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RESEARCH PAPER

In Vitro Skin Permeation and Retention of Paromomycin from Liposomes for Topical Treatment of the Cutaneous Leishmaniasis

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ABSTRACT

Paromomycin (PA), a very hydrophilic antibiotic, has been tested as an alternative topical treatment against cutaneous leishmaniasis (CL). Although this treatment has shown promising results, it has not been successful in accelerating the recovery in most cases. This could be attributed to the low skin penetration of PA. Liposomal formulations usually provide sustained and enhanced drug levels in skin. The aim of this study was to prepare liposomal formulations containing PA and to investigate their potential as topical delivery systems of this antileishmanial. Large multilamellar vesicles (MLVs) were prepared by conventional solvent evaporation method. Large unilamellar vesicles (LUVs) were prepared by reverse-phase evaporation method. The lipids used were soybean phosphatidylcholine (PC) and PC:cholesterol (CH) (molar ratio 1:1). The skin permeation experiments across stripped and normal hairless mice skin were performed in modified Franz diffusion cells. The PA entrapment in LUV liposomes (20.4±2.2%) was higher than that observed for MLV liposomes (7.5±0.9%). Drug entrapment was 41.9±6.2% and 27.2±2.4% for PC and PC:CH LUV, respectively. The skin permeation was 1.55±0.31%, 1.29±0.40%, 0.20±0.08%, and 0.50±0.19% for PC LUV, PC:CH LUV, empty LUV+PA and aqueous solution, respectively. Controlled topical delivery, across stripped skin, was observed for PA entrapped in LUV liposomes.

Key Words: Skin permeation; Paromomycin; Topical treatment; Cutaneous leishmaniasis; Tegumentary leishmaniasis.

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INTRODUCTION

Tegumentary Leishmaniasis is a complex group of diseases caused by a variety of *Leishmania* species. Each species is responsible for a certain group of clinical symptoms, which can vary from the presence of single localized ulcers [cutaneous leishmaniasis (CL)] to the diffuse or the disfiguring mucocutaneous forms of the disease.[1] Systemic administration of Pentavalent antimony (Sb) organic compounds, amphothericin B, or pentamidine has remained the conventional therapy for all forms of leishmaniasis, despite the side effects associated with the use of these compounds.[2]

In the past few decades, major emphasis has been given to the development of alternative therapies, including the identification of formulations for local treatment of CL. Local therapy can be of value in nondisseminated disease because of its obvious simplicity and the less toxic side effects. Petrolatum ointments containing paromomycin at 15% have been evaluated with favorable results, especially against *Leishmania major*, *L. tropica*, and *L. panamensis* infections.[3]

Paromomycin (PA), an aminoglycoside antibiotic, is a highly polar molecule and relatively lipid insoluble. Its chemical structure is similar to that of neomycin. Paromomycin has a broad spectrum of activity, including activity against protozoa, such as *Leishmania sp*., bacteria, and cestodes.[4] Paromomycin is associated with methylbenzethonium chloride (MBCL), an ammonium quaternary surfactant with antileishmanial activity, for topical therapy of the CL.[5] However, MBCL induces local irritation, which has prevented its use in some cases.[6] Considering the ointments containing only PA, they are not as effective as its association with MBCL.[7] This limitation could be attributed to low cutaneous penetration of PA. It has been argued that the addition of MBCL would enhance PA skin permeation, although this aspect has not been confirmed experimentally.

An important consideration in topical treatment of cutaneous leishmaniasis is the skin condition. Topical formulations may be applied either to open lesions, that have lost SC barrier property, or to thickened lesions, that represent an additional barrier to absorption.[8] Topical delivery of hydrophilic drugs through intact skin usually creates problems due to their inability to penetrate into stratum corneum (SC). Liposomal formulations have demonstrated the capacity to increase drug penetration across and into skin when compared to conventional formulations.[9,10] In addition, when applied to the stripped skin, liposomes provided targeted and sustained topical delivery.[11] Thus, we hypothesized that liposomal formulation containing entrapped PA could be an interesting alternative for topical treatment of CL. The aim of this study was to develop liposomal formulations containing PA and to investigate their potential as topical delivery systems of this antileishmanial.

MATERIAL AND METHODS

Materials

Paromomycin sulfate (PA), potency of 718 mg/g, was provided by from Pharmacia & Upjohn (Milan, Italy). Soybean phosphatidylcholine (PC) (Lipoid, Germany) and cholesterol (CH) were obtained from Merck (Germany). All other chemicals used for analysis were of analytical reagent grade.

Preparation of Liposomes

To investigate the influence of liposome type on PA entrapment, large multilamellar vesicles (MLV) and large unilamellar vesicles (LUV) were prepared. The MLVs were prepared by a conventional solvent evaporation method. Ten, 20, or 40 mM of PC was dissolved in 2 mL of chloroform in a round-bottomed flask. The solvent was then removed by vacuum evaporation using a rotary a evaporator in such a way that a thin lipid film was deposited on the wall of the flask. The film was hydrated with PA solutions (10, 20, or 40 μg/mL) in phosphate buffer (0.1 M, pH 8) and subsequently was shaken vigorously. The LUVs were prepared by reverse-phase evaporation method. The PC was dissolved in chloroform in a round-bottomed flask. Solvent was removed by vacuum evaporation using a rotary evaporator and the lipid film was dissolved in 9 mL of diethyl ether. Next, 3 mL of PA solution in phosphate buffer was incorporated. The mixture was sonicated for approximately 5 minutes. The resulting w/o emulsion was placed in a rotary evaporator for evaporation of the organic phase and consequent formation of LUVs. The LUVs composed of PC or PC:CH molar ratio 1:1 at 30 mM were also prepared.

Determination of Drug Entrapment

Encapsulated PA was separated from free PA by ultracentrifugation in a microcentrifuge (Denver Instrument Company, Denver, CO) at 14,000 g for 60 minutes. The washing number necessary for elimination of free PA (not encapsulated) was evaluated in previous

experiments. One washing was sufficient to separate the encapsulated PA from free PA. Then, liposomes were resuspended in phosphate buffer and a sample (100 μL) was added to equal volume of n-propanol to disrupt the vesicles. The PA concentration was evaluated by microbiological assay (see above).

In Vitro Skin Permeation

In vitro skin permeation of PA was determined with open-cap, ground-glass surface, modified Franz diffusion cells (membrane surface area of 1.77 cm^2 and volume of the receptor fluid of 6.7 mL). Dorsal skin was excised from 8–10-week-old hairless male mice. Animals were sacrificed by cervical rupture and the dorsal skin was used immediately after removing the subcutaneous fat. Experiments were conducted with intact (normal) or stripped skin. Removal of the *stratum corneum* (SC) was performed as previously described by Flynn, Durrheim, and Higuchi with some modification.[12] The SC was removed by stripping 15 times with cellophane tape (Scotch Magic no. 810, 3M) after sacrifice of the animals. The skin was kept horizontally, dividing the cell into two compartments: the donor and the receptor. Skin conditions were obtained with 0.05 M phosphate buffered saline (PBS) (pH 7.4), containing 0.01% of $HgCl_2$ as preservative, in the receptor compartment. It is well known that aminoglycoside antibiotics, such as PA, are very soluble in water. Receptor phase was continuously stirred with a small magnetic bar to ensure homogeneity. Experiments were conducted with a skin surface temperature at 32° C±1, achieved by maintaining the receptor phase, using cell jackets, at the correct temperature (37° C). The skin, mounted in the cell, was allowed to equilibrate with the environment for 1 hour. After this period, receptor fluid was totally removed and this compartment was filled with PBS without preservative in order to avoid interference with the microbiological assay. Previous experiments confirmed that the interference of the $HgCl_2$ was totally eliminated after rinsing the receptor chamber three times.

To simulate use conditions, the upper chamber (donor compartment) was left open and a relevant clinical dose of the preparations (100 μL) was applied on the skin using a pipette (Stepper 411, Scorex). Serial sampling was then performed at 2, 4, 6, 8, and 10 h through total removal of the receptor fluid and refilling with fresh solution. The PA concentration was determined by microbiological assay (see below).

At the end of the experiments, removal of excess formulation was determined by washing the treated surface two times with 500 μL of an aqueous solution of 1% polyoxyethylene 20 oleyl ether (Sigma, St. Louis, MO) and two times with 500 μL of water. Residue was removed with a cotton swab. Washing solution, pipette tips, and the cotton swab were added to a bottle containing 100 mL of distilled water. The PA concentration in this solution was determined by microbiological assay. Previous experiments, performed immediately after application of the preparations, were conducted to validate extraction procedure. The PA recovery from liposomes was of 96.7%±2.7 (n=3).

Epidermis and dermis were separated using a scalpel and maintained for 12 hours in 5 mL of 0.1 M phosphate buffer (pH 8) for PA extraction retained into skin. The biopsies of the skin (dermis and epidermis), in phosphate buffer, were homogenized using an Ultra-Turrax. Samples were then centrifuged and supernatant was filtered through a Millipore filter (0.45 μm). The PA concentration was determined by microbiological assay. Previous experiments, performed 4 hours after application of the preparations, showed that PA recovery from skin was of 93.0%±1.4 (n=3).

Formulations without PA were also submitted to all protocols described previously. These samples did not interfere with the analysis method.

Microbiological Assay

Microbiological assays are traditionally used for determination of the pharmacokinetic of aminoglycoside antibiotics. The PA concentration was determined by an agar-diffusion inhibition assay of the growth of *Bacillus subtilis* (ATCC 6633) as previously described.[13] Briefly, after overnight incubation of the plates at 35° C, the zone of inhibition was measured for each sample with an antibiotic zone reader (mm). A linear relationship between diameter of the inhibition zone and logarithm of the PA concentration was observed. Standard curve ($y=17.5750+7.1720x$; $R=0.999$) was obtained with the following PA concentrations: 0.2, 0.4, 0.8, 1.6, and 3.2 μg/mL in 0.1 M phosphate buffer (pH 8.1) according to the method described in the United States Pharmacopoeia (USP 23). Limits of detection and quantification were 0.15 and 0.2 μg/mL, respectively. Samples containing high concentrations of PA (>3.2 μg/mL) were diluted in phosphate buffer (pH 8.1) for a range of the standard curve.

Thin-Layer Chromatography Assay of PA

To investigate the stability of PA in the receptor compartment and in the skin homogenate, thin-layer chromatography experiments were conducted (USP 23,

modified). An aqueous solution of PA (1 mg/mL) was maintained in the receptor compartment or in contact with the skin for 12 hours. Then, samples of 5 μL of these solutions were applied with a microcapillary tube (Drummond Scientific Co., Broomall, PA) to a suitable thin-layer chromatographic plate (20 × 20 cm), coated with a 0.25 mm layer of chromatographic silica gel (Merck, Darmstadt, Germany), in duplicate. The spots were allowed to dry and the chromatographic plate developed in a solvent system, consisting of a freshly prepared mixture of chloroform: methanol: ammonium (1:3:2), until the solvent front has moved about three-fourths the length of the plate. The plate then was air-dried for 10 minutes and heated at 105°C for 1 hour. After cooling, a ninhydrin solution in ethanol (0.5%) was sprayed over the plate, which was heated again at 105°C for 10 minutes: PA appears as a brown spot over a yellow background.

Statistical Analysis

Student's t-test was used to compare the two groups. One-way analysis of variance (ANOVA) was used to evaluate differences between means. Differences were considered statistically significant when the P value was less than 0.05.

RESULTS

Preparation of Liposomes

The influence of PA and lipid concentration on the entrapped drug content in MLV liposomes was investigated. Increasing PA concentration of 10 to 40 μg/mL increased the encapsulation from 3.3%±0.48% to 4.5%±0.69% by using a lipid concentration of 10 mM. A similar phenomena was observed by using a lipid concentration of 40 mM: entrapped PA content increased from 5.2%±0.59% to 7.5%±0.96% when the drug concentration in hydration solution ranged from 10 to 40 μg/mL (Fig. 1). As expected, increase in lipid concentration (10 to 40 mM) also improves drug entrapment. Although these differences are significant, PA entrapment increased only about 1.5 times, while PA (in hydration solution) or lipid concentration increased by four times. Besides, encapsulation of PA in MLVs (7.5%) was considered low, although PA concentration in topical preparations is usually very high.

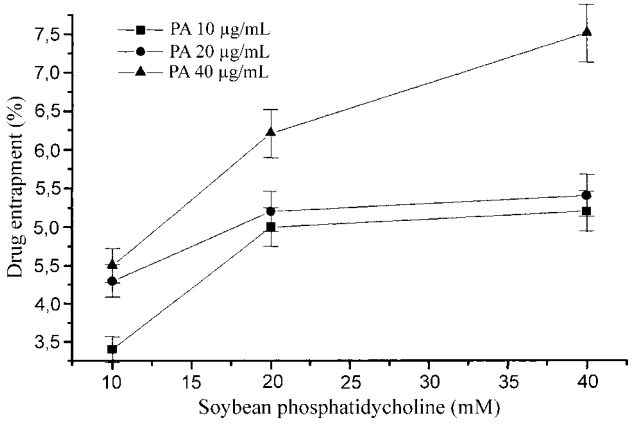

Figure 1. Influence of lipid and drug concentration in hydration solution on PA entrapment in PC MLV liposomes.

Table 1. Influence of lipid composition and PA concentration on PA entrapment in LUVs.[a]

Lipid composition (30 mM)	% Entrapment		
	10 mg/mL	40 mg/mL	100 mg/mL
PC	24.2±4.4	25.4±3.5	41.9±6.2
PC:CH	16.0±1.3	NR[b]	27.2±2.4

[a]Results represent average±SD (n=4).
[b]NR=Not realized.

To investigate the influence of liposome type, LUVs were prepared and compared to MLVs. The PA encapsulation in LUVs (20.4%±2.2%) was three times higher than that observed for MLV liposomes (7.5%±0.9%). Thus, LUV liposomes were used for further studies.

Influence of lipid composition (PC and PC:CH at 30 mM) on PA entrapment in LUVs was also investigated (Table 1). The PA encapsulation in PC LUV was significantly greater than that observed for PC:CH LUV independent of the drug concentration in hydration solution. In addition, increase of PA concentration from 10 to 100 mg/mL also increased drug entrapment. The PA entrapment in PC LUV and PC:CH LUV was 41.9%±6.2% and 27.2%±2.4%, respectively, when drug concentration in hydration solution was 100 mg/mL. Skin permeation studies were performed with LUV liposomes.

Skin Permeation of PA from Liposomes

Skin permeation experiments were conducted with normal (intact) and stripped skin. The permeation of PA across intact skin was low (0.2% to 1.5% of the dose applied) regardless of the formulation tested (Table 2). However, amounts of PA permeated from

Table 2. 24 h in vitro skin distribution of PA (% applied dose) from conventional and liposomal formulations with intact hairless mouse skin in various compartments.[a]

	Compartment				
Formulations	Receptor	Epidermis	Dermis	Surface	Recovery
PA solution	0.5±0.2	0.5±0.04	0.2±0.03	85.9±2.4	89.5±9.6
Empty LUV+PA	0.2±0.1	0.7±0.01	0.4±0.04	90.6±7.9	91.9±9.8
PC LUV	1.6±0.3	2.6±0.1	0.7±0.1	85.3±7.2	89.0±9.6
PC:CH LUV	1.3±0.4	3.0±0.1	0.7±0.3	89.6±11.7	94.2±13.9

[a]Formulations containing 100 mg/mL of PA were applied over biopsies of hairless mouse skin mounted in Franz cells. Each point represents average (n=3)±SD.

PC LUV (1.6%±0.3%) and PC:CH LUV (1.3%±0.4%) were significantly higher than those observed from physical mixture (empty LUV+PA) and aqueous solution. Differences between PC and PC:CH LUVs were not statistically significant. Additionally, PA skin permeation from the physical mixture (0.2%±0.1%) was lower than that observed for the aqueous solution (0.5%±0.2%), but this difference was not statistically significant. This indicates that the presence of lipids was not sufficient to induce a significant modification in skin permeability. Skin retention of PA (epidermis+dermis) from PC LUV (3.3%) and PC:CH LUV (3.7%) was about three times higher than that observed from the physical mixture (1.1%) and aqueous solution (0.7%). In these experiments, total recovery was from 89% to 94.2% of the applied dose.

As expected, PA skin permeation across stripped skin was higher than that observed across intact skin, regardless of the tested formulation (Fig. 2; Table 3). Total recovery was about of 100% of the applied dose. Skin retention of PA (epidermis+dermis) from PC and PC:CH LUV was significantly higher than that observed from the solution and empty LUV plus PA (Table 3). Permeation of PA after 10 hours for PC (71.6%±1.7%) and PC:CH (74.9%±2.3%) LUV, which contained free and encapsulated drug, was significantly lower than that observed for the solution (91.5%±3.7%) and empty LUV plus PA (91.0%±2.8%) (Fig. 2). However, PA flux from liposomes, as well as that for solution and physical mixture, was high, but dramatically decreased with time. This was attributed to the fact that the stripped skin offers negligible resistance to PA permeation and, therefore, penetration of the free drug is fast. Thus, another set of experiments was performed with PC LUV containing only encapsulated drug.

Skin permeation of PA after 10 hours from PC LUV (22.2%±0.5%) was significantly lower than that observed from solution and empty LUV plus PA (Fig. 3). The PA flux from preparations containing free drug (aqueous solution and empty LUV plus PA) was

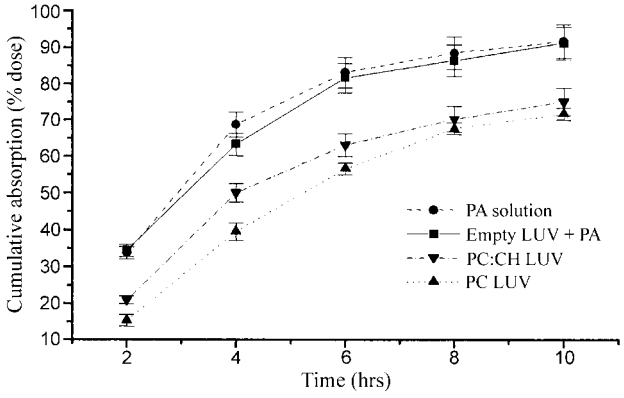

Figure 2. In vitro skin permeation of PA from PC and PC:CH LUV liposomes, aqueous solution, and empty LUV+PA across stripped hairless mice skin. Liposomes containing free and entrapped drug.

Table 3. 10 h in vitro skin distribution of PA (% applied dose) from conventional and liposomal formulations with stripped hairless mouse skin in various compartments.[a]

	Compartment			
Formulations	Receptor	Skin[b]	Surface	Recovery
PA solution	91.5±3.7	3.3±0.2	8.8±0.8	103.5±4.3
Empty LUV+PA	91.0±2.8	3.2±0.4	12.9±1.3	107.2±1.1
PC LUV	71.6±1.7	4.9±0.9	33.2±4.1	109.7±4.1
PC:CH LUV	74.9±2.3	4.0±0.4	23.5±1.2	102.4±1.3

[a]Formulations containing 10 mg/mL of PA were applied over biopsies of hairless mouse skin mounted in Franz cells. Each point represents average (n=3–5)±SD.
[b]Epidermis+dermis.

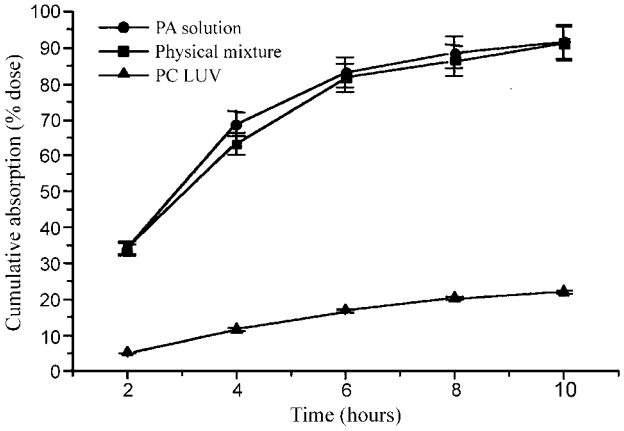

Figure 3. In vitro skin permeation of PA from PC LUV liposomes, aqueous solution, and empty LUV+PA across stripped hairless mice skin. Liposomes containing only entrapped drug.

high after the first 4 hours, but it decreased with time. The inflection observed in Fig. 3 could be attributed to a significant depletion of the PA concentration in the donor compartment. In contrast, steady-state flux of PA from PC LUV was observed after 10 hours (Fig. 3). Topical delivery of PA from PC LUV containing only encapsulated drug was sustained when compared to formulations containing free drug.

Thin-Layer Chromatography Assay

Thin-layer chromatography assay reveals that the principal spot, obtained for PA aqueous solution, was similar to that obtained for the receptor compartment or skin homogenate (data not shown). These results showed that the integrity of PA was maintained during the permeation experiments and that the inhibition of microorganism growth can be attributed to penetration of the target molecule.

DISCUSSION

The most efficient topical formulations for treatment of CL associate 15% PA to MBCL, since the effectiveness of PA alone is usually low.[2,3,7] The MBCL does enhance PA skin permeation, but its use is associated with adverse reactions. Liposomes would be an interesting alternative for increased skin penetration of PA. The aim of this study was, therefore, to prepare liposomes containing encapsulated PA and to investigate its in vitro skin permeation.

The PA concentration for topical treatment of CL is usually high, thus the content of drug entrapped is a determinant. Encapsulation of the hydrophilic PA in LUV was three times higher than that obtained in MLV. This could be attributed to the fact that the aqueous compartment in LUV is greater than in MLV.[14] Hydrophilic compounds such as PA are entrapped in the aqueous portion of the vesicles, therefore, their entrapment efficiency depends on maximizing liposome capture volume.

Encapsulation of PA in PC LUV was greater than that observed for PC:CH LUV. Cholesterol leads to high rigidity of the liposomal membrane in PC liposomes, in which the transition temperature is low, increasing their stability[14,15] and, consequently, formation of lower vesicles. Therefore, decrease of PA entrapment in PC:CH LUV when compared to PC LUV can be attributed to a reduction in aqueous compartment.

Liposomal entrapment of PA resulted in improved topical delivery into and across intact skin when compared with empty liposomes and aqueous solution of the drug. The results of our study showed that empty liposomes had little or no effect on PA skin permeation and retention when compared to aqueous solution. This can be explained by the fact that the reduced lipid concentration in these liposomes (about of 25 mg/mL) was not sufficient to induce significant change of skin permeability. The effect of skin pretreatment with empty lipid vesicles showed little or no effect on skin permeation of a hydrophilic[9] or lipophilic[16] drug. Besides, transdermal flux of oestradiol was improved by vesicles containing entrapped drug when compared with solutions containing the lipids used for preparation of the liposomes. These results emphasized the importance of preparing the phospholipids as vesicles for efficient skin delivery.[17]

Our data for permeation across intact skin are consistent with several studies previously published, in that dermal and transdermal delivery of hydrophilic drugs from loaded liposomes were greater than that observed for conventional formulations.[9,10,18,19] However, many groups have reported decreased skin permeation associated with improved retention of hydrophilic drugs from liposomal formulations.[20,21] Touitou et al. reported a high accumulation of caffeine in the skin with relatively low permeation as compared to the aqueous solution. These contradictory results can be attributed to different parameters involved in the experimental design such as origin of skin, volume applied, and lipid concentration.[22]

Liposomal entrapment of PA resulted in controlled topical delivery across stripped skin when compared to empty liposomes and aqueous solution of the drug.

These results indicate that, in the absence of a barrier for PA permeation, the liposomes showed ability to modulate PA delivery. The results of our study with stripped skin agree with data previously published in which topical delivery of hydrocortisone from liposomes across stripped skin was sustained and targeted when compared to conventional ointments.[11]

The dose currently used for topical treatment of cutaneous leishmaniasis is about 1 mg/mm^2, which corresponds to 100 mg/cm^2.[5] This dose is extremely high when compared to that usually applied on the skin for topical dosage forms. Thus, in our studies of skin permeation, a high dose (about of 57 mg/cm^2) was used. Furthermore, these experiments were carried out with an animal skin model. It has been shown that results obtained from hairless mouse skin cannot be easily extrapolated to human skin. However, this has not been completely investigated in the case of damaged skin. Additional studies are required to evaluate better the relevance of our findings for human skin.

CONCLUSIONS

The PA entrapment in LUV liposomes was higher than that observed for MLVs. The PA encapsulation in PC LUV was greater than that observed for PC:CH LUV. Improved skin permeation and retention across intact skin were obtained with encapsulation of PA in LUV liposomes in comparison with solution and empty LUV+PA. Controlled topical delivery across stripped skin was observed for PA entrapped in LUV liposomes. Our data suggest that PA entrapped in liposomes could be an interesting alternative for topical treatment of cutaneous leishmaniasis.

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