

## Original Article



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### Corresponding Author:

Sandra Milena Leal Pinto, PhD

Facultad de Ciencias de la Salud, Universidad de Santander, Bucaramanga, Colombia, 680006.

Tel: (7) 6516500 ext 1214


E-mail: sa.leal@mail.udes.edu.co


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### ORCID iDs


Sandra Milena Leal Pinto   
<https://orcid.org/0000-0002-0120-8060>


Luis Alexandre Muehlmann   
<https://orcid.org/0000-0003-4711-7583>


Lucía Liliana Mantilla Ojeda   
<https://orcid.org/0000-0002-5227-2855>

Angélica María Vera Arias   
<https://orcid.org/0000-0002-3352-2978>

Martha Viviana Roa Cordero   
<https://orcid.org/0000-0003-4588-0681>

María de Fátima Menezes Almeida Santos   
<https://orcid.org/0000-0003-0372-0567>

Ricardo Bentes Azevedo   
<https://orcid.org/0000-0002-2137-9588>

Patricia Escobar Rivero   
<https://orcid.org/0000-0002-7527-2364>

# Nanoemulsions with Chloroaluminium Phthalocyanine and Paromomycin for Combined Photodynamic and Antibiotic Therapy for Cutaneous Leishmaniasis

Sandra Milena Leal Pinto <sup>1</sup>, Luis Alexandre Muehlmann <sup>2</sup>,  
Lucía Liliana Mantilla Ojeda <sup>1</sup>, Angélica María Vera Arias <sup>3</sup>,  
Martha Viviana Roa Cordero <sup>1</sup>, María de Fátima Menezes Almeida Santos <sup>4</sup>,  
Ricardo Bentes Azevedo <sup>4</sup>, and Patricia Escobar Rivero <sup>3</sup>

<sup>1</sup>Universidad de Santander, Facultad de Ciencias de la Salud, Bucaramanga, Colombia

<sup>2</sup>Laboratory of Nanoscience and Immunology, Faculty of Ceilandia, University of Brasilia, Brasilia/DF, Brazil

<sup>3</sup>Centro de Investigación en Enfermedades Tropicales (CINTROP-UIS), Departamento de Ciencias Básicas, Escuela de Medicina, Universidad Industrial de Santander, Bucaramanga, Colombia

<sup>4</sup>Department of Genetics and Morphology, Institute of Biological Science, University of Brasilia, Brasilia/DF, Brazil

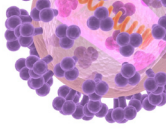
## ABSTRACT

**Background:** Photodynamic therapy (PDT) using chloroaluminium phthalocyanine (ClAlPc) and paromomycin sulfate (PM) can be effective against New World *Leishmania* species involved in cutaneous leishmaniasis (CL). The aim of this study is to assay the skin permeation and the antileishmanial effects of a nanoemulsion (NE) containing both ClAlPc and PM in experimental CL by *Leishmania (Viannia) braziliensis*.

**Material and Methods:** Cremophor ELP/castor oil-based NEs were prepared by a low-energy method and characterized for their physicochemical parameters. The NEs were used to deliver both ClAlPc and PM to leishmania cells. The *in vitro* toxicity of NEs were tested *in vitro* against *L. (V.) braziliensis* and THP-1 cells. The *in vivo* toxicity was assessed in non-infected BALB/c mice. *Ex-vivo* permeation and retention studies using healthy mice skin were also conducted. Finally, the *in vivo* activity of NE-PM+ClAlPc after PDT was tested in BALB/c mice infected with parasites.

**Results:** NEs are colloiddally stable with average droplet diameter of 30 nm, polydispersity index (PDI) below 0.2, and zeta potential near zero. Both promastigotes and intracellular amastigotes treated with NE-PM, NE-ClAlPc and NE-PM+ClAlPc were inhibited at >50%, >95%, >88%, respectively, after PDT with a phototoxic index (PI) >1.2. No skin ClAlPc permeation was observed. In contrast, PM skin permeation was 80-fold higher using PM-loaded NE formulation in comparison to aqueous PM solution. Topical treatment with NE formulations showed no signs of local toxicity or genotoxicity. In addition, concentrations of PM between 27.3 - 292.5 µM/25 mg of tissue were detected in different organs. *In vivo*, the NE-PM+ClAlPc treatment did not reduce skin lesions.

**Conclusion:** The Cremophor ELP/castor oil NE formulation increases the permeation of PM through the skin and can be used to co-deliver PM plus ClAlPc for combined PDT protocols. However, the lack of efficacy in the *in vivo* model evidences that the therapeutic scheme has to be improved.



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#### Conflict of Interest

No conflicts of interest.

#### Author Contributions

Conceptualization: PER. Data curation: SMLP, LLM. Formal analysis: SMLP, MVRC, PER. Funding acquisition: PER, RBA. Investigation: SMLP, LLM, AMV, MVRC, RBA. Methodology: SMLP, LAM, MVRC, PER. Project administration: PER, RBA. Resources: PER, RBA. Software: PER, MVRC. Supervision: PER, MFMAS, RBA, LAM. Validation: SMLP, PER, LAM. Visualization: SMLP, MFMAS, RBA, PER. Writing - original draft: SMLP, PER. Writing - review & editing: SMLP, PER, LAM, RBA.

**Keywords:** *Leishmania (Viannia) braziliensis*; Photodynamic therapy; Toxicity; Antileishmanial activity; Permeation

## INTRODUCTION

Leishmaniasis is a vector-borne disease produced by different species of *Leishmania* protozoa. Leishmaniasis constitutes a serious public health problem in endemic regions; approximately 350 million people are at risk of transmission, and 700,000 to one million new cases occur each year [1]. In 2018, the Americas reported a total of 46,041 cases of cutaneous and mucosal Leishmaniasis with 16,432 cases registered in Brazil, 6,362 in Colombia and 8,842 in Central America [2]. *L. (Viannia) braziliensis* is the most prevalent parasite, followed by *L. (V.) panamensis*, *L. (V.) guyanensis*, and *L. (L.) amazonensis* [3]. Leishmaniasis treatment is based on the use of pentavalent antimonials. Pentamidine isethionate, amphotericin B (liposomal forms) and miltefosine can be used as alternative treatments in the cases of therapeutics failure or contraindications. However, adverse effects, prolonged therapies, parenteral administration, resistant strain emergence, availability and therapeutic failure are the main concerns [4]. Topical treatments such as thermotherapy, photodynamic therapy (PDT) and semisolid formulations with antibiotics (such as paromomycin sulphate/gentamicin) can be used for non-severe cutaneous leishmaniasis (CL) disease [4]. PDT is based on the use of a photosensitizer (PS) and visible light in the presence of molecular oxygen, leading to the production of cytotoxic reactive oxygen species (ROS), such as singlet oxygen ( $^1O_2$ ) and free radicals, which can damage cellular organelles and cause cell death and tissue destruction irreversibly [5, 6].

PDT can be an alternative treatment for CL. Exogenous photosensitizers such as phenothiazine, porphyrins and phthalocyanine (Pc) or endogenous photosensitizers such as  $\delta$ -aminolevulinic acid (ALA)-induced protoporphyrin IX have given interesting results in CL models [5, 7-9]. Aluminium-phthalocyanine chloride (ClAlPc) is a chemically stable and commercially available photosensitizer active against different *Leishmania* species after PDT [9-11]. The poor solubility of unsubstituted metal-phthalocyanines and their high molecular weight ( $574.96 \text{ g}\cdot\text{mol}^{-1}$  for ClAlPc), however, are some of the major concerns regarding its topical use [12]. Thus, ClAlPc has been encapsulated in different nanocarriers, such as liposomes, polymeric nanoparticles and nanoemulsions (NEs), and tested against different eukaryotic cells, including *Leishmania*, showing higher activity in terms of parasite inhibition [10, 11, 13, 14].

Drugs for topical treatment need to pass biological barriers such as the skin and be internalized by macrophages in order to kill intracellular parasites. Research on non-invasive routes of application to allow drug permeation across the *stratum corneum* and to deliver it into the dermis is required [15]. Nanoemulsions are very interesting drug delivery systems for topical treatments due to their kinetic stability, rapid permeability, small drop size and easy dermal absorption [16]. This kind of formulation has already been used for the delivery of a number of drugs, such as cyclosporine and pentyl gallate, for the treatment of dermatological conditions such as psoriasis and herpes [17, 18]. NEs loaded with zinc-phthalocyanine or containing other compounds, such as chalcones and Amphotericin B, have also been tested as treatments of CL [19-21].

The inhibitory effect of ClAlPc and PM in *Leishmania* has motivated us to evaluate a novel NE containing ClAlPc and PM intended for the treatment of CL. The efficacy and safety of this formulation was evaluated in *in vitro* and *in vivo* experimental models, using mammalian cells and *L. (V.) braziliensis* infected mice, respectively.

## MATERIALS AND METHODS

### 1. Nanoemulsion preparation

Four oil-in-water NEs were prepared: NE-ClAlPc, NE-ClAlPc-15% PM, NE-15% PM and NE without compound (vehicle). All NEs were prepared using a spontaneous emulsification method as described by Muehlmann et al 2015 [22]. Briefly, Cremophor ELP® (9 g, Sigma-Aldrich, São Paulo, Brasil) and castor oil (3 g, Sigma-Aldrich) were mixed together with mild magnetic stirring (300 rpm) at room temperature, forming the organic phase. For ClAlPc (Sigma-Aldrich) formulations, ClAlPc ( $444 \mu\text{mol}\cdot\text{kg}^{-1}$ ) was first dissolved in ethanol and then added to the organic phase with magnetic stirring. For PM-containing formulations, 15 g of PM (Sigma-Aldrich) were added to the aqueous phase of NE-ClAlPc, and then the pH was adjusted to 7.4 with 0.1 M NaOH. Control NEs containing only one compound (ClAlPc or PM) or without any compound (vehicle) were also prepared. The ClAlPc concentration was measured fluorometrically using a spectrofluorometer (Spectramax® M2; Molecular Devices LLC, Sunnyvale, CA, USA) at excitation/emission wavelengths of 400/682 nm. A calibration curve of ClAlPc diluted in ethanol at concentrations ranging from 0.2 - 50.0  $\mu\text{M}$  was previously performed. The PM concentration was measured using high-performance liquid chromatography (HPLC, Agilent 1,100 Series HPLC system, Waldbronn, Germany) using a C18 octadecylsilane column obtained from Khymós (Eclipse XDB-C18, Agilent Technologies distributor, Bogotá, Colombia) PM was derivatized with 1-fluoro-2, 4-dinitrobenzene [23]. For the PM calibration curve, drug concentrations ranging from 10 to 1,000  $\mu\text{M}$  were prepared.

### 2. Nanoemulsion characterization

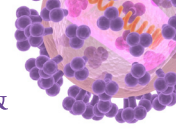
The droplet size, polydispersity index (PDI) and zeta potential were measured by dynamic light scattering (DLS) and electrophoretic laser Doppler velocimetry using a Zetasizer Nano ZS® (Malvern Instruments, Malvern, UK). Samples were diluted 1:50 (v:v) in PBS before readings. All measurements were performed in triplicate, and the results are presented as the mean  $\pm$  SD. The pH was assessed with an indicator strip (Merck, Darmstadt, Germany, 0 - 14). The physicochemical stability of the NEs was evaluated each month for at least four months after preparation. Samples were stored at 4°C, and PDI, zeta potential and ROS generation after PDT were measured following the protocols described above.

### 3. Irradiation systems

Different irradiation systems were used in this work. For the *in vitro* assays a biological photoreactor (Luzchem Research Inc, Ontario, Canada) with emission of light in the visible spectrum (400 - 700 nm) and under controlled temperature (37°C: non-infected mammalian cells, 32°C: infected mammalian cells and 28°C promastigote forms) was used. The cells were irradiated for 32 min at a fluency of 5 J/cm<sup>2</sup>. For the *in vivo* safety assays on non-infected mice, a LumaCare® LC-122A lamp equipped with a fibre optic filter at  $672 \pm 40$  nm was used at the site of treatment, animals were irradiated for 7 min at a fluency of 20 J/cm<sup>2</sup> after of 30 min of applying the formulation onto the skin. For the *in vivo* efficacy assays, infected mice were irradiated at a wavelength of 660 nm, for 7 min (50 J/cm<sup>2</sup>), power of 119.1 mw with a light-emitting diode (LED). This equipment was designed and produced by the Institute of Physics of the Brasilia University (Research Group of Semiconductor and Magnetic Nanostructures, Brasilia DF, Brasil).

### 4. Antioxidant activity

The photo-induced generation of ROS by PDT was determined indirectly using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPBF) assay as previously described by Muehlmann et al [22]. Briefly, serially diluted NE samples (200  $\mu\text{L}$ ) were incubated with 10  $\mu\text{L}$  of



ethanolic DPBF (1 g/mL) solution in 96-well plates and then irradiated with a light energy dose of 5 J/cm<sup>2</sup> by 20 min in 2-min intervals (Luzchem). The absorbance at 410 nm was monitored using a spectrophotometer (Thermo Scientific, Multiskan, GO, MA, USA) before and after each irradiation. The decay in benzofuran-specific absorption at 410 nm was related to ROS generation.

### 5. Parasites and mammalian cells

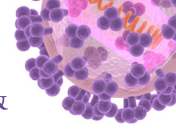
Promastigotes of *L. (V.) braziliensis* (MHOM/BR/75/M2903) were maintained in Schneider's insect medium with 10% FCS at 28°C. Human monocytes (THP-1, TIB-202, ATCC, Manassas, Virginia, Estados Unidos) were cultured in RPMI medium supplemented with 10% FCS at 37°C, 5% CO<sub>2</sub> and 95% humidity. Cells were transformed to an adherent phenotype using PMA for 72 h. Intracellular amastigotes were obtained after transformed THP-1 cell infection with latent-phase promastigotes.

### 6. In vitro assays

Promastigotes ( $5 \times 10^5$  cells/mL), as well as infected and non-infected transformed THP-1 cells ( $8 \times 10^5$  cells/mL), were incubated with serial dilutions (1:2 to 1:1,000) of each NE, vehicle or ClAlPc (0.04 to 20 μM) and PM (244.0 to 121,827.0 μM) solutions. Control cells received no treatment. After 24 h, cells and parasites were irradiated and then incubated in the dark for additional 24 h. Additional plates were left without irradiation. THP-1 cell viability was determined using a colorimetric MTT assay. Parasite inhibition was evaluated microscopically by counting with eosin yellow for promastigotes and counting infected and uninfected cells stained with Giemsa for intracellular amastigotes. The percent THP-1 toxicity or parasite inhibition was calculated by the equation: cytotoxicity (%) = (optical density [OD] of parasite count control group – OD of parasite count treatment group) × 100/OD of parasite count control group. The activities of the compounds are expressed as the cytotoxic or inhibitory concentration of 50 and/or 90% of the cells or parasites (IC<sub>50</sub> and IC<sub>90</sub>, respectively), calculated by sigmoidal regression using the statistics software XLfit4 (ID Business solution, Guildford, UK). Additionally, the selectivity indices (SIs) were calculated as follows: % parasite photo-inhibition/% phototoxicity in THP-1 cells, and the phototoxicity index (PI) was calculated by dividing the % parasite inhibition at 0 J/cm<sup>2</sup>/ % parasite inhibition at 5 J/cm<sup>2</sup>.

### 7. Ex vivo permeation test

A Franz diffusion cell (PermeGear, Hellertown, PA, USA) with a diffusion area of 0.196 cm<sup>2</sup> was used according to the protocol described by Rico et al. [24]. NEs were added to the donor compartment. PBS/2% SDS for ClAlPc or Trizma buffer (pH 7.8) for PM was used as the receptor diluent. Samples were collected at 0, 3, 6, 12, and 24 h. The medium was replaced with the same amount of fresh medium each time a sample was removed. The ClAlPc or PM concentration was determined as described before. The results are expressed as μM/cm<sup>2</sup>. After the end of the experiment, skin membranes were collected and washed. For tape stripping, 15 pieces of adhesive tape (Scotch Magic 3M, Bogotá, Colombia) were individually applied to the skin pieces under a controlled weight of 520 g for 10 seg [25]. The pieces of tape were separated in groups 1 - 5, 6 - 10 and 11 - 15 and processed separately. The remaining skin was cut into pieces, macerated, and treated with ethanol or Trizma buffer for ClAlPc or PM extraction, respectively. The results are expressed as μM/cm<sup>2</sup>. The enhancement ratio (ER), defined as the permeation difference between encapsulated (NE with drugs) and non-encapsulated (free compound), was calculated as follows: Enhancement ratio (ER) = mass/average area of compound present in the NE system/mass/average area of free compound (PBS).



## 8. *In vivo* assays

### 1) *Mice*

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) and the ethics committee of the Universidad Industrial de Santander, Bucaramanga, Colombia (Act 13/2014). Female BALB/c mice aged 7 - 9 weeks were obtained from the National Health Institute (Bogotá, Colombia) and maintained on a 12 h light/dark cycle at 23°C, with water and mouse food pellets *ad libitum* according to the internationally valid guidelines.

### 2) *Toxicity tests*

Healthy mice were treated topically at the base of the tail with one dose of 50 µL of NE every three days for 15 days. Four experimental groups (N = 3) were tested and treated as follows: group 1, NE-ClAlPc; group 2, NE-15% PM; group 3, NE-ClAlPc/15% PM; and group 4, vehicle. After treatment, all animals were kept in the dark for 30 min. Animals from groups 1 and 3 were irradiated as describe above. Six days after treatment, the animals were euthanized with anaesthetics. Clinical signs of skin toxicity, such as irritation, redness or lacerations, were visually evaluated each day after treatment was started.

In addition, a micronucleus assay was also performed following protocol TG474 from the Organisation for Economic Co-operation and Development (OECD/OCDE). Briefly, bone marrow of the euthanized mice was extracted from the femurs, and slides were made, fixed and coloured with Giemsa. The frequency of micronuclei was determined microscopically with 1000× magnification by counting 2000 polychromatic erythrocytes. The results are expressed as the percentage of micronuclei present in the polychromatic cell erythrocytes.

### 3) *Quantification of ClAlPc and PM in organs after treatment in healthy animals*

Six days after the end of the previously described treatment, the animals were euthanized, and liver, spleen, kidney and treated skin zone (20 - 25 mg) samples were collected. The samples were macerated, sonicated and centrifuged for 10 min at 10,000 rpm. Supernatants were dissolved in a mixture of 85% ethanol: 10% DMSO:1% acetic acid for ClAlPc or in Trizma buffer (pH 7.8) for PM detection. The compound concentration was determined as previously described. The final concentration of PM in each organ sample was calculated as follows:  $PM = PM\ initial / PM\ in\ sample \times 100$ .

### 4) *In vivo antileishmanial activity*

Animals were infected subcutaneously into their shaven rumps with  $2 \times 10^6$  stationary-phase promastigotes. After 10 - 14 weeks (with the appearance of CL lesions), the animals were divided into three experimental groups (N = 3) and treatment was started: group 1 NE-ClAlPc-15%PM + PDT; group 2: NE-ClAlPc-15%PM without PDT; and group 3: 0.5% miltefosine (Cayman, MI, USA) gel. The formulations were applied topically onto the entire lesion (ulcer + nodule) each day for 18 days. Every two days and 30 min after the application of the formulation, the lesions were irradiated (50 J/cm<sup>2</sup> for 7 min). The formulation of 0.5% miltefosine gel was applied topically for 20 days and used as the reference drug [26]. Lesion sizes were measured weekly during treatment using digital callipers and the area (mm<sup>2</sup>) was calculated. Animals were anaesthetized and euthanized by cervical dislocation 10 days after the end of treatment.

### 9. Statistical analysis

For *in vitro* assays, the data were analysed by ANOVA with Bonferroni's post-test and a *t* test for independent data. The PM and ClAlPc tissue distribution results after treatment were analysed by the Kruskal-Wallis test for independent samples, with multiple comparisons evaluated by the Whitney *U* test. All analyses were performed with a 95% confidence interval using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). Values of *P* < 0.05 were considered statistically significant.

## RESULTS

### 1. NE characterization

The final concentrations of ClAlPc and PM in all NEs were  $39.2 \pm 0.3 \mu\text{M}$  and  $145.2 \pm 5.8 \text{ mM}$ , respectively. The physicochemical characteristics of NE formulations are expressed in **Table 1**. The average values of the hydrodynamic diameter were below 30 nm, with a PDI < 0.25. Blank NEs (without ClAlPc or PM; vehicle) presented smaller droplets in comparison to the other formulations (*P* < 0.05). The zeta potentials were slightly negative in all systems. All NEs were stable for at least four months after preparation. Fourteen months in the case of NE-ClAlPc (data not showed). A reduction of 60 to 80% of the absorption of benzofuran after PDT (ROS positive) was observed in the NEs containing ClAlPc compared to vehicle and NE-PM, respectively (**Table 1**, *P* < 0.05).

### 2. NEs toxicity on parasites and mammalian cells

The results are presented in **Table 2**. In general, all prepared NEs (vehicle and others) were toxic at dilutions of 1: 2 and 1: 10. Non-encapsulated ClAlPc (0.4  $\mu\text{M}$ , 1: 100 dilution) showed inhibition rates of 100 and 65.8% for promastigotes and intracellular amastigotes, respectively, after PDT. Moreover, encapsulated ClAlPc (0.4  $\mu\text{M}$ ) inhibited >94% of both parasitic forms, presenting a 1.4-fold higher effectiveness compared to non-encapsulated ClAlPc against intracellular amastigotes after PDT (*P* < 0.05). On the other hand, non-encapsulated PM (1.5%) showed antiparasitic effects against both promastigotes (>99%) and amastigotes (>44%) independent of PDT application, as expected, with PI values of >1, being more active for the free forms compared to the intracellular form (*P* < 0.05). The parasite inhibition was similar between encapsulated and non-encapsulated PM (*P* > 0.05) when the concentration was 0.05%. In the case of NE-ClAlPc/PM, more than 88.0% inhibition was observed at the concentrations tested after PDT. Selectivity indices of 1.55 and 1.38 were determined after PDT in promastigotes and amastigotes, respectively, with NE-ClAlPc/15%PM concentrations of 0.15% PM and 0.4  $\mu\text{M}$  ClAlPc.

**Table 1.** Physicochemical characteristics of NE at different times after preparation (up to 4 months, at 4°C)

Formulation	Size (nm)		PDI		Zeta potential (mV)		pH		ROS generation under photoactivation	
	Onset time	4 months after	Onset time	4 months after	Onset time	4 months after	Onset time	4 months after	Onset time	4 months after
NE	25.3 ± 0.4	25.2 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	-4.9	-3.8	7	7	-	-
NE-ClAlPc	27.2 ± 0.28	27.1 ± 0.7	0.1 ± 0.0	0.1 ± 0.0	-7.3	-4.4	7	7	+	+
NE-15% PM	29.7 ± 0.4	27.3 ± 0.02	0.3 ± 0.0	0.2 ± 0.0	-3.6	-4.9	7	7	-	-
NE-ClAlPc-15% PM	29.8 ± 1.3	29.2 ± 0.4	0.2 ± 0.0	0.2 ± 0.0	-0.6	-1.8	8	8	+	+

Size and PDI were indicated by Mean ± SD.

NE, nanoemulsions; nm, nanometer; PDI, polydispersity index; ROS, reactive oxygen species; ClAlPc: chloroaluminium phthalocyanine; PM, paromomycin.

**Table 2.** Susceptibility of *Leishmania (Viannia) braziliensis* and mammalian cells to nanoemulsion and controls

Formulations	PDT	% of inhibition (mean ± SD)											
		THP-1 cell				Promastigotes				Intracellular amastigotes			
		Dilutions				Dilutions				Dilutions			
		2	10	100	1,000	2	10	100	1,000	50	100	300	500
ClAlPc (s)	-	4.5 ± 1.7	0	0	0	0.3 ± 0.7	1.0 ± 1.0	0	0	2.8 ± 0.3	0.5 ± 0.8	0.4 ± 0.0	0
	+	97.8 ± 0.6	97.6 ± 0.5	49.1 ± 4.8	13.8 ± 4.1	100	100	100	0	93.4 ± 2.8	65.8 ± 4.7	21.8 ± 7.0 <sup>a</sup>	6.9 ± 2.1 <sup>a</sup>
NE-ClAlPc	-	86.8 ± 1.7	72.3 ± 7.1	0	0	100	96.9 ± 0.1	0.2 ± 0.4	1.4 ± 1.8	0	0	0	0
	+	89.3 ± 3.3	70.1 ± 8.0	59.6 ± 5.7	20.6 ± 6.7	99.1 ± 0.9	100	100	100	100	94.6 ± 3.2	76.8 ± 21.8 <sup>a</sup>	64.2 ± 23.1 <sup>a</sup>
PM (s)	-	79.2 ± 3.0	33.1 ± 3.5	17.9 ± 8.0 <sup>a</sup>	0.3 ± 0.6	78.5 ± 3.3	98.4 ± 1.6	99.9 ± 0.0	100	60.6 ± 2.4	48.5 ± 5.8	27.3 ± 8.1	2.3 ± 1.1
	+	78.7 ± 5.3	34.3 ± 5.2	18.6 ± 5.1	0.7 ± 1.0	78.7 ± 5.9	98.5 ± 1.4	99.9 ± 0.0	100	54.1 ± 2.6	46.6 ± 2.8	30.3 ± 2.8	0
NE-15% PM	-	90.3 ± 1.5	84.8 ± 1.8	0.6 ± 0.1 <sup>a</sup>	0	100	100	98.0 ± 0.1 <sup>a</sup>	0.35 ± 0.2	70.7 ± 6.7	44.3 ± 9.9 <sup>a</sup>	36.3 ± 6.9	18.5 ± 8.9
	+	89.7 ± 1.5	78.8 ± 8.4	3.3 ± 0.5	0	100	100	97.8 ± 0.1	0	61.9 ± 10.0	56.4 ± 8.4	30.3 ± 8.7	12.3 ± 1.5
NE-ClAlPc+15% PM	-	89.3 ± 1.3	86.4 ± 1.2	1.6 ± 3.1	0.3 ± 0.0	100	100	97.5 ± 0.1	0	75.6 ± 14.2	53.0 ± 8.9	12.9 ± 1.5	6.2 ± 1.7
	+	91.2 ± 1.7	88.3 ± 3.1	64.7 ± 5.5 <sup>a</sup>	0.4 ± 0.0 <sup>a</sup>	100	100	100	100	91.3 ± 2.9	89.6 ± 3.7	88.5 ± 1.6	85.7 ± 3.1
Vehicle	-	84.9 ± 1.0	57.9 ± 6.5	9.8 ± 1.0	0	98.9 ± 1.2	98.2 ± 1.9	0.7 ± 1.0	0	3.0 ± 0.2	0.5 ± 0.0	0.2 ± 0.0	0.7 ± 0.0
	+	91.8 ± 0.9	90.8 ± 1.2	4.1 ± 2.5	1.2 ± 0.2	100	99.2 ± 0.9	0	0	7.8 ± 1.0	2.7 ± 0.5	0.7 ± 0.0	0.3 ± 0.0

<sup>a</sup>P < 0.05.

SD, standard deviation; PDT, photodynamic therapy; ClAlPc, chloroaluminium phthalocyanine; (s), solution; NE, nanoemulsion; PM, paromomycin.

### 3. Ex vivo ClAlPc and PM skin permeation

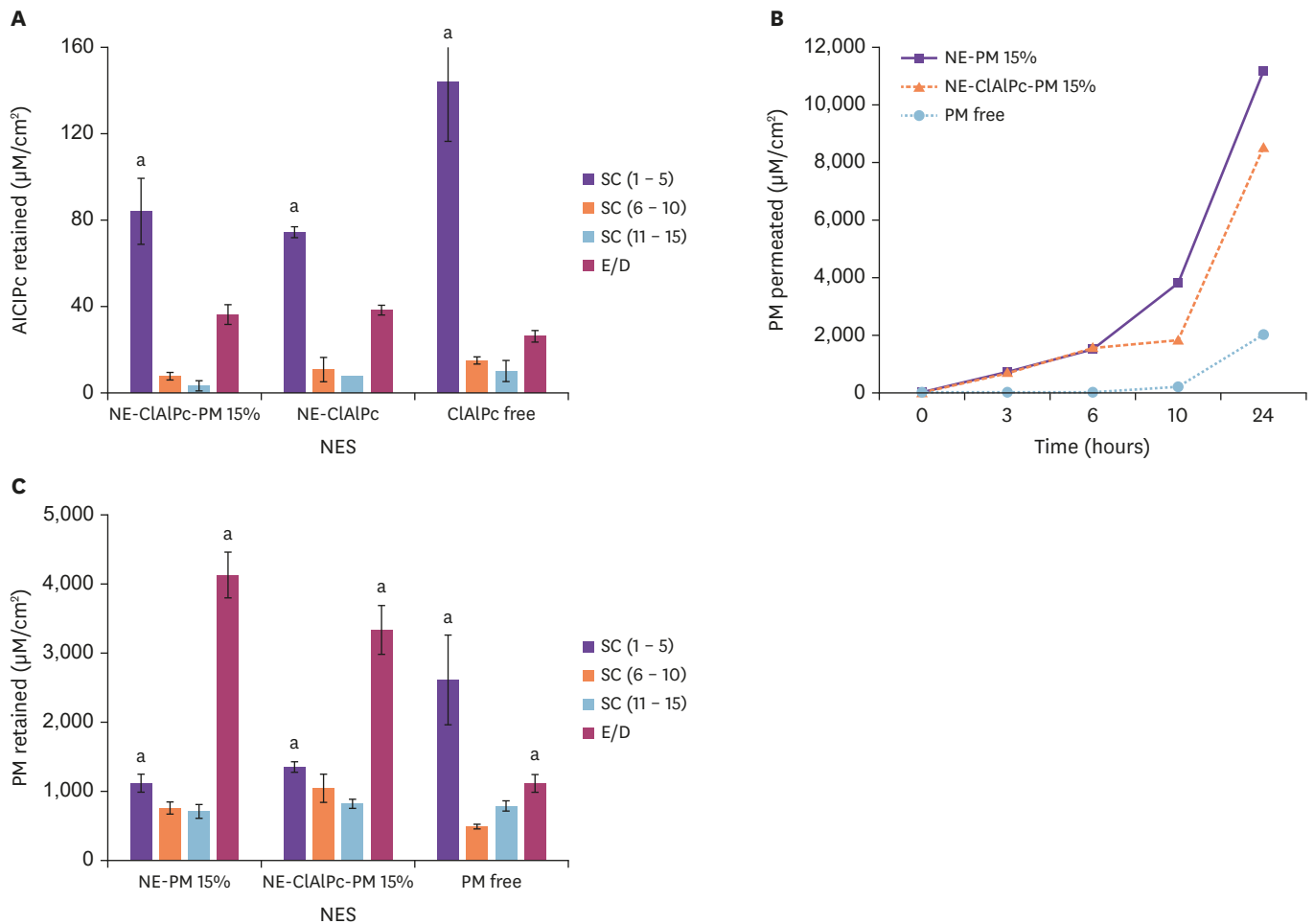
After the treatment of the skin with ClAlPc-containing formulations, ClAlPc was not detected at the receptor compartment at any time point during the assay. However, ClAlPc was found to be mainly retained by the *stratum corneum* in a rank order of ClAlPc (S) > NE-ClAlPc/15% PM > NE-ClAlPc with values of 143.97, 84.07 and 74.10 μM/cm<sup>2</sup>, respectively, and in the epidermis/dermis in a rank order of NE-ClAlPc > NE-ClAlPc/15% PM > ClAlPc (S) with values of 38.19, 36.21 and 26.27 μM/cm<sup>2</sup>, respectively (Fig. 1A). In contrast, the NE system markedly increased the permeation of PM through the skin. PM was detected in the receptor container, and significant differences (P < 0.05) in PM permeation were found when this drug was administered in the NEs and in solution. At 6 h, the permeation of PM vehiculated by the NEs (NE-15% PM and NE-15% PM/ClAlPc) was both faster (at three hours) and greater (almost 80-fold) than that of PM in solution (Fig. 1B). At the end of the experiment (24 h), differences in PM permeation/retention (P < 0.001) were observed. The ranking of both PM skin permeation and skin retention on the tested PM systems was NE-PM 15% > NE-PM/ClAlPc > PM in solution with values of 11,152, 8,544.31, 2,023.83 μM/cm<sup>2</sup> for permeation at 24 h and 4,115.22, 3,316.45, 1,089.36 μM/cm<sup>2</sup> for retention in the dermis, respectively (P < 0.001) (Fig. 1C).

### 4. In vivo toxicities

In addition to body weight, clinical signs of irritation, redness or lacerations at the site of application were also monitored (data not shown). The average body weight of the mice before the experiment was 25.8 ± 0.5 g, and after the experiment, it was 27.9 ± 0.5. A minimal frequency of micronuclei in the range of normal values (0.06 ± 0.02 to 0.16 ± 0.02) was detected (data not shown).

### 5. Detection of ClAlPc and PM in organs after treatment

No ClAlPc was detected in any of the extracted organs using the established methodology. In contrast, concentrations of PM between 27.26 - 292.49 μM/25 mg of organ were detected (Table 3). After NE-ClAlPc/15% PM treatment, 197.70 μM PM was observed in 25 mg of spleen. In the case of NE-PM 15% treatment, the PM accumulation was similar in the pieces taken from different organs (P = 0.33).



**Figure 1.** Skin permeation and retention of compounds. (A) ClAlPc skin retention, (B) PM skin permeation, (C) PM skin retention. <sup>a</sup>*P* < 0.05.

The results are the mean and standard deviation of three experiments.

ClAlPc, chloroaluminium phthalocyanine; PM, paramomycin; AICIPc, aluminum phthalocyanines; NE, nanoemulsions; NES, Nanoemulsion system; SC, stratum corneum; E/D, epidermis/dermis.

**Table 3.** Paromomycin detection in BALB/c mouse organs after topical treatment with different nanoemulsion systems for 15 days

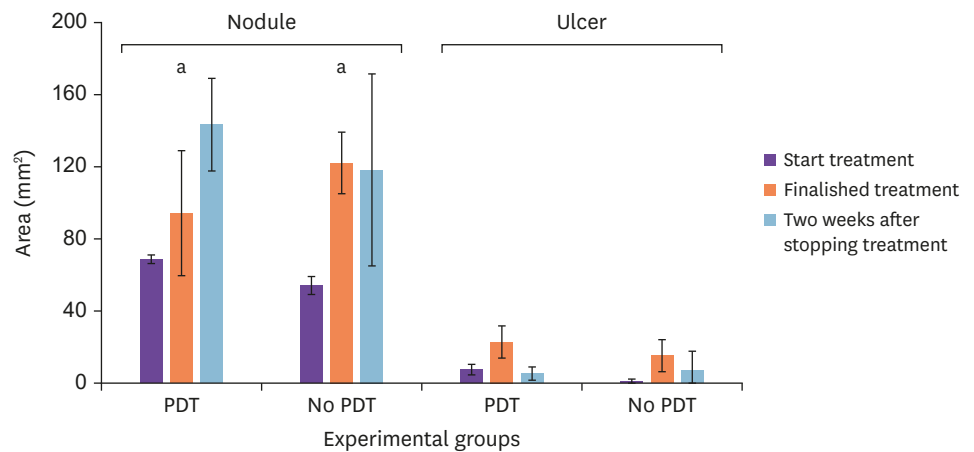
Treatment	Organ	Concentration of PM (µM), Mean ± SD
NE-ClAlPc-15% PM (Group 2)	Liver	113.91 ± 44.28
	Spleen	197.70 ± 8.70
	Skin	63.34 ± 46.17
	Kidney	72.69 ± 38.48
NE-15% PM (Group 3)	Liver	116.79 ± 67.76
	Spleen	121.67 ± 18.59
	Skin	91.42 ± 43.31
	Kidney	121.65 ± 53.78

PM, paramomycin; SD, standard deviation; NE, nanoemulsion; ClAlPc, chloroaluminium phthalocyanine.

### 6. Antileishmanial activity of NE-ClAlPc-15% PM in mice

The topical treatment of lesions with ClAlPc and PM nanoemulsion was ineffective in the *in vivo* model with BALB/c mice infected with *L. (V.) braziliensis*. An increase in the nodule size from  $68.43 \pm 2.02$  to  $143.24 \pm 25.79$  mm<sup>2</sup> was observed in the group treated with NE-ClAlPc-15%PM + PDT, and an increase from  $54.00 \pm 5.29$  to  $118.01 \pm 57.77$  mm<sup>2</sup> was observed in the group of animals treated without PDT. Decreases ranging from 5.18 to 22.5 mm<sup>2</sup> in the ulcer





**Figure 2.** Skin lesion size in BALB/c mice infected with *Leishmania (Viannia) braziliensis* and treated with ClAlPc-PM 15%-NE. The error bars represent the standard deviation of measurements for 3 individuals from each treatment.

<sup>a</sup>P < 0.05.

ClAlPc, chloroaluminium phthalocyanine; NE, nanoemulsions; PDT, photodynamic therapy.

size were observed in some cases (**Fig. 2, Supplementary Fig. 1**). Intracellular amastigotes were observed in skin biopsies of all animals (data not shown). Miltefosine gel was used as the reference drug, causing a 91% decrease in the size of the lesion 15 days after the end of the treatment [26].

## DISCUSSION

Based on previous studies [9, 10], this work describes the design and biological evaluation of a NE system containing both ClAlPc and PM as an experimental antileishmanial topical treatment. Nanoemulsions were prepared using the simple spontaneous emulsification method producing a stable system in terms of size, homogeneity and charge. Although the neutral zeta potential of the nanodrop can be a concern in some nanosystems [27], the NE system showed to be stable at the interfacial level probably due to the presence of a solvation layer stabilized by the highly hydrophilic groups of the surfactant used [22]. Comparable physicochemical characteristics and stability were obtained with a NE system containing only ClAlPc [22]. In regard to the PM, it is remarkable that a considerably high concentration (15%, w/v) of this drug was dissolved in the continuous phase of NE, which did not affect the stability of the formulation.

The ability of NE loaded with ClAlPc to generate ROS after PDT was confirmed in this work. At all evaluated time points after preparation, ROS were generated under photoactivation, suggesting that the photoactivity of the ClAlPc was maintained by the formulation. Irradiation of a photosensitizer triggers type I and type II reactions, which induce production of reactive species as hydroxyl radicals (OH), superoxide anions (O<sub>2</sub><sup>-</sup>) and mainly singlet oxygen [5, 6]. ROS can react with cellular components such as membrane lipids, proteins and DNA leading to cell death [5, 6]. In *Leishmania*, the role of ROS in the destruction of the parasite has been previously demonstrated [28]. Overall, it was shown that all formulation exhibited a selective antileishmanial effect after PDT with phototoxic index (PDT+/PDT-) above 3.0 in the most diluted samples. The highest activity of NE-ClAlPc-15%PM was expressed against intracellular amastigotes after PDT. The antileishmanial activities of ClAlPc (free or encapsulated or

combined with others molecules such as uroporphyrin) against different mammalian cells and *Leishmania* species has been previously demonstrated [10, 11, 29].

The addition of PM to the formulation was an important strategy due to the known clinical effectiveness of this drug against leishmaniasis. Its activity remained at similar levels even after irradiation and NEs-PM prepared allowed both PM permeation on healthy skin and PM distribution on internal organs after topical treatment (see below). Paromomycin has been tested as an antileishmanial agent in both CL preclinical and clinical trials [4, 30-32]. *In vitro* PM activities displayed higher values ( $IC_{50} > 50 \mu M$ , low activity) and variable clinical efficacy (healing rates ranging from 53.3 to 90.0%) using different CL topical formulations alone or combined [4, 30-32]. In this work, PM activities, both in solution and in NE, on intracellular amastigotes were also low.

The ability of the NEs system designed in this study to allow drug (CIAIPc and PM) permeation across the skin was determined. CIAIPc did not permeate through the skin, and was retained by the stratum corneum. Therefore, no CIAIPc was detected on internal organs after topical treatment. Same results have been reported in dorsal skin of Wistar rats and abdominal skin of human. In these studies, the photosensitizers did not permeate the skin but were retained by the *stratum corneum* [24, 33]. Conversely, significant amounts of PM were found in the spleen and in the liver of both irradiated and non-irradiated mice, treated with topical PM-loaded NE. *Ex vivo* trials showed an 80-fold increase in the PM skin permeation using NE-PM than non-encapsulated PM (solution) treatment. Taking into account that PM is a highly hydrophilic compound, this is a remarkable result. This increased permeation of the skin by PM can be due to disturbances caused by NE ingredients on the *stratum corneum*. Paromomycin permeation and skin retention studies have been performed by other authors. Liposomal PM induced lower skin permeation compared to PM in solution (71.6 - 74.9% liposomal PM versus 91.5% PM in solution) [30]; nanogel PM allowed the diffusion of PM after 1.5 h on synthetic membranes but not through the skins of mouse and pig ears [31]. The results obtained by Gaspar et al using Liposomal-PM formulation given intravenously, in healthy BALB/c mice, are comparable to those registered by us during *in vivo* assays [32]. The authors showed PM accumulation of up to 10% of the injected dose in the spleen, liver and lungs. This formulation had a better therapeutic effect in a murine model infected with *L. infantum*.

Some studies about NE toxicities were also performed. As expected, *in vitro* studies evidenced that high concentrations of blank NE were toxic against cells in monolayers. The components of the NE, especially surfactants, can be involved in toxic reactions [34], which is more evident in culture conditions due to the direct exposure of cells to the components of the formulation. In healthy animals, however, the treatment with topical NE-CIAIPc-15% PM during 15 days with three PDT sessions did not show any clinically detectable sign of toxicity or skin irritation. In previously published clinical studies, Cremophor used in a parenteral paclitaxel formulation has been associated with acute hypersensitivity [34]. Moreover, methylbenzothonium chloride used in PM formulations displayed some adverse effects such as pruritus, burning, redness, oedema, and pain in some CL-treated patients [35, 36]. Thus, the topical formulation proposed in this work seems to be of acceptable safety.

Finally, an explorative study using topical NE-CIAIPc+15%PM/15 days plus or without PDT was unable to reduce CL lesions on *L. (V.) braziliensis* infected mice. Similar results were obtained with CIAIPc associated to liposomes in BALB/c mice infected with *L. braziliensis* [11]. It is possible that the poor CIAIPc permeation through the skin limited the efficacy of the PDT

*in vivo*. The use of ClAlPc as the only therapy for CL has not had positive effects, which could be due to factors related to the permeability and concentration of PS, the parasite or the host immune response [37]. In contrast, the combination of local ClAlPc-based PDT and systemic therapy with glucantime (20 mgSb<sup>3+</sup>/kg/day) exhibited clinical and parasitological efficacy in C57BL/6 mice infected with *L. (L.) amazonensis* [38]. Although the combination of PDT and PM proposed in this work did not reduce the size of the leishmanial lesions, it is reasonable to suggest that modifications on the therapeutic protocol of even combination of PDT with other antileishmanial agents can give better results.

In conclusion, the NE system proposed in this work can be used to significantly increase the percutaneous permeation of PM, but not of ClAlPc. We demonstrate that NE-ClAlPc+15%PM/15 have the best anti-*Leishmania* effect *in vitro* on intracellular amastigotes, after PDT. Although the *in vivo* results evidenced a lack of antileishmanial efficacy of the proposed protocol, the use of a different photosensitizer in this same formulation or modifications on the treatment protocols can be studied in an attempt to have better outcomes.

## SUPPLEMENTARY MATERIAL

### Supplementary Figure 1

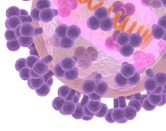
Efficacy of the nanoemulsion containing ClAlPc/PM 15% in BALB/c mice infected with *Leishmania (Viannia) braziliensis*. Animals were treated topically with the NE (N = 3, M1 - M3) for 18 days. PDT was applied on days 1, 4, 8, 11 and 15. Mice without PDT were kept in the dark.

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