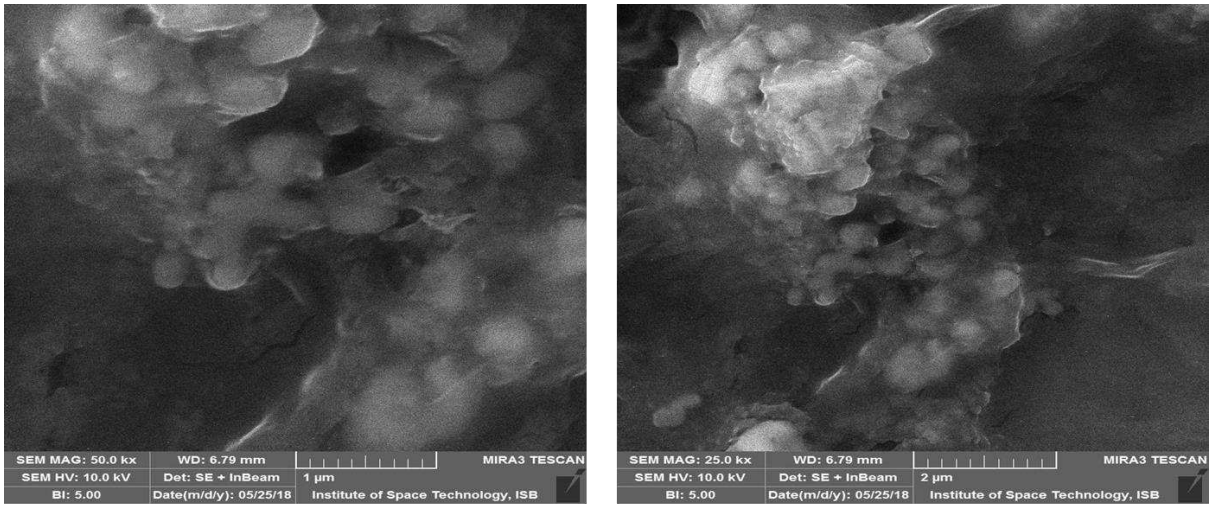

(a) (b)

Figure 1: SEM images of AmB-NLCs under two resolutions (a) 1µm (b) 2µm.

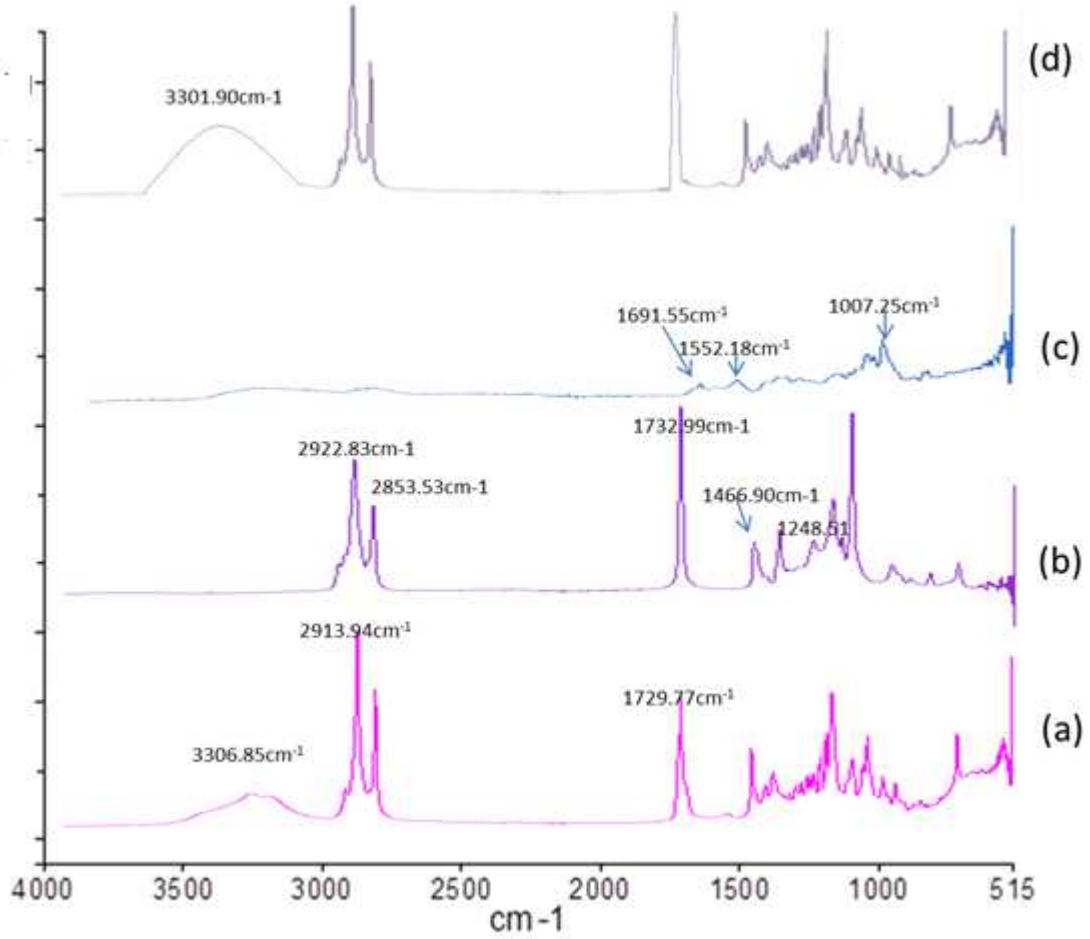

Figure 2: FTIR spectra of (a): GMS, (b): IPM, (c): AmB and (d): AmB-NLCs

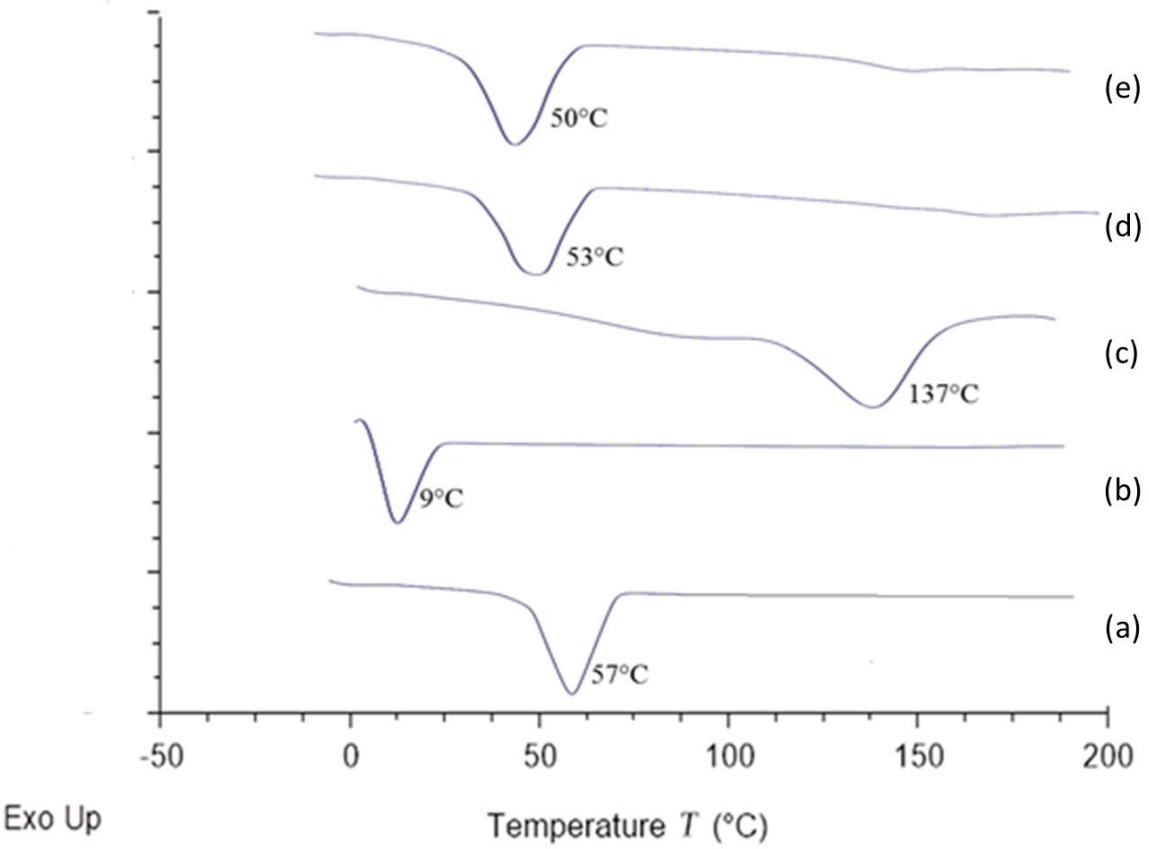

Figure 3: DSC thermograms for (a): GMS, (b): IPM, (c): AmB, (d): Blank NLCs and (e): AmB-NLCs.

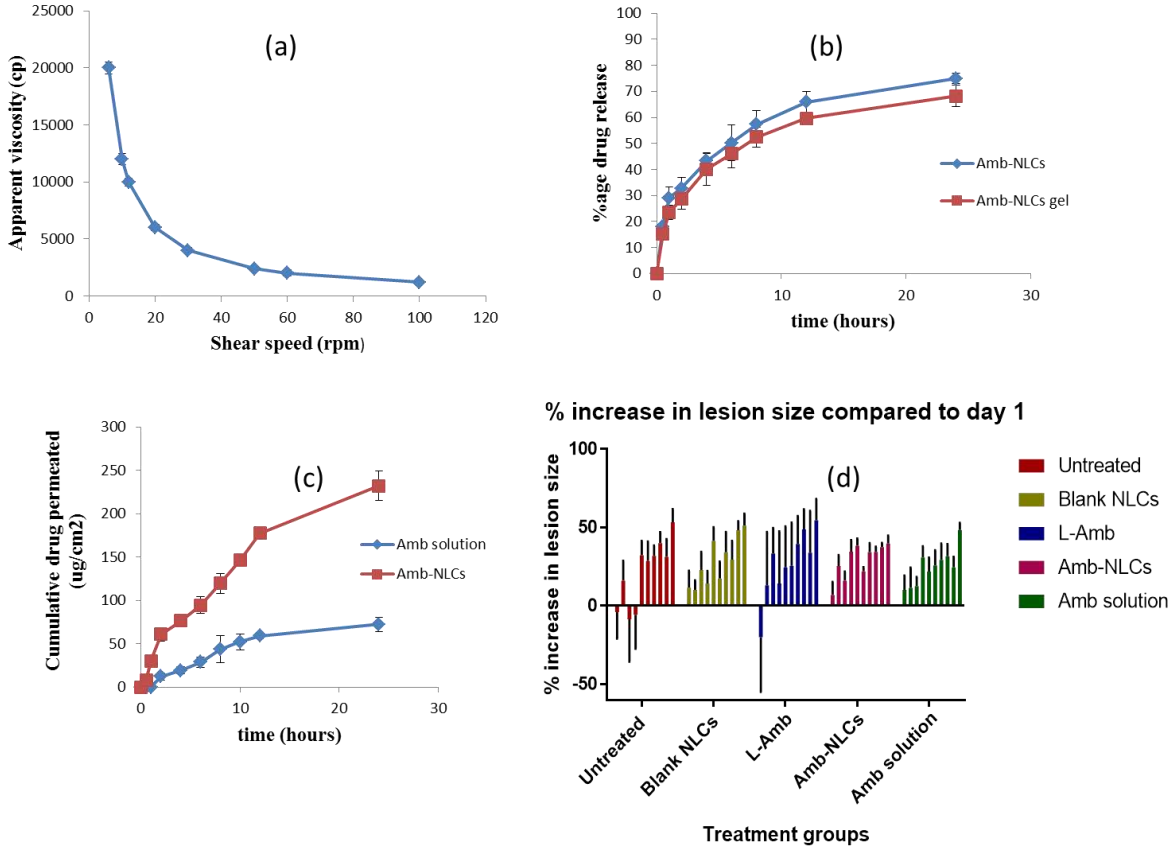

Figure 4: (a): Apparent viscosity vs shear rate for AmB-NLCs gel. (b): Drug release (%) from AmB-NLCs and AmB-NLCs gel. (c): Cumulative drug permeated through skin from AmB RG and AmB-NLCs gel. (d): Average increase in lesion size (%) compared to day 1 among mice of various groups used for *in vivo* study.

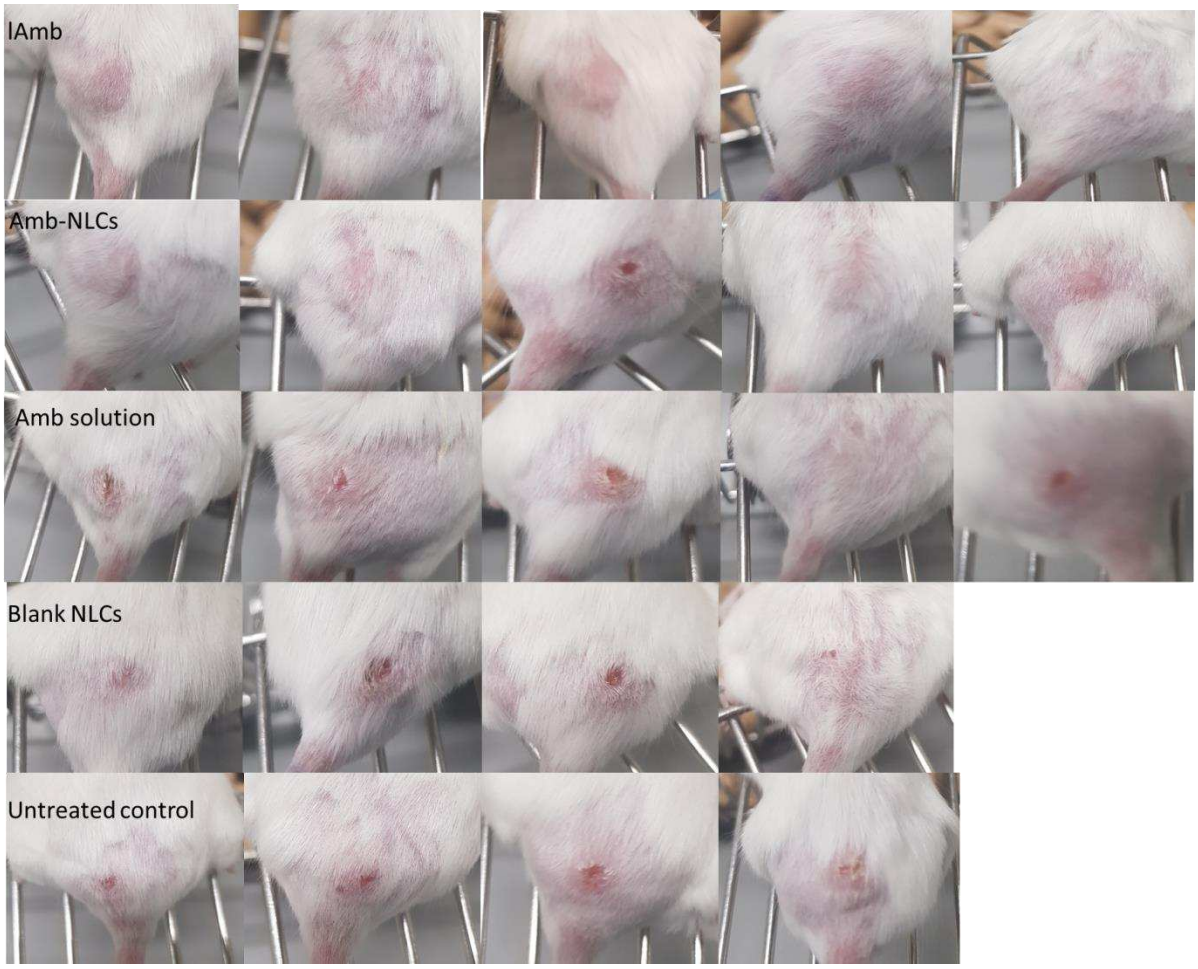

Figure 5: Clinical picture of lesions among the animals of the various treatment groups at day 11 before animals were euthanized.

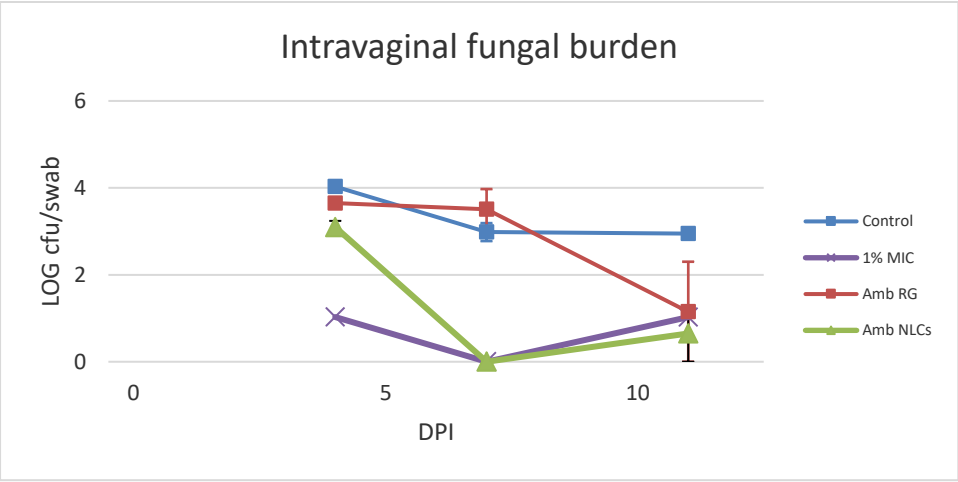

Figure 6: Average intravaginal fungal burden at various time points for animals of different groups.

Table S1: Kinetic modelling of drug release data using DDSolver software.

Formulations	Kinetic models							
	Zero order $F=k_0*t$		**First order $F=100*[1-Exp(-k_1*t)]$**		**Higuchi $F=k_H*t\hat{}0.5$**		**Korsmeyer-Peppas $F=kK_P*t\hat{}n$**	
	K_o	R^2	K_1	R^2	K_H	R^2	R^2	n
Amb-NLCs	4.310	0.066	0.113	0.755	18.351	0.887	0.988	0.331
Amb-NLCs gel	3.915	0.137	0.091	0.728	16.624	0.905	0.986	0.346

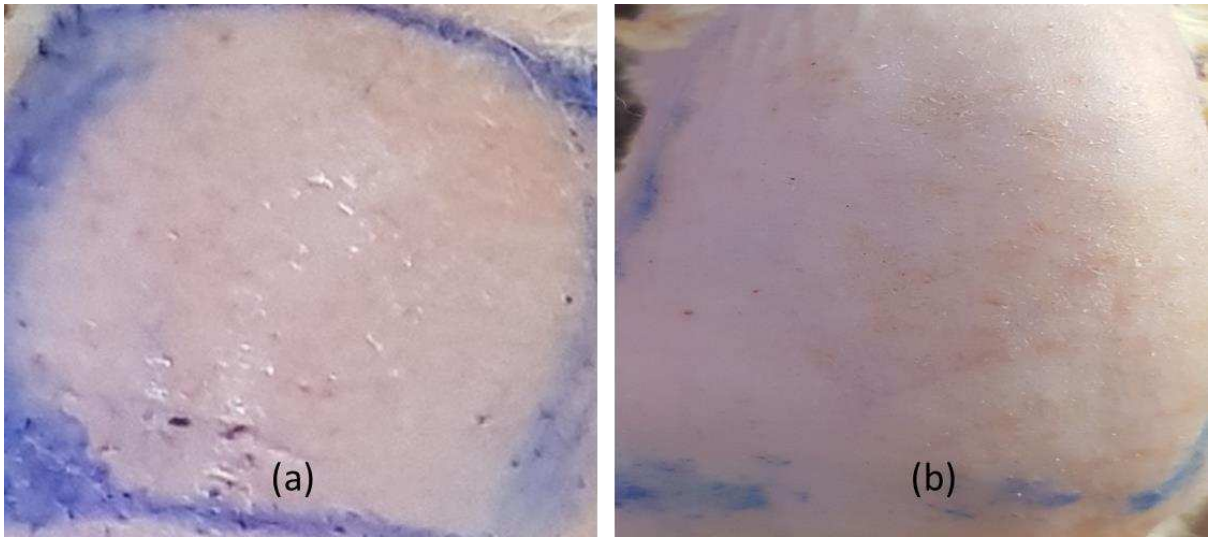

Figure 5: Skin condition of animals at 24 h representative of control group (a) and treatment group (b).