Gavin Publishers
Gavin Journal of Dermatology Research and Therapy
Volume 2016; Pages 7
Bicho J et al.

Research Article

Lipid Nanocarriers for Ketoconazole Topical Delivery

Joana Bicho[1], Joana Marto[1], Ana Salgado[1], Sara Raposo[2], Sandra Simões[1]* and Helena Margarida Ribeiro[1]

[1]Research Institute for Medicines and Pharmaceutical Sciences (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal

[2]Laboratório Edol, Produtos Farmacêuticos, Portugal

***Corresponding author:** Sandra Simões, Research Institute for Medicines and Pharmaceutical Sciences (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Estrada do Paco do Lumiar, 22 Edificio F, R/C 1649-038 Lisboa, Portugal, Tel: +351 217500769; Fax:+351 217946470; E-mail: ssimoes@ff.ulisboa.pt

Citation: Bicho J, Marto J, Salgado A, Raposo S, Simões S, et al. (2016) Lipid Nanocarriers for Ketoconazole Topical Delivery. Gavin J Dermatol Res Ther 2016: 7-13.

Received: 24 March, 2016; **Accepted:** 14 April, 2016; **Published:** 28 April, 2016

Abstract

Skin's superficial fungal infections are diseases that affect most people worldwide. About 20 to 25% of world's population is infected and the incidence continues to increase. Superficial dermatomycoses caused by *Trichophyton rubrum*, among other dermatophytes, and yeasts, such as *Candida spp* and *Malassezia spp*, has increased considerably, especially among pediatric and geriatric populations. Common treatment strategies are generally well tolerated and effective. However, frequent relapses following antifungal therapy cessation are described. The search for new treatment modalities and drugs is hampered by the lack of understanding of basic pathophysiological mechanisms that underlie these frequently encountered infections. Ketoconazole (KTZ) is a synthetic, broad-spectrum antifungal drug. It is a potential inhibitor of ergosterol (a main lipid membrane of fungi) synthesis. It is poorly soluble in water and unstable, especially in aqueous media. The purpose of this work was to develop new systems for the percutaneous delivery of KTZ. A nanoemulsion with 0.13% (w/w) and liposomes with 0.094% (w/w) KTZ were developed and fully characterized. Although the percentage of KTZ incorporated was quite low when compared with more conventional commercial formulations (2%), the results obtained showed an increased microbiologic efficacy. These results are likely associated with dissolution of KTZ in the formulations and the presence of a skin enhancer, ethoxydiglycol. Although liposomes presented higher efficacy they also showed higher skin permeation as they were retained in the deeper layers of the skin, while nanoemulsion was mainly retained in the *stratum corneum* where retention of KTZ is essential. Carrier-mediated skin transport seems to play a role on KTZ efficacy and developed innovative formulations (nanoemulsion and liposomes) showed promising results to be considered on superficial fungal infections management.

Keywords

Ketoconazole; Liposomes; Nanoemulsions; Skin permeation

Introduction

Superficial infections of skin are diseases that affect more people worldwide. Skin fungal infections are classified as superficial or cutaneous and mucocutaneous, subcutaneous, and systemic or deep [1]. Superficial fungal infections are believed to affect 20 to 25% of the world's population, and the incidence continues to increase [2-4]. These infections vary with age, gender and socio-cultural habits, affecting some areas of the body as the top layer of skin, hair, nails and mucous membranes [5]. These superficial fungal infections known as dermatomycoses are usually caused by dermatophytes, *Malassezia spp* (pityriasis versicolor and seborrhoeic dermatitis) and *Candida albicans* (candidiasis) [5]. For the treatment of these superficial infections, topical administration

of antifungals presents advantages over oral administration. After topical application, exposure to the drug is limited to the affected skin and the drug entry into the blood flow is limited or minimized, which limit the drug adverse effects [6,7]. In this type of infection, wherein the pathogenic microorganism acts inside the outermost layer of the skin, the antifungal must achieve the *Stratum Corneum* (SC) in sufficient concentration to inhibit the growth of the pathogenic agent [8].

Ketoconazole (KTZ) is a synthetic, broad-spectrum antifungal drug. It inhibits the ergoesterol synthesis (a main lipid membrane of fungi). It is poorly soluble in water (logP = 4.34) and it is a weak base with two pKa values 6.75 and 3.96. It is unstable by oxidation and hydrolysis, if not properly formulated, especially in aqueous media.

The currently marketed products may not be ideal for successful therapy. Severe liver toxicity is a known risk associated with oral KTZ treatment. In the case of topical KTZ, the amount of KTZ absorbed systemically is expected to be very low; however, an ideal KTZ formulation would promote successful skin retention. The development of alternative approaches for treatment of fungal skin infections by topical application includes new vehicles systems for antifungal agents such as colloidal systems, nanoparticles and vesicular transporters [7]. The aim of this study was the development of innovative systems applying nanoencapsulation as an alternative for topical delivery of KTZ and to elucidate the underlying mechanism of permeation enhancement. Topical formulations with increased patient compliance, shortening treatment period and promoting drug penetration into the SC, nails and hair in order to maintain a clinically relevant concentration of the drug in the affected area, are the requirements established for a new topical medicine [6]. Nanoemulsions present superior properties compared to macroemulsions: small droplet size, uniform distribution on the skin, high surface area, better occlusivity and pleasant sensation when applied topically [9]. Liposomes have been used as drug carriers for the topical treatment of dermatological diseases [10]. Liposomes can incorporate hydrophilic or hydrophobic active substances, allowing a sustained and/or controlled release as well as higher drug retention in the skin. The incorporation of KTZ in these vehicles may cause a prolonged drug delivery and minimize side effects [11].

A nanoemulsion containing 0.13% (w/w) KTZ and liposomes containing 0.094% (w/w) KTZ were developed and fully characterized. *In vitro* studies (permeation and retention) and microbiologic efficacy against *Candida albicans* were assessed.

Materials and Methods

Materials

KTZ was obtained from Laboratório Edol, Portugal. Sucrose stearate (Sisterna® SP 70-C), "Pro-liposome"- a preparation able to form liposomes spontaneously with only an addition of water (Pro-lipo™ Duo) - and ethoxydiglycol (Transcutol®CG) were kind gifts from Sisterna (Roosendaal, Netherlands), Lucas Meyer Cosmetics (Champlan, France) and Gattefossé (Saint Priest Cedex, France), respectively. KTZ-commercial cream (Nizoral 20mg/g cream, a product of Janssen Farmacêutica, Portugal) was purchased from a local pharmacy.

Hydroxypropyl methylcelullose (HPMC), Phosphotungstic Acid (PTA), propylene glycol, phosphate buffer and methanol were purchased from Sigma Aldrich (Missouri, USA). All other reagents were HPLC grade. Purified water was obtained by reverse osmosis (Millipore, Elix® 3).

Nanoemulsion Preparation

Sucrose stearate was mixed with purified water, and the KTZ dissolved in ethoxydiglycol was added, mixed and homogenized using an Ultra$^{Turrax®}$ Basic 10 at 30.000 rpm, during 10 min at room temperature (25±2°C). HPCM was added to this system and stirred using a magnetic stirrer (250 rpm) for 12 h. Qualitative and quantitative compositions are described in (Table 1).

Nanoemulsion Formulation		Liposomal Formulation	
Ingredients	**Quantitative Composition (%, w/w)**	**Ingredients**	**Quantitative Composition (%, w/w)**
Sucrose stearate	5.00	Pro-liposome preparation containing lecithin, glycerin and ethanol	3.00
Ethoxydiglycol	5.00	Ethoxydiglycol	88.00
HPMC	1.00	Purified Water for liposomes	7.00
Purified water	88.87	Purified Water residual	1.91
KTZ	0.13	KTZ	0.094

Table 1: Qualitative and quantitative composition (%, w/w) of final formulations.

Liposomes preparation

Pro-liposome preparation was mixed with KTZ previously dissolved in ethoxydiglycol, using a homogenizer (Heidolph, Essex, UK), at 800-1000 rpm. Water was added drop wise and liposome formulation was left to agitate for additional 20 min, at the same speed. Qualitative and quantitative compositions are described in (Table 1).

Carrier size quantification

Nanoemulsion droplet size distribution was measured by laser diffraction using Malvern Mastersizer 2000 equipment (Malvern Instruments Ltd, Worcestershire, UK) in conjunction with the accessory 2000 Hydro S. The parameters used for the analysis of samples were: an obscuration range of 10%-20%, water as dispersant, stirring 750 rpm without ultrasound. The sample size used was enough to get an obscuration in the selected range. The data are expressed in terms of relative volume distribution of particles in the size range class (mean ± SD, n = 5 independent batches).

The mean size of liposomes was measured by dynamic light scattering using Zetasizer Nano ZS equipment (Malvern Instruments Ltd., Worcestershire, UK). The liquid used for dispersing the particles was water, in appropriate cells. The sample (20 µL) was diluted in 2 mL of water. The data are presented as mean ± SD (n = 3 independent batches).

Transmission Electron Microscopy (TEM) analysis

Particle morphology analysis was performed by Transmission Electron Microscopy (TEM). Briefly, the suspension sample was applied to the cooper grid (Formvar/carbon, 200 mesh Cu) and dried at room temperature. After drying completely, a drop of a 1% aqueous solution of PTA was added for negative staining. Forty-five seconds later, the excess solution was wiped with filter paper and sample was analysed on a Hitachi 8100 equipment (Tokyo, Japan) with Thermo Noran light elements EDS detector and digital image acquisition (accelerating voltage of 75 kV).

Determination of pH

The pH was determined for all developed formulations using a potentiometer (Mettler Toledo®) and an electrode In Lab® Expert Pro pH (Mettler Toledo®), at 25±2°C.

HPLC method for the quantification of KTZ

High Performance Liquid Chromatography (HPLC) with UV detection was used to determine the KTZ concentration in the formulations. A chromatograph Beckman (detector, pump and software) and a Midas auto sampler with a column Lichrospher 100 RP-18 5 µm (250 mm x 4 mm) were used. The analysis was performed at room temperature. Test conditions were: isocratic mode; acetonitrile: phosphate buffer (55:45, v/v) as the mobile phase; flow rate of 1.5mL/min; injection volume of 20 µL; UV detection at 254 nm.

Incorporation Efficiency (IE)

To quantify the amount of KTZ incorporated in liposomes, non-incorporated fraction was separated from the loaded one by ultra-centrifugation. Samples were centrifuged in an ultra-centrifuge (Optima XL90, Beckman) at 180000×g for 2 h, at 15°C. After centrifugation, the supernatant was removed and the pellet was suspended in water. The quantification of the drug was performed after extraction with methanol under vigorous stirring. Samples were analyzed by UV spectrophotometry at 242.6 nm (U-2001 Spectrophotometer, Hitachi). Samples were analyzed in duplicate. IE(%) = A/(A+B) × 100 where A is the amount of KTZ in the pellet and B is amount of KTZ in the supernatant.

In vitro permeation of KTZ

The skin permeation of formulations was measured using Franz diffusion cells and full-thickness newborn pig skin obtained from a local slaughter house was used as the membrane. The skin was cut into sections (1 cm^2 permeation area). Based on a preliminary study for the search of the appropriate acceptor fluid, propylene glycol: ethanol mixture (1:1) was used as the receptor phase that assured perfect sink conditions during all experiment period. The cells were immersed in a bath system at 37±2°C under stirring (200 rpm). The formulation samples were applied (0.2±0.1 g, at infinite dose) on the skin surface in the donor compartment further sealed by Parafilm® to prevent formulation components evaporation. A commercial cream with 2% KTZ was used as the control. Samples were collected from the receptor fluid at pre-determined time points (2, 4, 8, 12 and 24 h) and replaced with an equivalent amount (200 µL) of fresh receptor medium. The KTZ content in the withdrawn samples was analyzed by HPLC. For each formulation, 6 replicates were used.

The cumulative amount of KTZ permeated (Q_t) through newborn pig skin was plotted as function of time and determined based on equation 1.

$$Q_t = \frac{V_r \times C_t + \sum_{t=0}^{t-1} V_s \times C_i}{S} \quad \text{(Eq. 1)}$$

Where, C_t is the drug concentration of the receiver solution at each sampling time, C_i the KTZ concentration of the sample applied on the donor compartment, and V_r and V_s the volumes of the receiver solution and the sample, respectively. S represents the skin surface area (1 cm^2).

The slope and intercept of the linear portion of the plot, for each formulation, were derived by regression using the Prism1, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). KTZ fluxes (J, $\mu g cm^{-2} h^{-1}$) through the skin were calculated from the slope of the linear portion of the cumulative amounts (M(t), $\mu g cm^{-2}$) permeated through the pig skin per unit surface area versus time plot. The permeability coefficients (Kp, $cm h^{-1}$) were obtained by dividing the flux (J) by the initial drug Concentration (C_0) in the donor compartment applying the Fick's 2nd law of diffusion (Equation 2), and it was assumed that under sink conditions the drug concentration in the receiver compartment is negligible compared to that in the donor compartment.

$$M(t) = KLC_0 \left[\frac{D\,t}{L^2} - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(-Dn^2 \pi^2 \frac{t}{L^2}\right) \right] \quad \text{(Eq. 2)}$$

where D is the diffusion coefficient, K is the partition coefficient between membrane and vehicle and L is the thickness of the diffusion membrane.

In vitro tape stripping

In vitro skin retention or penetration study was performed by tape stripping according to the method described by OECD Guideline [12]. The formulations (0.2±0.1 g) were spread over the newborn pig skin (1 cm^2) in contact with 4 mL of receptor phase as described before. A commercial cream with 2% KTZ was used as a control. Twenty-four hours later, the skin samples

(n=3 for each formulation tested) were rinsed to remove the excess formulation and dried with filter paper. After each skin sample had been attached and fixed on a smooth surface, the SC was removed using 20 adhesive tapes (Scotch® 3M, UK). In order to improve the reproducibility of the tape stripping technique, a cylinder (2 kg) on foam and an acrylic disk were used and the pressure was applied for 10 sec for each tape. All the tapes (excluding the first one) with the SC removed and the remaining skin (viable epidermis and dermis, ED) were cut into small pieces used for the extraction process previously validated [13]. In this extraction process, 3 mL of mobile phase and 0.5 mL of methanol were added to the SC tapes and ED pieces. Both samples were vigorously stirred for 2 min in a vertical mixer (Kinematica AG, Luzern, Switzerland), and sonicated for 20 min in order to obtain the cell lysis. The final solution was centrifuged (30000 rpm, 10 min) and the supernatant was filtered (0.2 µm) and injected into the HPLC to quantify the amount (%) of KTZ retained in these skin layers (SC + ED).

Microbiological efficacy of formulations

For this study two types of methods were used: Etest® method and Disk Diffusion Susceptibility Test, which is generally used in *in vitro* assays for the determination of microbial sensitivity. For both methods, *Candida albicans* (ATCC 10231) was inoculated on Sabouraud Dextrose Agar plate (SDA) (Thermo Scientific™ Oxoid™, UK).

After the inoculation, the Etest® tape impregnated with KTZ was added to plate and incubated at 37 °C for 24 h and then observed the Minimum Inhibitory Concentration (MIC).

Disk Diffusion Susceptibility Test was performed using standard diffusion disks and newborn pig skin disks, as a skin adapted agar diffusion test. The last one is an adaptation of the well-known *in vitro* assay to verify the effectiveness of an antibiotic product existing in topical formulations. Three standards with KTZ concentrations of 5 µg/mL (S1), 15 µg/mL (S2), and 38 µg/mL (S3) were used. These standards were selected according to the concentration of KTZ in the developed formulations: liposomal formulation (5 µg/mL) and nanoemulsion (35 µg/mL). Three tests were performed and the number of replicates of standard discs was two and the number Briefly, KTZ-impregnated small-paper discs are dropped in different zones of the culture on SDA. The diameter of the inhibition zone is proportional to the sensitivity of the microorganism and the efficacy of the antifungal agent. In the adapted test, instead of antifungal impregnated discs, newborn pig skin discs were place on cultured agar plates and 15 µL of test formulations were applied on the skin surface and incubated 24 h at 37 °C (Figure 1). Skin discs were made with 13mm biopsy punches and newborn pig skin was obtained from a local slaughter house. After incubation time, efficacy of tested formulation was determined by measuring the inhibition zone diameter from back of plate using a caliper.

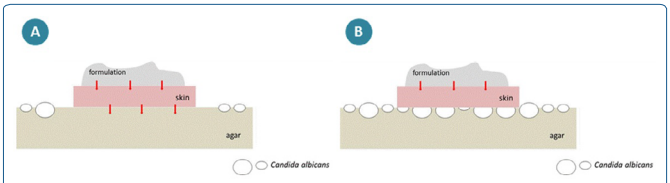

Figure 1: Skin adapted agar diffusion test cross sections.

A - Drug skin permeation (red arrows) inhibits Candida growth and an inhibition zone is observed around the skin disk,

B - Drug skin retention (red arrows) does not inhibit Candida growth. No inhibition zone is observed under the skin or around the skin disk.

Statistical analysis

The data were expressed as mean and standard deviation (mean ± SD) of experiments. Statistical evaluation of data was performed using one-way Analysis of Variance (ANOVA). An alfa error of 5% was chosen to set the significance level.

Results and Discussion

Characterization of formulations

Carrier size and incorporation efficiency are key parameters in the development of carrier-mediated skin transport. Nanoemulsions can enhance the dermal and transdermal permeation of drugs via their finely dispersed oil droplet phase and due to the enhancement of the drug thermodynamic activity favoring its partitioning into the skin [14]. Liposomes have been used with success for the incorporation of both hydrophilic and hydrophobic drugs and to promote drug skin deposition [15]. Liposomes have also been used to enhance the associated drug stability and by inference the formulation stability. Pro-liposome mixture used in this work is a preparation able to spontaneously form liposomes with the addition of water. We have used instant liposomes in an attempt to test a platform that can be easily scalable and adapted for industry.

The nanoemulsion prepared in this study showed white and homogeneous appearance while liposomal formulation presented a translucent yellow color and homogeneous aspect. The formulations exhibited different pH, about 7.16 and 5.97 for the nanoemulsion and liposomes, respectively. Both formulations indicated adequate pH for topical application.

Mean droplet size for the nanoemulsion formulation was 375±5 nm. The last step of nanoemulsion preparation included the addition of a polymer. The incorporation of HPMC increased the droplet size from 375 nm to 90 µm. As nanoemulsion had very low viscosity and viscosity plays an important role in promoting the formulation to contact to the skin, gelling agents are introduced to achieve the appropriate viscosity and to improve topical applicability. Liposome's average size was 213±3 nm and can be considered a suitable size for skin drug delivery by means of Nanocarriers [15]. This result is in accordance to the Pro-liposomes manufacturer description.

Liposomal formulation was further examined by TEM in order to get a clear image of liposomes (Figure 2). Liposomes present a multilamellar structure and the vesicles diameter appears to be homogeneous but smaller than that measured by dynamic light scattering.

Commercially available KTZ topical formulations contain, in general, 2% (w/w) KTZ. The maximum amount of KTZ possible to incorporate in the nanoemulsion was 0.13% (w/w). In the case of liposomal formulation, only 0.09% (w/w) KTZ was incorporated and the amount of KTZ associated to the liposomes (IE) was 72±1%. Other authors [16,17] developed various types of liposomes with the purpose of incorporating KTZ and maximum entrapment efficiency was found to be 54% with higher initial KTZ amount.

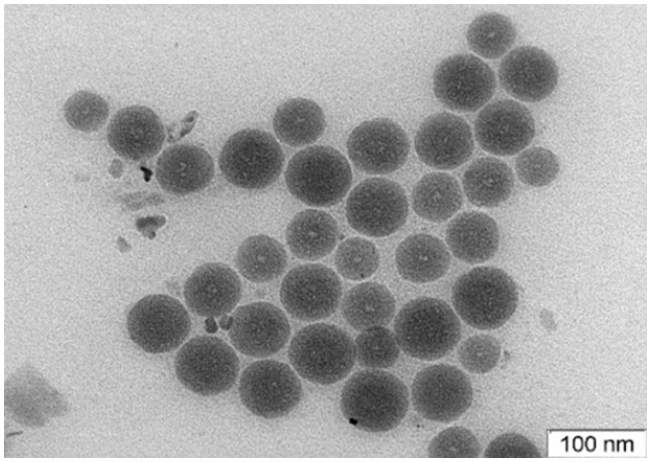

Figure 2: Microscopic appearance of KTZ-liposomes obtained by transmission electron microscopy.

In vitro permeation and skin retention of KTZ

The diffusional barrier of the skin may be modified depending on the composition of the formulation. In order to evaluate the influence of the carrier, *in vitro* permeation studies of KTZ-nanoemulsion, KTZ-liposomes and KTZ commercial cream through newborn pig skin were performed and the results are shown in Figure 3. After 24 h, nanoemulsion, liposomes and commercial cream permeated 0.20±0.22%, 0.02±0.03% and 0.19±0.07% of KTZ, respectively. ANOVA statistical analysis, showed no significant differences between nanoemulsion and the commercial formulation. These results indicate that the addition of the skin enhancer ethoxydiglycol increased drug dissolution and allowed results comparable to those of the commercial cream. The permeation parameters (Table 2) show that the innovative formulations present lower flux comparing to the commercial cream, indicating that KTZ on these formulations had more difficulties in permeating the SC. Ethoxydiglycol is a powerful solubilizing agent and is miscible with polar and nonpolar solvents with optimal solubilizing properties for a number of drugs. It is often used as a penetration enhancer due to its non-toxicity and biocompatibility with the skin [17]. In the present study, it seems that ethoxydiglycol increased the solubility of KTZ in the skin and drug partitioning into the SC. The results obtained for the lag time are in accordance with such conclusion. If a drug is retained longer in the SC it permits a more lasting action in the case of skin superficial infections. Liposome formulation presented the lower flux value comparatively to the conventional KTZ form tested. This prolonged drug release may be related to presence of several lipid bilayers present in multilamellar liposomes.

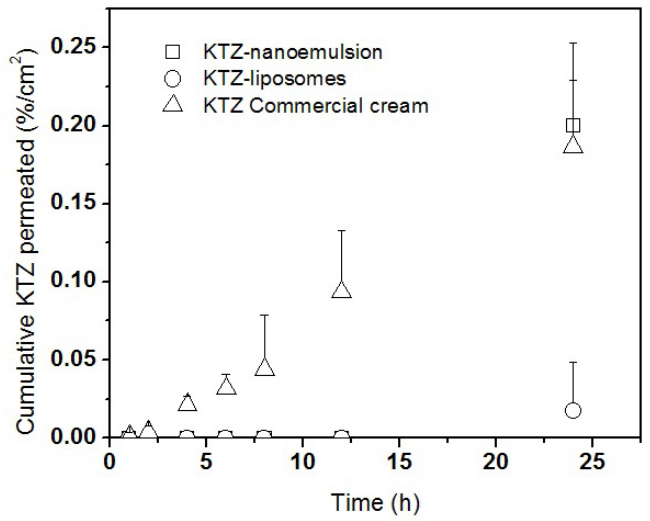

Figure 3: In vitro skin permeation of KTZ from KTZ-nanoemulsion, KTZ-liposomes and KTZ Commercial cream through newborn pig skin (mean ± SD, n=6).

	Flux (μgcm^{-2}h^{-1})	Kp (cmh^{-1})	Lag time (h)
Nanoemulsion	0.050 ± 0.050	2.9x10^{-5} ± 3.3x10^{-5}	4.75 ± 0.00
Liposome	0.002 ± 0.004	2.5x10^{-6}± 4.4x10^{-6}	4.75 ± 0.01
Commercial cream	0.570 ± 0.180	2.9x10^{-5}± 8.9 x10^{-6}	1.55 ± 0.71
Kp - Permeability coefficient			

Table 2: Permeation parameters of KTZ innovative and commercial formulations (mean ± SD, n=6).

Skin retention studies were performed 24 h after KTZ from different formulations contacted to the skin. A tape-stripping technique was employed to determine the drug retention on the SC and in the viable skin. The results of KTZ retention in the SC and in the viable layers are shown in (Figure 4). The results obtained for SC were 0.8% (4.0 μg/mL), 0.4% (1.1 μg/mL), and 0.1% (6.1 μg/mL) for nanoemulsion, liposome and commercial cream, respectively. For viable skin layers, the results were 0.3% (1.8 μg/mL), 1.0% (3.1 μg/mL), and 0.04% (2.6 μg/mL) for nanoemulsion, liposome and commercial cream, respectively.

Liposomal formulation showed higher amount of drug in the epidermis and dermis than in the SC. The other two tested formulations showed higher accumulation of KTZ in the SC. Probably, the results obtained for liposomes are associated to the high amount of ethoxydiglycol present in the composition. Conventional liposomes do not penetrate deep into the skin, but remain confined to the upper layer, the SC, with minimal

penetration into the deeper tissues [16]. However, liposomal formulation used in this work contains a high concentration of ethoxydiglycol necessary to solubilize KTZ. Manconi et al., [18] suggested a possible mechanism of vesicle-skin interaction for liposomes containing ethoxydiglycol. Nevertheless, developed formulations presented higher skin retention than commercial KTZ cream.

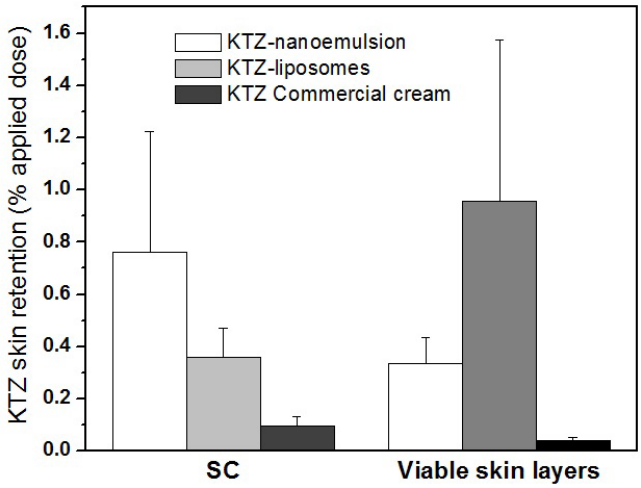

Figure 4: Percentage of KTZ retained in SC and in viable skin layers from KTZ-nanoemulsion, KTZ-liposome and KTZ Commercial cream (mean±SD, n= 3).

Microbiological efficacy of formulations

Etest® permitted to determine 0.047 µg/mL as the MIC that inhibits microbial growth of *Candida albicans*. In the paper discs, the results obtained in terms of the inhibition zone for the standards were approximately 25 mm, 28 mm and 32 mm for S1, S2, and S3, respectively (Figure 5a). For the formulations, the inhibition zone obtained was 32 mm, 35 mm and 39 mm for nanoemulsion, commercial cream and liposomes, respectively (Table 3). Concerning placebos, the yeast growth inhibition occurred only in the commercial cream placebo, probably related to the presence of a preservative (Figure 5a).

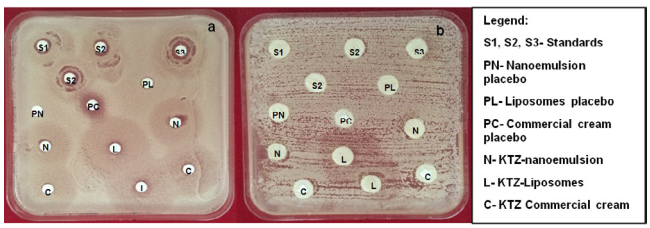

Figure 5: Inhibition halo zones using Candida albicans in (a) disc diffusion test and in (b) skin adapted agar diffusion test.

	Halo (mm)	MIC (µgml^{-1})
KTZ-nanoemulsion	32	34
KTZ-liposomes	39	67
KTZ Commercial cream	35	47

Table 3: Inhibition halo diameters and MIC of KTZ formulations against Candida albicans, as evaluated in disc diffusion test (n=2).

Given that the standard S1 has a KTZ concentration similar to that of liposomes (5 µg/mL), comparing the inhibition halos is perceptible that there was a higher diffusion from liposomal formulation. Nanoemulsion presented the same inhibition halo as S3 standard. Commercial cream, which has 421 µg/mL of KTZ could only produce a 35 mm inhibition zone, being this result very similar to that obtained for nanoemulsion with a much lower concentration of KTZ.

The skin adapted diffusion test revealed drug skin retention and no inhibition zone was detected (Figure 5b) for standards, placebos, commercial cream and nanoemulsion. However, for liposomes the growth inhibition occurred.

All formulations studied have a KTZ concentration above the MIC obtained in Etest® assay for *Candida albicans*, which may indicate that the formulations studied were effective against the yeast used. The results obtained in the disc diffusion method using paper discs, indicated that liposomes, despite being the preparation with less KTZ in their composition, was the one with a higher zone of inhibition. In the case of the nanoemulsion, this system showed a similar zone of inhibition as the commercial cream, however, there is a significant difference in the amount of KTZ, which is significantly lower in the case of nanoemulsion. Thus, innovative formulations, although having less amount of antifungal agent, showed better efficacy results. These results suggest that the use of a skin enhancer like ethoxydiglycol improved KTZ solubilization, influencing the efficacy.

For effective treatment of superficial skin infections, drug retention in the superficial skin layers is crucial. Using the skin adapted diffusion test it was possible to observe that KZT from KTZ-loaded liposomes permeated the entire skin structure producing the higher inhibition zone. The absence of an inhibition hole for KTZ-nanoemulsion is an indication that this carrier enhances KTZ skin retention. Additionally, this nanosystem was effective against *Candida albicans* using a KTZ lower concentration than that present in the commercial form. These results were obtained with pig skin and a good correlation with human skin is expected as similarities between porcine and human skin are well-described [19]. The overall results suggest that a nanoemulsion formulation of KTZ can be of actual value for improving its clinical effectiveness in topical treatment of fungal infections.

Conclusion

A nanoemulsion and a liposome formulation containing KTZ were developed and characterized. The *in vitro* permeation studies and the evaluation of the skin retention revealed their profile to be used as topical dosage forms. In both systems, the presence of a skin enhancer increased drug solubility in the formulation and improved skin drug bioavailability. Evaluation of KTZ-nanosystem antimicrobial activity presented higher microbiologic efficacy against *Candida albicans* for liposomes, and nanoemulsion was mainly

retained in the SC, promoting drug accumulation in superficial skin region. The present study demonstrated that the tested lipid nanocarriers held immense promise to target KTZ at the desire sites at clinically useful rates.

Acknowledgments

Authors gratefully acknowledge the support of aboratório Edol Produtos Farmacêuticos S.A. The work as funding, in part, by iMed.ULisboa (UID/DTP/04138/2013) from Fundação, para a Ciência e a Tecnologia (FCT), Portugal.

References

1. Hay RJ (2006) Fungal infections. Clin Dermatol 24: 201-212.
2. Brennan B, Leyden JJ (1997) Overview of topical therapy for common superficial fungal infections and the role of new topical agents. J Am Acad Dermatol 36: 3-8.
3. Ameen M (2010) Epidemiology of superficial fungal infections. Clin Dermatol 28: 197-201.
4. Havlickova B, Czaika V, Friedrich M (2008) Epidemiological trends in skin mycoses worldwide. Mycoses 4: 2-15.
5. Hay R (2013) Superficial fungal infections. Medicine 41: 716-718.
6. Kaur IP, Kakkar S (2010) Topical delivery of antifungal agents. Expert Opin Drug Deliv 7: 1303-1327.
7. Güngör S, Erdal MS, Aksu B (2013) New formulation strategies in topical antifungal therapy. Journal of Cosmetics, Dermatological Sciences and Applications 3: 56-65.
8. Pershing LK, Corlett J, Jorgensen C (1994) In vivo pharmacokinetics and pharmacodynamics of topical ketoconazole and miconazole in human stratum corneum. Antimicrob Agents Chemother 38: 90-95.
9. Yukuyama MN, Ghisleni DD, Pinto TJ, Bou-Chacra NA (2016) Nanoemulsion: process selection and application in cosmetics - a review. Int J CosmetSci 38: 13-24.
10. Patel RP, Patel H, Baria AH (2009) Formulation and evaluation of liposomes of ketoconazole. International Journal of Drug Delivery Technology 1: 16-23.
11. Patel RP, Patel HH, Baria AH (2009) Formulation and evaluation of carbopol gel containing liposomes of ketoconazole (Part-II). International Journal of Drug Delivery Technology 1: 42-45.
12. OECD (2004) OECD guideline for the testing of chemicals. Skin absorption: in vitro method. Pg: 1-8.
13. Ascenso A, Raposo S, Batista C, Cardoso P, Mendes T, et al. (2015) Development, characterization, and skin delivery studies of related ultradeformable vesicles: transfersomes, ethosomes, and transethosomes. Int J Nanomedicine 10: 5837-5851.
14. Shakeel F, Baboota S, Ahuja A, Ali J, Aqil M, et al. (2007) Nanoemulsions as vehicles for transdermal delivery of aceclofenac. AAPS PharmSciTech 8: 104.
15. El Maghraby GM, Barry BW, Williams AC (2008) Liposomes and skin: from drug delivery to model membranes. Eur J PharmSci 34: 203-222.
16. Guo F, Wang J, Ma M, Tan F, Li N (2015) Skin targeted lipid vesicles as novel nano-carrier of ketoconazole: characterization, in vitro and in vivo evaluation. J Mater Sci Mater Med 26: 175.
17. Barthélémy P, Farah N, Laforet JP (1995) Transcutol - product profile. Product Information. Gattefossé. Pg. 1-10.
18. Manconi M, Caddeo C, Sinico C, Valenti D, Mostallino MC, et al. (2011) Ex vivo skin delivery of diclofenac by transcutol containing liposomes and suggested mechanism of vesicle-skin interaction. Eur J Pharm Biopharm 78: 27-35.
19. Jung EC, Maibach HI (2015) Animal models for percutaneous absorption. J Appl Toxicol 35:1-10.