proceedings

MDPI

Proceedings

Topical Delivery of Amphotericin B Utilising Transferosomes for the Treatment of Cutaneous Leishmaniasis †

Raquel Fernández-García [1,*], Larry Sttats [2], Jessica A. de Jesus [3], María Auxiliadora Dea-Ayuela [4], Francisco Bolás-Fernández [5], M. Paloma Ballesteros [6], Marcia Dalastra Laurenti [7], Luiz F.D. Passero [8], Aikaterini Lalatsa [9] and Dolores R. Serrano [10]

[1] Departamento de Farmacia Galénica y Tecnología Alimentaria, Facultad de Farmacia. Universidad Complutense de Madrid, Plaza Ramón y Cajal, s/n, 28040 Madrid, Spain
[2] Biomaterials, Bio-engineering and Nanomedicines (BioN) Laboratory, Institute of Biomedical and Biomolecular Sciences, School of Pharmacy and Pharmaceutical Sciences, University of Portsmouth. St. Michael's Building, White Swan Road, Portsmouth, UK; larry.statts@myport.ac.uk
[3] Laboratory of Pathology of Infectious Diseases (LIM-50), Medical School, University of São Paulo, Avenida Dr. Arnaldo, 455, 01246903 Cerqueira César, Brazil; jessicaa@usp.br
[4] Departamento de Farmacia, Facultad de Ciencias de la Salud, Universidad CEU Cardenal Herrera, Carrer Santiago Ramón y Cajal, s/n, 46113 Valencia, Spain; mdea@uchceu.es
[5] Departamento de Microbiología y Parasitología. Facultad de Farmacia. Universidad Complutense de Madrid. Plaza Ramón y Cajal, s/n., 28040 Madrid, Spain; francisb@ucm.es
[6] Departamento de Farmacia Galénica y Tecnología Alimentaria/Instituto Universitario de Farmacia Industrial (IUFI), Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal, s/n, 28040 Madrid, Spain; pballesp@ucm.es
[7] Laboratory of Pathology of Infectious Diseases (LIM-50), Medical School. University of São Paulo, Avenida Dr. Arnaldo, 455, 01246903 Cerqueira César, Brazil; mdlauren@usp.br
[8] São Paulo State University (UNESP), Institute for Biosciences São Vicente, Praça Infante Dom Enrique, s/n, 11390-900 São Vicente/Institute for Advanced Studies of Ocean São Vicente. Av. João Francisco Bernsdorp, 1178, 11350-011 São Vicente, Brazil; felipe.passero@unesp.br
[9] Biomaterials, Bio-engineering and Nanomedicines (BioN) Laboratory, Institute of Biomedical and Biomolecular Sciences, School of Pharmacy and Pharmaceutical Sciences, University of Portsmouth. St. Michael's Building, White Swan Road, Portsmouth, UK; katerina.lalatsa@port.ac.uk
[10] Departamento de Farmacia Galénica y Tecnología Alimentaria/Instituto Universitario de Farmacia Industrial (IUFI), Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal, s/n, 28040 Madrid, Spain; drserran@ucm.es
* Correspondence: raqfer01@ucm.es; Tel.: +34-91394-1620
† Presented at the 1st International Electronic Conference on Pharmaceutics, 1–15 December 2020; Available online: https://iecp2020.sciforum.net/.

Received: date; Accepted: date; Published: date

Abstract: Amphotericin B (AmB) is a high-molecular weight poorly soluble drug with a high efficacy in the treatment of infectious caused by *Leishmania* spp. parasites, which possesses a very low topical bioavailability. Transferosomes (TFs), which are ultradeformable vesicles consisting on, drugs lipids, an edge activator and a low amount of ethanol (<10%), have been engineered and optimised to enhance the permeation of AmB across the skin and, thus, its antiparasitic activity. Drug loading of the formulation resulted in 0.086%, while a good physicochemical stability for 6 months under desiccated conditions was seen. AmB-TFs illustrated a flux of 4.91 ± 0.41 μg/cm²/h across mouse skin. *In vivo* studies demonstrated a good permeation of the drug after topical application on healthy mouse skin which increased by using microneedles at early times of exposure, while *in vivo* efficacy studies demonstrated that parasite load was decreased in a 98.24 ± 1.54%.

Keywords: amphotericin B; transferosomes; ultradeformable lipid vesicles; leishmaniasis

1. Introduction

Nowadays, cutaneous leishmaniasis remains a common public health problem due to the lack of an effective and safe treatment which ensures healing and prevents parasite dissemination, while avoiding undesired systemic effects. The main challenge resides in the utilisation of parenteral and oral treatments, which should be suitable for visceral and mucocutaneous leishmaniasis but become highly toxic in the treatment of skin infections.

Amphotericin B (AmB) is a macrolide which possesses a high molecular weight (924 g/mol) and is naturally synthetised by the fungi *Streptomyces nodosus* [1,2]. This molecule has a broad spectrum antifungal drug which also presents antileishmanial activity due to its ability to binding the ergosterol in the cell membranes [1]. However, the systemic utilisation of AmB would result undesirable taking into account its narrow therapeutic window [3], but also due to its low solubility in aqueous media. As a result, AmB could present a wide variety of adverse effects, but also a poor permeability across biological barriers [1,4].

Transferosomes (TFs) are lipid vesicles which consists on a mixture of drug, lipids, and edge activator with very reduced ethanol content (below 10%) that are capable of squeezing themselves, losing their original structure to permeate across the skin [5,6]. Thus, TFs result useful to enhance the permeability of poorly soluble drugs, such as AmB, promoting drug targeting, and reducing the risk of undesired effects [5,6]. In this work, AmB-TFs were engineered and optimised via Quality by Design (QbD) tools to ensure a high entrapment efficiency (EE), appropriate particle size distribution, and good colloidal and physicochemical stability. This formulation was in vitro and in vivo tested to understand drug permeation across synthetic and biological membranes, but also the efficacy of this drug delivery system in *Leishmania amazonensis* and L. *braziliensis* promastigotes and amastigotes. *In vitro* toxicity was also evaluated in terms of cytotoxicity and haemolysis to ensure the safety of the developed formulation.

2. Materials and Methods

2.1. Materials

AmB was purchased from the North China Pharmaceutical Huasheng Co. (Hebei, China). Phosphatidylcholine (Lipoid S100) was kindly provided from Lipoid S100 GmBH (Ludwigshafen, Germany). Sodium deoxycholate (NaDC), Roswell Park Memorial Institute 1640 medium (RPMI-1640), and other products were obtained from Sigma-Aldrich Chemie GmbH (Madrid, Spain) and were at least ACS grade. The rest of the materials were purchased as follows: ethanol 96° (Alcoholes Aroca S.L., Madrid, Spain), Strat-M® membranes (Merck KGaA, Darmstadt, Germany), Triton® X-100 (Sigma-Aldrich Co., St. Louis, MO, USA), Sabouraud dextrose agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), Müller-Hinton agar (Laboratorios Conda S.A., Madrid, Spain), glucose (Panreac Química S.A.U., Barcelona, Spain) and sterile 0.9% sodium chloride (Laboratorios ERN, S.A., Barcelona, Spain).

2.2. Methods

2.2.1. Optimisation and Manufacturing of AmB-TFs

A three-level Box-Behnken model was employed to optimise the AmB-TFs formulation. DesignExpert 10 software (Stat-Ease Inc., Minneapolis, MN, USA) was used to establish the high and low levels of the design. Selected independent and dependent variables and their ranges are shown in table 1.

Table 1. Selected independent, dependent variables to implement the Box-Behnken model to optimize AmB-TFs.

Independent Variables			
	Levels		
Factor	Low	Medium	High
Concentration of NaDC/edge activator (%)	6	13	20
Amount of drug (mg)/2 mL batch	0.5	1.25	2
Concentration of ethanol in the reconstituted buffer (%)	0	4	8.5
Dependent Variables			
Entrapment efficiency (%) Particle size (nm) Zeta-potential (mV)			
Other Variables			
Batch size	200 mg in 2 mL		
Amount of lipids (mg)	Amount remaining from the 200 mg total amount after substracting the amounts of sodium deoxycholate and drug		
Rotaevaporation time	30 min		
Reconstitution volume	2 mL		
Probe sonication time	2 cycles × 10 s		

AmB-TFs were manufactured by the thin film hydration method. 1.82 mg of AmB as well as 171.74 mg of Lipoid S100 and 28.26 mg of NaDC were dissolved in 20 mL of methanol in a round-bottom flask. The solvent was evaporated using a RV 06-ML rotaevaporator (Janke & Kunkel Ika-Labortechnik, Staufen, Germany) and a Vac V-500 vacuum pump (Büchi Labortechnik, Flawil, Switzerland) at 40–45 °C and 250 rpm. After evaporation, a film was obtained and reconstituted using a mixture of ethanol and phosphate buffered saline (PBS) at pH 7.4 (91.5:8:5 v:v). The mixture was homogenised for 30 min and then probe sonicated at 10 W (Branson Sonifier 250, Emerson Industrial, East Granby, CT, USA).

Solid-state AmB-TFs were also obtained by freeze-drying. Mannitol was added to liquid AmB-TFs as a cryoprotectant agent. Samples were frozen at −40 °C overnight and freeze-dried at −50 °C and 0.2 bar for 24 h utilising a LyoQuest freeze-drier (Azbil Telstar, S.L., Terrasa, Spain).

2.2.2. Characterisation of AmB-TFs

AmB-TFs were characterised in terms of particle size, zeta-potential, and EE. A Zetatrac Ultra Analyser (Microtrac Inc., Montgomery, PN, USA) was employed to measure particle size and zeta-potential by dynamic light scattering (DLS).

On the other hand, AmB-TFs were centrifuged (6000 rpm, 15 min) to separate entrapped and unencapsulated AmB. The supernatant was diluted using methanol (1/100 *v*/*v*) and, then, absorbance was measured by UV-visible spectrophotometry (PharmaSpec, Shimadzu, Kyoto, Japan) at 406 nm. EE was calculated as follows (Equation (1)):

$$EE(\%) = \frac{A-B}{A} \times 100 \quad (1)$$

where A was the absorbance at 406 nm immediately after probe sonication, while B was the absorbance at 406 nm after centrifugation.

2.2.3. Physicochemical Stability of AmB-TFs

Freshly prepared liquid AmB-TFs were diluted using PBS pH 7.4 and later divided into different vials containing 1 mL of sample each. Half of the vials were freeze-dried as above described. Then,

both batches were stored at 4 and 25 °C, while relative humidity (RH) was also controlled for the solid-state AmB-TFs (10 and 60% RH) using different saturated salt solutions. For this purpose, uncapped vials were placed into stability chambers at the above-mentioned conditions, and samples were analysed at different time points. Samples were reconstituted using 1 mL of deionised water to measure particle size (physical stability) and with 1 mL of HPLC mobile phase (chemical stability) [7].

2.2.4. In Vitro Permeability Studies

Diffusion studies took place using AmB-TFs and AmB dissolved in dimethyl sulfoxide (DMSO). Franz cells (Soham Scientific, Loughborough, UK) were employed to understand in vitro permeation through two different membranes: synthetic Strat-M® membranes (Merck KGaA, Darmstadt, Germany) and mouse skin. These membranes were located on top of the receptor compartment, while PBS pH 7.4 supplemented with 4% *w/v* NaDC was placed in the receptor compartment to ensure sink conditions. AmB concentration at different time points was quantified using a previously validated HPLC method [7] (Jasco Inc., Easton, MD, USA).

Once the study was finished, the remaining amount of AmB was extracted and analysed by the above described HPLC method [7].

2.2.5. Ex Vivo Red Blood Cells (RBCs) Haemolysis Studies

RBCs were taken from blood samples from a human donor. The process took place as previously described with some modifications [8]. Cells (4%) were diluted using PBS pH 7.4 and placed in a 96-well plate followed by AmB-TFs at different concentrations and compared with blank TFs and AmB-DMSO, while PBS pH 7.4 and Triton®-X 100 were used as negative and positive controls, respectively. Results of haemolytic toxicity were expressed as HC_{50}.

2.2.6. In Vitro Antiparasitic Activity and In Vitro Macrophage Cytotoxicity

Leishmania amazonensis and *L. braziliensis* were cultured in Schneider's insect medium (Sigma-Aldrich, St. Louis, MO, USA) at 26 °C supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in culture flasks. Two different antiparasitic activity studies were carried out for promastigotes and amastigotes as previously described [9]. Cytotoxicity was also evaluated in J774 murine macrophages following previously described protocols [9,10]. Results of efficacy and toxicity were expressed as IC_{50} and CC_{50}, respectively.

2.2.8. In Vivo Pharmacokinetics and Pharmacodynamics

Both experiments were carried out in CD-1 male mice (8 weeks-old, 28 g). All *in vivo* processes, as well as approved ethical committees, are described in more detail in an already published article from the research group [2].

3. Results and Discussion

3.1. Optimisation and Characterization of AmB-TFs

Box-Behnken model determined that higher amounts of AmB and NaDC provided a larger particle size, while the smallest particle size would be achieved with low to intermediate amounts of NaDC are higher amounts of AmB. In terms of EE, the largest EE was achieved when intermediate amounts of edge activator and drug were utilised. However, none of the tested variables showed a high impact on zeta-potential.

The optimised formulation resulted in a 14:86 (w:w) edge activator : lipids ratio, with a final drug loading of 0.086%, while the reconstitution buffer consisted of 91.5% PBS pH 7.4 and 8.5% ethanol. This formulation had a particle size of 149 ± 22 nm, showed good colloidal stability with a zeta-potential of −35.02 ± 2.71 mV and an EE of 93.3 ± 1.9%.

3.2. Physicochemical Stability of AmB-TFs

AmB-TFs remained physicochemically stable for at least one month in the liquid state at 4 °C, while freeze-dried AmB-TFs were stable for at least 6 months at 4 and 25 °C under desiccated conditions (10% RH). However, the stability of AmB-TFs was highly affected at 60% RH.

3.3. In Vitro Permeability Studies

Both AmB-TFs and AmB-DMSO showed a linear permeation of the drug across Strat-M® membranes, with a lag time of 9.99 ± 4.59 and 4.86 ± 2.87 min and a steady-state flux value of 41.18 ± 1.39 and 211.06 ± 15.33 μg/cm²/h for AmB-TFs and AmB-DMSO, respectively, which means that AmB-DMSO had a 5-fold higher permeation across synthetic membranes. However, the behavior of AmB was very different when permeation was evaluated across mouse skin. The drug did not show any lag time as it started to permeate immediately, while flux resulted in 4.91 ± 0.41 and 5.49 ± 1.18 μg/cm²/h for AmB-TFs and AmB-DMSO, respectively, indicating there are no significant differences when permeation is evaluated across biological barriers (Figure 1a). This would suggest that testing *in vitro* permeation through mouse skin should be more reliable compared with *in vivo* results.

3.4. Evaluation of Haemolytic Toxicity

Haemolytic studies concluded that AmB-TFs showed 10-fold higher values of HC_{50} than AmB dissolved in DMSO. This would suggest that entrapping AmB with TFs reduces drug toxicity, while the utilised excipients are safe for RBCs (Figure 1b).

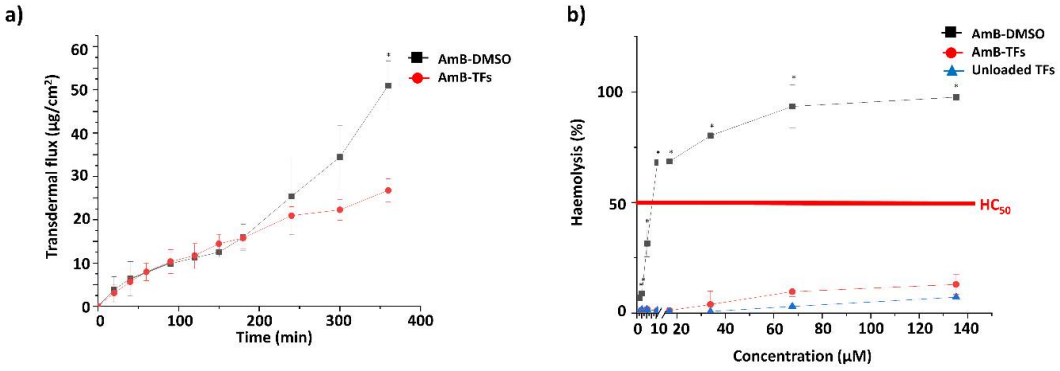

Figure 1. In vitro permeability and toxicity of AmB-TFs: (**a**) Permeability of AmB-Table 0. when comparing AmB-DMSO with the other formulations.

3.5. In Vitro Antiparasitic Activity and Cytotoxicity against Macrophages

The IC_{50} values of AmB-TFs against promastigotes were 2.00 ± 0.24 μM and 3.42 ± 0.15 μM for *L. amazonesis* and *L. braziliensis*, respectively, while higher IC_{50} values (5.37 ± 0.19 and 7.08 ± 0.34 μM) were observed for the amastigotes. The CC_{50} against J774 murine macrophages was 33.98 ± 4.02 μM, indicating an appropriate selectivity index of AmB-TFs for both species of *Leishmania.*

3.6. In Vivo Pharmacokinetics and Pharmacodynamics

AmB-TFs were able to permeate to deeper regions on the skin. The application of microneedles before the topical administration of the formulation resulted useful to increase drug permeation during the first hours, especially in the deepest layers. The utilisation of these solid metallic microneedles formed transient micropores that enhanced drug permeability. However, micropores tend to reseal within 2 hours when the skin is not occluded, indicating microneedles could not result useful at later times (Figure 2a).

Regarding in vivo efficacy, the administration of AmB-TFs once a day for 10 consecutive days resulted in a decrease of parasite load of 98.24 ± 1.54%, which resulted in similar to the intralesional

administration of Glucantime® (99.78 ± 0.31%) (Figure 2b). The utilisation of AmB-TFs could result in many advantages taking into account that the administration of pentavalent antimonials has been described as painful, but also they can produce severe adverse effects, including cardiotoxicity, pancreatitis, and nephrotoxicity [11].

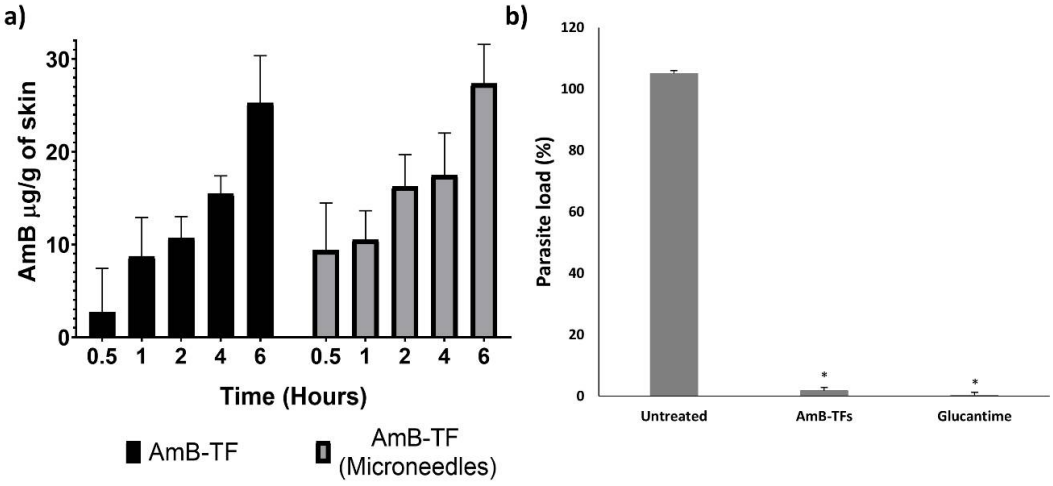

Figure 2. In vivo permeability and efficacy of AmB-TFs: (**a**) Amount of AmB remaining in the skin after topical AmB-TFs administration without and with the application of microneedles; (**b**) *In vivo* antiparasitic activity (parasite load) of AmB-TFs and Glucantime® in experimental cutaneous leishmaniasis *(L. amazonensis).* Statistically significant differences between treated and untreated animals are represented by * ($p < 0.05$, Mann-Whitney U test).

4. Conclusions

AmB-TFs enabled the permeation of AmB after topical administration that allowed therapeutically relevant amounts to be uptaken and accumulated within the dermis where parasites accumulate. The low toxicity of the formulation allows for safe and effective non-invasive formulations for the treatment of these parasitic infections.

Author Contributions: Methodology, R.F.-G., L.S., J.A.d.J., M.A.D.-A., L.F.D.P., A.L., D.R.S.; formal analysis, R.F.-G., L.S., J.A.d.J., M.A.D.-A., L.F.D.P., A.L., D.R.S.; investigation, R.F.-G., L.S., J.A.d.J., M.A.D.-A., L.F.D.P., A.L., D.R.S.; data curation, R.F.-G., L.S., J.A.d.J., M.A.D.-A., M.D.L., L.F.D.P., A.L., D.R.S.; writing original draft, R.F.-G., L.S., J.A.d.J., M.D.L., L.F.D.P., A.L., D.R.S.; visualization, R.F.-G., L.S., A.L., D.R.S.; review and editing, R.F.-G., M.P.B., A.L., D.R.S.; validation, L.S., J.A.d.J., M.A.D.-A., L.F.D.P., A.L., D.R.S.; supervision, F.B.-F., M.P.B., M.D.L., L.F.D.P., A.L., D.R.S.; funding acquisition, F.B.P., M.D.L., L.F.D.P., A.L., D.R.S.; resources, M.D.L., L.F.D.P., A.L., D.R.S.; project administration and execution, M.D.L., L.F.D.P., A.L., D.R.S.; conceptualization, A.L., D.R.S.; data analysis, A.L. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The authors are grateful to FAPEST (2016/0048-0) and HC-LIM 50. L.S. was supported with an Erasmus+ grant to undertake a placement at UCM, Madrid, Spain. This work was funded by an Ibero-American Universities Union Research Collaboration Fund (Unión Iberoamericana de Universidades; ENF03-2017), allowing a student mobility exchange between UCM (Madrid, Spain) and USP (Sao Paulo, Brazil).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AmB	amphotericin B
TFs	transferosomes
QbD	quality by design

EE	entrapment efficiency
NaDC	sodium deoxycholate
PBS	phosphate buffered saline
DLS	dynamic light scattering
RH	relative humidity
DMSO	dimethyl sulfoxide
HPLC	high-performance liquid chromatography
RBCs	red blood cells
HC_{50}	haemolytic concentration 50
IC_{50}	inhibitory concentration 50
CC_{50}	cytotoxic concentration 50

Appendix A

This research has been already published in: Fernández-García, R.; Statts, L.; de Jesús, J.A.; Dea-Ayuela, M.A.; Bautista, L.; Simao, R.; Bolás-Fernández, F.; Ballesteros, M.P.; Dalastra-Laurenti, M.; Passero, L.F.; Lalasta, A.; Serrano, D.R. Ultra-deformable lipid vesicles localize amphotericin B in the dermis for the treatment of skin infectious diseases. *ACS Infect Dis.* **2020**, *6*, 2647–2660.

References

1. Fernandez-Garcia, R.; de Pablo, E.; Ballesteros, M.P.; Serrano, D.R. Unmet clinical needs in the treatment of systemic fungal infections: The role of amphotericin B and drug targeting. *Int. J. Pharm.* **2017**, *525*, 139–148, doi:10.1016/j.ijpharm.2017.04.013.
2. Fernández-García, R.; Statts, L.; de Jesus, J.A.; Dea-Ayuela, M.A.; Bautista, L.; Simão, R.; Bolás-Fernández, F.; Ballesteros, M.P.; Laurenti, M.D.; Passero, L.F.D.; et al. Ultradeformable Lipid Vesicles Localize Amphotericin B in the Dermis for the Treatment of Infectious Skin Diseases. *ACS Infect. Dis.* **2020**, *6*, 2647–2660, doi:10.1021/acsinfecdis.0c00293.
3. Serrano, D.R.; Hernandez, L.; Fleire, L.; Gonzalez-Alvarez, I.; Montoya, A.; Ballesteros, M.P.; Dea-Ayuela, M.A.; Miro, G.; Bolas-Fernandez, F.; Torrado, J.J. Hemolytic and pharmacokinetic studies of liposomal and particulate amphotericin B formulations. *Int. J. Pharm.* **2013**, *447*, 38–46, doi:10.1016/j.ijpharm.2013.02.038.
4. Torrado, J.J.; Espada, R.; Ballesteros, M.P.; Torrado-Santiago, S. Amphotericin B formulations and drug targeting. *J. Pharm. Sci.* **2008**, *97*, 2405–2425, doi:10.1002/jps.21179.
5. Cevc, G.; Blume, G. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochim. Biophys. Acta* **1992**, *1104*, 226–232.
6. Fernández-García, R.; Lalatsa, A.; Statts, L.; Bolás-Fernández, F.; Ballesteros, M.P.; Serrano, D.R. Transferosomes as nanocarriers for drugs across the skin: Quality by design from lab to industrial scale. *Int. J. Pharmaceut.* **2020**, *573*, 118817, doi:10.1016/j.ijpharm.2019.118817.
7. Espada, R.; Josa, J.M.; Valdespina, S.; Dea, M.A.; Ballesteros, M.P.; Alunda, J.M.; Torrado, J.J. HPLC assay for determination of amphotericin B in biological samples. *Biomed. Chromatogr.* **2008**, *22*, 402–407, doi:10.1002/bmc.947.
8. Evans, B.C.; Nelson, C.E.; Yu, S.S.; Beavers, K.R.; Kim, A.J.; Li, H.; Nelson, H.M.; Giorgio, T.D.; Duvall, C.L. Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. *J. Vis. Exp.* **2013**, 10.3791/50166, e50166, doi:10.3791/50166.
9. Bilbao-Ramos, P.; Sifontes-Rodriguez, S.; Dea-Ayuela, M.A.; Bolas-Fernandez, F. A fluorometric method for evaluation of pharmacological activity against intracellular Leishmania amastigotes. *J. Microbiol. Methods* **2012**, *89*, 8–11, doi:10.1016/j.mimet.2012.01.013.
10. Dea-Ayuela, M.A.; Castillo, E.; Gonzalez-Alvarez, M.; Vega, C.; Rolon, M.; Bolas-Fernandez, F.; Borras, J.; Gonzalez-Rosende, M.E. In vivo and in vitro anti-leishmanial activities of 4-nitro-N-pyrimidin- and N-pyrazin-2-ylbenzenesulfonamides, and N2-(4-nitrophenyl)-N1-propylglycinamide. *Bioorg. Med. Chem.* **2009**, *17*, 7449–7456, doi:10.1016/j.bmc.2009.09.030.
11. Alvar, J.; Croft, S.; Olliaro, P. Chemotherapy in the treatment and control of leishmaniasis. *Adv. Parasitol.* **2006**, *61*, 223–274, doi:10.1016/s0065-308x(05)61006-8.