European Journal of Pharmaceutical Sciences 203 (2024) 106925

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Development and evaluation of nanostructured systems for cutaneous delivery of H_2S-releasing corticosteroids for skin inflammatory diseases

Daniel A.G. Miranda [a], Anderson R.A. Cerqueira [a], Marcelo N. Muscará [a], Beatrice Severino [b], Giuseppe Caliendo [b], Angela Corvino [b], Giorgia Andreozzi [b], Antonia Scognamiglio [b], Marlus Chorilli [c], Francesco Frecentese [b], Soraia K.P. Costa [a], Luciana B. Lopes [a,*]

[a] Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil
[b] Department of Pharmacy, School of Medicine, University of Naples Federico II, Naples 80131, Italy
[c] School of Pharmaceutical Sciences, São Paulo State University, Araraquara, SP, Brazil

ARTICLE INFO

Keywords:
Hydrogen sulfide (H_2S) donors
Psoriasis
Lamellar phase
Skin
Topical delivery

ABSTRACT

Psoriasis is an immune-mediated chronic inflammatory disease that causes major psychosocial impact. Topical corticosteroids represent the standard pharmacological treatment for mild-to-moderate disease, but their local and systemic adverse effects reinforce the need for treatment innovations. Here we developed lamellar phase-based formulations for topical delivery of a hybrid dexamethasone and hydrogen sulfide (H_2S) donor molecule (Dexa-TBZ), aiming to potentiate the effects of the glucocorticoid with H_2S. They offer the possibility to obtain precursor formulations free of water that originate lamellar phases upon water addition, preventing drug hydrolysis during storage. Two groups of formulations were developed varying the surfactants and oil phase types and content. Systems containing 20 and 70 % of water formed, respectively, bulk lamellar phase and a more fluid formulation consisting of dispersed droplets (< 1000 nm) stabilized by lamellar phase. Both presented pseudoplastic behavior. Dexa-TBZ was incorporated at 1 %, remaining stable for 8 h. Drug content decreased to ~80 % after 1 week in precursor formulations free of water, but remained stable after that. Without causing changes to the cutaneous barrier function *ex vivo* or to the histological structure of the skin *in vivo*, the formulation containing phosphatidylcholine as surfactant and 70 % of water promoted 1.8- and 2.7-fold increases in Dexa-TBZ penetration in the stratum corneum and epidermis+dermis, respectively, compared to a control solution, demonstrating their potential applicability as topical delivery systems.

1. Introduction

Psoriasis is a chronic, inflammatory, immune-mediated disorder that affects the skin of approximately 1 to 3 % of the population (Parisi et al., 2013; Szepietowski and Reich, 2016), with age, gender, geography and ethnicity influencing the prevalence of the disease (Naldi, 2013; Parisi et al., 2013). Psoriasis presents itself in many forms, and among them, plaque psoriasis is the most common, representing 85 to 90 % of cases, with 60 to 90 % of patients presenting itching and inflammatory effects on the skin (Krueger and Bowcock, 2005; Nestle et al., 2009; Szepietowski and Reich, 2016). Plaque psoriasis is characterized by the formation of dry, reddish skin with silver/white scales, and can affect any part of the body (Nestle et al., 2009).

Treatment depends on the form and severity of the disease (Mrowietz et al., 2011; Schon, 2005), with corticosteroids representing the most frequently employed pharmacological agents. However, even when topically employed, its chronic use results in both local and systemic adverse effects that include skin atrophy, telangiectasia, hypothalamus-pituitary-adrenal inhibition and Cushing's syndrome (Castela et al., 2012). Thus, combination of these drugs with other agents capable of complementing or potentiating their pharmacological effects represents an interesting strategy to maintain efficacy and reduce corticosteroid dose compared to its individual use, reducing adverse effects especially in the long-term maintenance treatment (Castela et al., 2012; Devaux et al., 2012; Lamba and Lebwohl, 2001).

Recent studies demonstrate that the endogenous mediator hydrogen sulfide (H_2S) participates in various physiological and pathophysiological processes (Alshorafa et al., 2012; Li et al., 2011; Łowicka and

* Corresponding author: Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, *Av*. Prof. Lineu Prestes 1524, São Paulo - SP, Brazil.
E-mail address: lublopes@usp.br (L.B. Lopes).

https://doi.org/10.1016/j.ejps.2024.106925
Received 21 May 2024; Received in revised form 30 August 2024; Accepted 4 October 2024
Available online 5 October 2024

Bełtowski, 2007). H_2S is a gas generated by mammalian cells by enzymatic and non-enzymatic pathways, with the highest production rates found in the brain, cardiovascular system, liver and kidneys (Łowicka and Bełtowski, 2007; Wang, 2012). Lower levels of H_2S have been detected in psoriasis compared to healthy patients, suggesting a role of this mediator in the disease (Alshorafa et al., 2012). To understand H_2S participation in inflammatory processes in a physiologically and pharmacologically relevant manner, several H_2S donors, varying in terms of release rates and triggers, have been developed (Powell et al., 2018). These molecules modulated inflammation and nociception in a concentration-dependent manner, and reduced leukocyte infiltration, edema and pruritus (Cunha et al., 2008; Li et al., 2011; Łowicka and Bełtowski, 2007; Rodrigues et al., 2017; Whiteman et al., 2010). They can be hydrolyzed and release H_2S in aqueous environments, including the serum (Hasegawa and van der Vlies, 2014). Combinations of H_2S donors and non-steroidal anti-inflammatory drugs (NSAIDs) have been demonstrated to prevent severe injury and bleeding in the small intestine induced by repeated administration of NSAIDs (Fiorucci et al., 2005). In a similar combinatorial approach, association of corticosteroids and H_2S donors increased macrophage sensitivity to the former and inhibited TNF-α and IL-8 release in cigarette smoke-exposed U937 cells (Sun et al., 2015), suggesting a potential advantage of this combination in terms of corticoid dose reduction for treatment of inflammatory disorders.

In the face of these facts, this study aimed at developing a nanostructured formulation based on liquid crystalline phases for topical delivery of hybrid H_2S-corticosteroid molecules to enable potentiation of anti-inflammatory effects without an increase in the corticosteroid dose. We focused on dexamethasone linked to an H_2S donor molecule (Dexa-TBZ, **Supplementary Figure 1**), and hypothesized that, through a careful formulation design, it would be possible to increase the hybrid molecule delivery into viable skin layers (where the disease develops) and protect the molecule against hydrolysis/degradation during storage.

Lamellar liquid crystalline phases were selected for two reasons. First, they have been demonstrated to increase the skin penetration of lipophilic compounds in a manner that depends on the aqueous phase and penetration enhancers content and type (Hosmer et al., 2013; Hosmer et al., 2011; Mojeiko et al., 2022). Given its high molecular weight (627.23 g/mol) and predicted lipophilicity (logP 4.13 ± 0.75), Dexa-TBZ is not an optimum permeant and its delivery across the stratum corneum can benefit from penetration-enhancing formulations (Moser et al., 2001). Second, these systems enable the obtainment of extemporary formulations: they can be obtained as water-free precursor formulations than can form the lamellar phase upon addition of water and minimum input of energy (agitation), which might protect Dexa-TBZ from early hydrolysis during storage. For formulation screening and selection, we assessed the phase behavior of various combinations of surfactants, oil phase components and water. After selecting 4 formulations with differences in the internal structure, content of water and types of surfactants, we compared their rheological behavior, bioadhesive properties, Dexa-TBZ incorporation and stability. Subsequently, the formulations ability to affect the cutaneous barrier function, histological organization and increase Dexa-TBZ penetration was evaluated. With this study, we aimed to demonstrate the possibility to formulate and deliver this novel hybrid molecule using nanostructured systems that are simple to prepare and capable of increasing topical delivery of drugs.

2. Material and methods

2.1. Material

Oleic acid (Synth, São Paulo, Brazil), phytosphingosine (Avanti Polar Lipids, Alabama, USA), soy phosphatidylcholine 95 % (Avanti Polar Lipids, Alabama, SUA), shea butter (Engenharia das Esências, São Paulo, Brazil), sunflower oil (Cargill, São Paulo, Brazil), polysorbate 80 (Synth, Sao Paulo, Brazil), poloxamer 407 (Sigma-Aldrich, Missouri, USA) and tricaprylin (kindly provided by Abitec Corporation, Janesville, USA) were employed to produce the formulations. Acetonitrile (Mallinckrodt Baker, NJ, USA), ethanol (Synth, São Paulo, Brazil) and methanol (Merck, Darmstadt, Germany) were employed for Dexa-TBZ extraction from the skin and quantitative analysis. The design, synthesis and characterization of glucocorticoid-H_2S donors, including Dexa-TBZ was previously described (Corvino et al., 2022).

2.2. Methods

2.2.1. Design of formulations

Two groups of formulations were designed, differing in the selected components. They will be referred to as SB (surfactant blend) and PC (phosphatidylcholine) groups based on the compounds employed as surfactants. Surfactants and chemical penetration enhancers (polysorbate 80, poloxamer 407, oleic acid and glycerol) were employed for SB group formulations, while milder components previously described to aid skin integrity (shea butter, sunflower oil and phytosphingosine) were selected for formulations of the PC group (Danby et al., 2013; Lin et al., 2017; Lopes et al., 2015; Lopes et al., 2006; Školová et al., 2017).

In the SB (surfactant blend) group of formulations, we employed a surfactant blend composed of soy phosphatidylcholine, polysorbate 80 and glycerol (2:0.5:0.5 w/w/w), an oil phase consisting of tricaprylin and oleic acid (5:1 w/w), and a solution of poloxamer 407 (1 % w/w) as aqueous phase. The surfactant blend and oil phase were combined at 8:2 - 2:8 (w/w), and the aqueous phase was added at 5–80 % to assess the relationship between composition and phase behavior, and define the proportions of components. The relationship between the type of phase formed and composition was demonstrated in pseudo-ternary phase diagrams.

In the second group (PC formulations), soy phosphatidylcholine (PC) was employed as surfactant, and a mixture of tricaprylin, shea butter, sunflower oil and phytosphingosine (49.3:15:10:0.7, w/w/w/w, respectively) was used as oil phase; purified water (without additional surfactants) was employed as aqueous phase. Based on results from SB formulations, PC and oil phase were mixed at 6:4 (w/w), and water was added at 5–80 % to assess its influence on phase behavior.

For formulation preliminary characterization and identification of isotropic and anisotropic systems, an aliquot of the formulations was visualized under a polarized light microscope (Leica Microsystems DM 2500, Heerbrugg, Germany). Lamellar and hexagonal mesophases are anisotropic and present specific microscopic structures, which include Maltese crosses or a fan like-texture, while microemulsions and cubic phases are isotropic (Hosmer et al., 2011; Hyde, 2001). Based on the phase diagrams, formulations composed of surfactant:oil phase at 6:4 (w/w) containing 20 and 70 % of water (SB-20 or SB-70, and PC-20 or PC-70, respectively) were selected for further study. Additionally, because the formulations are supposed to be extemporaneous, precursor formulations (mixtures of oil and surfactants from SB and PC groups (without water) were also produced. Table 1 details the composition of the formulations.

2.3. Characterization of selected formulations

2.3.1. Rheological properties

Rheological properties were studied using a cone and plate type rheometer (Brookfield, Middleboro, USA) with shear rate up to 500 1/s under at 25 °C. Data were fitted using the power law model to classify the behavior of the fluid (Hosmer et al., 2013): $\tau = ky^n$; where τ = shear stress, k = consistency index, y = shear rate and n = fluid behavior index. In this model, $n=1$ indicates a Newtonian fluid, n>1 indicates a dilating fluid and n <1 indicates a pseudoplastic fluid (Hosmer et al., 2013; Gabbanelli et al., 2005) The analysis was performed using the software Origin 2018 (OriginLab Corporation, Wellesley Hills, USA).

Table 1
Composition of the selected SB (surfactant blend) and PC (phosphatidylcholine) formulations.

Formulations	Oil phase	Surfactant	Aqueous phase
Group SB	Tricaprylin: oleic acid (5:1, w/w)	PC: polysorbate 80: glycerol (2:0.5:0.5, w/w/w)	Poloxamer solution (1 %, w/w)
Precursor SB	60 %	40 %	0 %
SB-20	48 %	32 %	20 %
SB-70	18 %	12 %	70 %
Group PC	Tricaprylin:shea butter: sunflower oil: phytosphingosine (49.3:15:10:0.7, w/w/w/w)	PC	water
Precursor PC	60 %	40 %	0 %
PC-20	48 %	32 %	20 %
PC-70	18 %	12 %	70 %

2.3.2. Droplet size and zeta potential

Because the formulations SB-70 and PC-70 displayed small droplets under the microscope, average hydrodynamic diameter and zeta potential were assessed using a Zetasizer NanoZS90 equipment (Malvern Panalytic, Malvern, UK) after dilutions of 1:2000 and 1:10,000 (v/v) in deionized water.

2.3.3. Assessment of in vitro bioadhesive properties

The influence of composition on the bioadhesive properties of the formulations was assessed with a TA-XTplus texture analyzer (Stable Micro Systems, Surrey, UK) and porcine ear skin. Using a rubber ring, skin sections were attached to the lower end of a cylindrical probe with the *stratum corneum* facing outside for contact with formulations (Carvalho et al., 2019; Costa-Fernandez et al., 2021). The probe was lowered at a constant speed (1 mm/s), maintained in contact with the formulation surface for 60 s, and subsequently raised (0.5 mm/s). The bioadhesive force was measured as the maximum detachment force or the resistance to probe withdrawal. This procedure was repeated 6 times for each sample, and three samples of each formulation were assessed. Because PC-70 and SB-70 have low viscosity and high aqueous content, the force necessary to detach water was measured for comparison.

2.4. Incorporation of Dexa-TBZ and its influence on phase behavior

Dexa-TBZ was incorporated in the formulations depicted in Table 1 to obtain final concentrations of 0.5, 1 and 2 %. The hybrid molecule was initially added to the surfactant:oil phase mixture (referred to as precursor formulations, Table 1), which was then bath sonicated (model Q3350, Quimis, Diadema, Brazil) for 4 cycles of 5 minutes (with intervals of 1 min between the cycles) to dissolve Dexa-TBZ prior to aqueous phase addition (at 20 or 70 %). Visual inspection and polarized light microscopy were employed to assess formulation homogeneity, phase separation and drug precipitation. The formulations were also subjected to assessment by optical and polarized light microscopy to verify phase behavior and presence of non-dissolved Dexa-TBZ particles immediately after aqueous phase addition and after 24 -72 h.

2.5. Assessment of Dexa-TBZ physicochemical stability in the formulations

Because the formulations were conceived as extemporaneous systems, Dexa-TBZ content in the formulations containing 20 or 70 % of water was assessed for 24 h to ensure its stability in contact with water during the skin penetration assay. In addition, drug content was assessed for 28 days in precursor formulations free of water (see Table 1 for compositions). The formulations containing 1 % of the drug were prepared in triplicate and kept in closed vials protected from light at room temperature (maintained by air conditioning set at 25 °C). At 0, 2, 4 and 8 h, 7, 14, 21 and 28 days post-preparation, aliquots of the formulations were withdrawn and diluted with methanol to obtain a theoretical Dexa-TBZ concentration of 50 μg/mL. Quantification of the molecule was performed by HPLC (see section *Dexa-TBZ quantification method*).

2.6. Effect of the formulations on cutaneous permeability and skin penetration

2.6.1. Skin and diffusion cells

Porcine ear skin was employed as model tissue to assess the drug penetration and changes in transepidermal water loss mediated by the selected formulations. The ears were obtained from a local slaughterhouse and rinsed with water prior to the excision of the skin from the cartilage. After removal of excess subcutaneous tissue and hair, skin sections were wrapped in aluminum foil and frozen at -80 °C for up to 6 months.

Skin sections were mounted on the top of Franz diffusion cell (area of 1.77 cm^2, receptor phase volume of 7 mL, Hanson Research, Chatsworth, USA) and the receptor phase was maintained at 37° C under stirring (350 rpm) (Carvalho et al., 2019; Pepe et al., 2013). The receptor phase composition varied with the experiment: it was composed of phosphate-buffered saline (PBS) in experiments designed to assess changes on transepidermal water loss (in which unloaded formulations were employed), while ethanol (20 % v/v) was added for assessment of Dexa-TBZ penetration and permeation to aid its dissolution in the receptor phase (to >0.9 μg/mL), and avoid to artificially hinder its permeation across the skin (Pepe et al., 2013; Pepe et al., 2012).

2.6.2. Transepidermal water loss

Changes in the transepidermal water loss (TEWL) were measured as an index of barrier function disruption using a closed-chamber evaporimeter (Vapometer, Delfin, Kuopio, Finland) (Passos et al., 2020). The skin was mounted on the Franz diffusion cell and equilibrated for 10 min before basal TEWL was measured. The skin sections were then treated with the selected unloaded formulations (150 mg) for 8 h. After treatment, formulations were carefully removed using tissue paper, the skin was rinsed, reassembled in the Franz diffusion cell, and equilibrated for 10 min before TEWL was measured (Carvalho et al., 2017; Thomas et al., 2014). The results were expressed as the difference between TEWL at the end of treatment (8 h, after formulation removal) and before treatment (basal) (Thomas et al., 2014). PBS and water were used as controls.

2.6.3. Evaluation of cutaneous penetration of Dexa-TBZ

For assessment of in Vitro Dexa-TBZ penetration, skin sections were assembled in the diffusion cells and treated with 100 mg of formulations containing Dexa-TBZ (at 1 %) or a Dexa-TBZ solution in sunflower oil (control) for 8 h. At the end of the experimental period, the skin was subjected to tape stripping for separation of the stratum corneum (SC) (Carvalho et al., 2019). Fifteen pieces of adhesive tape were used; the first piece was discarded and the others were placed in a conical tube with 4 mL of methanol (employed as extracting solvent). The remainder of the skin (epidermis without SC + dermis, referred to as ED) was cut into small pieces and placed in a tube with 2 mL of methanol for extraction of Dexa-TBZ. Samples were vortexed for 30 s and bath sonicated (model Q3350, Quimis, Diadema, Brazil) for 15 min. Then, homogenates and receptor phase were filtered on a PTFE membrane with 0.45 μm pore size prior to Dexa-TBZ quantification by HPLC. The drug in the skin layers (SC and ED) was used as an index of retention or skin localization, while its content in the receptor phase was used as an index of transdermal or percutaneous permeation.

To verify whether Dexa-TBZ was stable in the aqueous receptor phase (and thus, could be quantified in this compartment), a PBS-ethanol (20 % of ethanol) solution of the molecule (20 μg/mL) was

prepared fresh and incubated at the receptor compartment for 8 h at 37 °C under stirring (350 rpm). Aliquots were withdrawn for 1–8 h, and subjected to HPLC analysis for quantification of the hybrid molecule.

2.7. Dexa-TBZ quantification method

The quantification method was developed using parameters previously established by Español Mariño (Español Mariño, 2015) for dexamethasone. Separation was performed using a C18 column (Synergi 4 µm 150 × 4.6 mm) with acetonitrile:water (55:45 v/v) at a flow rate of 1.0 mL/min as mobile phase, sample injection volume of 20 µL and wavelength of detection set at 250 nm. The equipment consisted of a Shimadzu apparatus equipped with degasser (DGU-14A), pumps (LC-10AD VP), detector (SPD-10 A VP), injector (SIL-10AD VP) and column oven (CTO-10AS VP). The concentrations used for the linearity assay were 0.35 - 50 µg/mL, and the quantification limit was 0.35 µg/mL.

2.8. Formulation effects on the integrity of murine dorsal skin

Balb/c mice were housed in the Department of Pharmacology animal facility with free access to food and tap water. The animal room was kept under a 12:12 h light–dark cycle (lights on at 7:00 am), and temperature was maintained between 22 and 23 °C. The protocol was conducted in accordance with the guidelines from the Brazilian Council for Control of Animal Experimentation (CONCEA), and approved by the Animal Care and Use Committee at the Institute of Biomedical Sciences of the University of São Paulo (protocol number 67/2017, São Paulo, Brazil).

The hair from the dorsal skin of the 6–7 weeks old anaesthetized (isoflurane in O_2; 3:97) naïve mice was removed with the aid of a depilatory cream. After 72 h, mice were randomly divided into 5 groups of 4–5 animals each, according to the treatment approach: 1- control

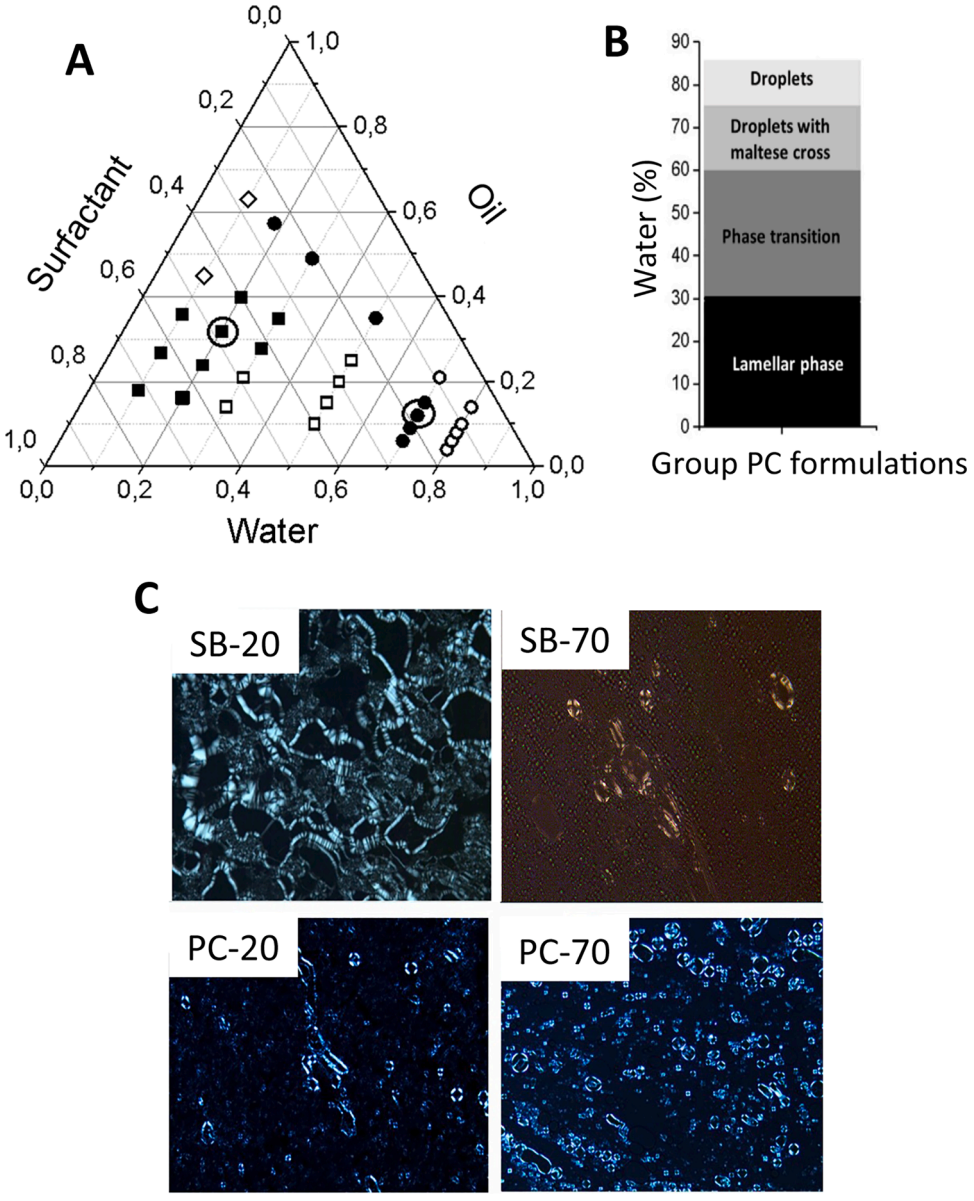

Fig. 1. Development and characterization of formulations. A: Pseudoternary phase diagrams obtained at room temperature demonstrating the relationship between composition of SB group formulations and phase behavior: black squares: lamellar phase, white diamond: fluid systems; white squares: mixtures; black circles: emulsified systems with lamellar phases; white circles: emulsions. The circles indicate the composition of SB-20 and SB-70. B: abbreviated diagram depicting the phase behavior of PC group formulations when the surfactant and oil phase was mixed at 6:4 (w/w) and titrated with various amounts of water. C: Polarized light microscopy images of selected PC and SB formulations containing 20 or 70 % of water.

(vaseline), 2-formulation SB-20, 3- formulation SB-70, 4- formulation PC-20 or 5- formulation PC-70, at a dose of 65 mg. The formulations were applied once a day for four consecutive days (day 1 to 4). On the 5th day, the animals were submitted to euthanasia under anesthetic overdose following exsanguination, the animal skin was removed and fixed in formaldehyde (4 %) for 24 h at 8 °C, followed by dehydration sessions in 70 %, 95 % and 100 % ethanol and xylol (I, II and III, 40 min), and then included in Paraplast®. Five-micrometer sections were obtained with the aid of a microtome (3 sections separated by a distance of 10 cuts) and mounted on poly-L-lysine coated slides (3 slides/mouse). The slides were stained with Hematoxylin and Eosin (H&E) and assembled for visualization under optical microscopy (Leica Microsystems DM 2500, Heerbrugg, Germany equipped with LAS V4.6 program for image capture) (Ekundi-Valentim et al., 2010). Three fields per section were photographed and analyzed using the Image Pro Plus program (MediaCybernetics Co., MD, USA) to determine the thickness of the epidermis.

2.9. Statistical analyses

The results are reported as means ± standard deviation. Data were statistically analyzed using ANOVA followed by Tukey post-test (GraphPad Prism software). Values were considered significantly different when $p < 0.05$.

3. Results

3.1. Design of formulations

Pseudo-ternary phase diagrams were constructed to demonstrate the relationship of the components of formulations from the SB group, their content and type of phases formed (Fig. 1A). Mixtures containing surfactant:oil phase at 5:5 and 3:7 (w/w) and 10 % of the aqueous phase formed fluid, single phase and isotropic system (white diamonds), which are compatible with microemulsions. Increasing the ratio of surfactant while maintaining the aqueous content low (10 %) originated lamellar phases. Using surfactant:oil phase ratios up to 6:4, the lamellar phase could be observed with water at 30 % or less. As the amount of water increased, a change in the structure of the system was observed: at 70 %, a turbid system in which very small droplets could be observed in the presence of Maltese crosses was produced, indicating an emulsified system containing the lamellar phase. Addition of water at 80 % precluded the formation of lamellar phases, and only a milky, emulsified system with large droplets was observed. Based on these results, the 6:4 (w/w) surfactant:oil phase ratio was selected to study the phase behavior of the PC group formulations.

As can be observed in Fig. 1**B**, the types of systems formed and phase transitions in the PC group were very similar to that observed for SB formulations, suggesting that the removal of polysorbate 80 from the surfactant mixture and the inclusion of Shea butter and phytosphingosine did not hinder the formation of the lamellar phase with water up to 70 %.

Based on these results, two formulations containing 20 or 70 % of water from each group were selected for further study: SB-20, SB-70, PC-20 and PC-70. Their composition is depicted in Table 1, while representative images of their structure under polarized light are represented in Fig. 1**C**. They were selected for two reasons. First, they differed in terms of components employed, and second, they varied in the terms of aqueous content, and thus, structural organization: while bulk lamellar phases were observed at 20 % of water, an emulsified system containing very small droplets that coexist with lamellar phases were obtained with 70 % of water. Therefore, their comparison enabled an assessment of the influence of composition, aqueous content and structural organization on the stability and penetration of the Dexa-TBZ, as well as their effects on the skin barrier and integrity.

3.2. Characterization of selected formulations

Only SB-70 and PC-70 were evaluated for size and zeta potential as they contained very small droplets under the microscope. Average hydrodynamic diameter, PDI and zeta potential are represented in Table 2**,** while a representative size distribution pattern is presented in **Supplementary Figure 2.** The results demonstrate that the formulations have two populations of particles, both with average diameter within the nanoscale. This polydispersity is also reflected in the PDI values above 0.3. This is not unexpected since high-energy methods for droplet size reduction were not employed (Mojeiko et al., 2022). Nevertheless, these results indicate that the selected combination of components enabled formation of nanostructured systems, in which dispersed droplets seem to be stabilized by or dispersed within the lamellar phase formed in the continuous phase of the emulsified system. Zeta potential was in the range of -10.6 to -20.0 mV. The less negative value for PC-70 might be justified by the presence of phytosphingosine, a cationic ceramide and/or absence of oleic acid.

All four formulations had similar rheological behavior: non-linear relationships between shear rate and shear stress and a decrease in viscosity with increases in the rate of shear were observed, which is consistent with pseudoplastic behavior (Fig. 2). This behavior can be ratified by the fluid behavior index (n), calculated using power law, which varied from 0.710 (for PC-70) to 0.332 (for PC-20). Formulations from the PC group were more viscous, most likely because of shea butter, which is solid at room temperature. As expected, increasing aqueous content to 70 % reduced the viscosity of formulations of both groups.

The influence of components and aqueous content on the bioadhesive properties of the formulations was evaluated with a tensile test to determine the maximum force to detach the formulation from the skin. As expected, the force necessary to detach formulations containing 20 % of water was higher compared to those containing 70 % (Fig. 2**B**), which is consistent with their higher viscosity. Among all formulations investigated, the highest detachment force was observed for PC-20 (2.2-fold higher than water, $p < 0.001$). A comparison between the formulations containing 70 % of water demonstrated that only the detachment force for PC-70 was significantly higher than that required for water (1.45-fold, $p < 0.05$). These results demonstrate that, comparing formulations with the same structure and amount of water, those from the PC group displayed more pronounced effects.

3.3. Incorporation and stability of the Dexa-TBZ in the formulation before and after incorporation of water

Because the formulations were designed as extemporaneous systems, we assessed the maximum amount of drug that could be incorporated in the precursor formulations, and whether water addition at 20 and 70 % would induce drug precipitation or degradation. The only difference in composition between the precursor formulations and the others was the absence of water. The maximum concentration of Dexa-TBZ that could be dissolved in all formulations was 1 %; at 2 % it precipitated after 24 h when the aqueous content was 70 %. At 1 %, the drug did not hinder liquid crystalline phase formation and did not induce phase transformation as demonstrated in Fig. 3**A**.

Having determined the maximum amount of Dexa-TBZ that could be incorporated, we next assessed its physicochemical stability for 30 days

Table 2
Size and polydispersity index (PDI) of selected formulations.

Formulations	Peak 1 (nm)	Peak 2 (nm)	PDI	Zeta potential (mV)	pH
SB-70	68.6 ± 30,2	371.4 ± 96.6	0.48 ± 0,07	- 20.0 ± 1.2	5
PC-70	183.4 ± 2.5	595.7 ± 70.2	0.32 ± 0.01	- 10.6 ± 1.5	6

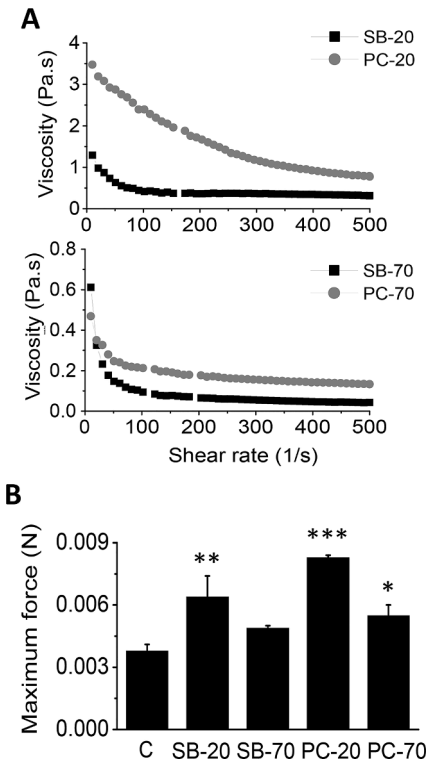

Fig. 2. Characterization of the viscosity and detachment force (as an index of bioadhesion) of the selected formulations from the PC or SB groups containing 20 or 70 % of water. A. Viscosity changes as a function of shear rate at 25 °C. B: Maximum detachment force assessed at 25 °C; *p < 0.05, **p < 0.01 and *** p < 0.001 compared to water.

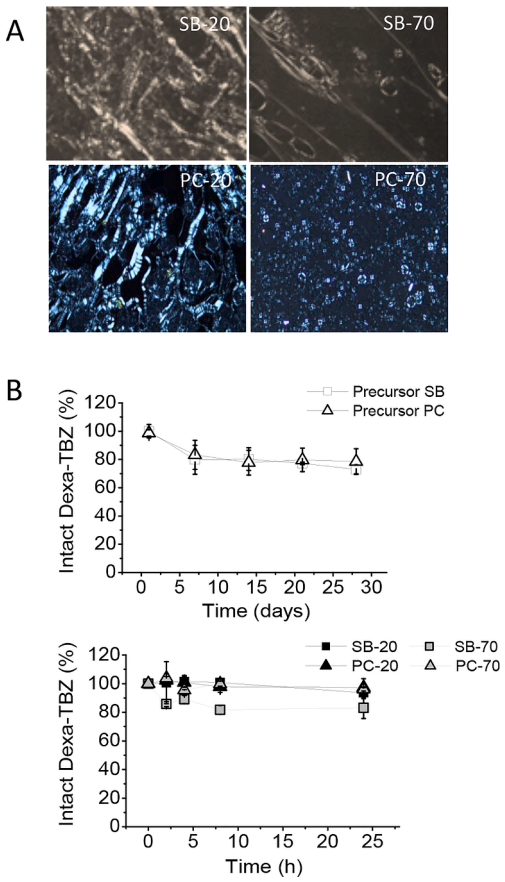

Fig. 3. Influence of Dexa-TBZ incorporation on the phase behavior of the selected formulations and variations on its content as a function of time. A: Polarized light microscopy images showing the lamellar structure of the selected PC or SB formulations containing 20 or 70 % of water and Dexa-TBZ. B. Variations in the content of Dexa-TBZ as a function of time. in the precursor PC and SB formulations (without water, top) and in the formulations containing 20 or 70 % of water (bottom).

in the precursor formulations. As can be observed in Fig. 3**B (top panel)**, the content of Dexa-TBZ dropped approximately 20 % during the first week, but remained stable for the next 21 days, without significant differences between the precursors PC and SB formulations.

Subsequently, Dexa-TBZ content was assessed in the formulations containing 20 % (SB or PC-20) or 70 % (SB or PC-70) of water for 24 h to ensure that the drug would remain intact during a daily application and during the penetration assay conducted here. As can be observed in Fig. 3**B (bottom panel)**, no pronounced changes on drug content were observed in PC-20, PC-70 and SB-20 formulations, suggesting that the drug is stable during this period of time and that the percentage of water, and consequently the internal structure, did not influence drug stability. At 8 h and longer, degradation was the highest in SB-70 (~80 % remaining), although not significantly different at 24 h.

3.4. Effect of the formulations on the cutaneous barrier and skin penetration

3.4.1. Evaluation of cutaneous penetration of the Dexa-TBZ

We assessed the concentration of Dexa-TBZ retained in the SC and epidermis without SC+dermis, which was also referred to as viable skin layers even though, in the case of the model employed here, it is not expected to be viable (Fig. 4). Formulations from the SB group failed to increase drug retention in SC or viable skin layers compared to the control solution. On the other hand, formulations from the PC group promoted increases in Dexa-TBZ retention in the SC (1.8 – 2.0-fold) and ED (2.3 – 2.7 fold) compared to the control, with PC-70 displaying more pronounced effects on the penetration into ED layers. These results demonstrate that PC formulations are superior in terms of promoting the cutaneous penetration of the drug into skin layers beyond the stratum corneum.

Dexa-TBZ concentration in the receptor phase was below the limit of detection of the method, which could result from either low transdermal delivery or its instability in PBS. To verify whether Dexa-TBZ is stable in the aqueous receptor phase, an aqueous solution of the molecule (20 μg/mL) was prepared fresh and incubated at the receptor compartment for 8 h (Fig. 4**E**). After incubation, only 9 % of the molecule remained, demonstrating that the lack of drug in the receptor phase does not necessarily mean that no permeation occurred, but degradation might have reduced drug concentration to levels below the detection limit of the quantification method.

3.4.2. Evaluation of transepidermal water loss

To establish relationships between formulation composition, penetration enhancement and changes in the barrier function, we evaluated the effects of the formulations on transepidermal water loss (TEWL). Only SB-20 produced a significant (4.5-fold) increase in TEWL compared to the controls (water and PBS) and to the other formulations (Fig. 5). There was a tendency of formulation PC-70 to decrease TEWL compared to the controls, but the effect was not significant.

3.5. Assessment of the effect of selected formulations on the integrity and histological characteristics of murine dorsal skin

Changes in the epidermis thickness and histological characteristics of the skin were evaluated *in vivo* after treatment during 5 days. This assay enabled us to assess whether multiple applications of the formulation, which would be necessary for treatment, could lead to pronounced

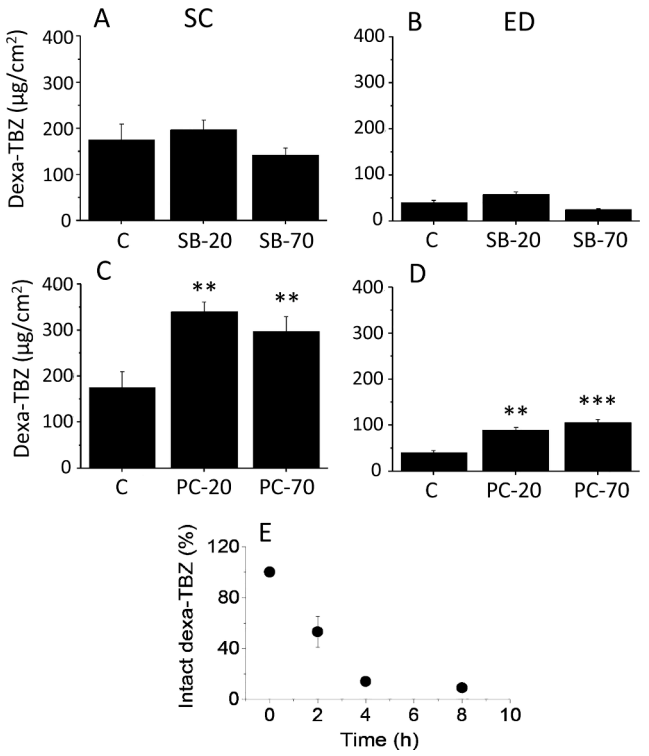

Fig. 4. Skin penetration of Dexa-TBZ after 8 h of treatment with SB-20, SB-70, PC-20 and PC-70. A and B: penetration of Dexa-TBZ mediated by SB formulations compared to a control solution in the stratum corneum (SC, A) and epidermis without stratum corneum+dermis (ED, B). C and D: penetration of Dexa-TBZ mediated by PC formulations compared to a control solution in SC (C) and ED (D). **$p < 0.005$ and ***$p < 0.001$ compared to control. E: variations in the content of Dexa-TBZ added to the receptor phase during 8 h in the same conditions as the skin penetration experiment.

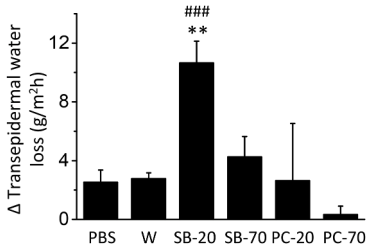

Fig. 5. Effect of cutaneous treatment for 8 h with the selected formulations on variations of transepidermal water loss (ΔTEWL) compared to the control vehicles (PBS and water, W). $^{\#\#\#\#}p < 0.001$ compared to water, **$p < 0.01$ compared to SB-70.

tissue damage. PC-70 was the only formulation that did not induce acanthosis or any other changes to the skin architecture (Fig. 6). Compared to the control, both SB-20 and SB-70 increased more pronouncedly (~1.7-fold) the epidermis thickness than PC-20 (~1.5-fold). These results demonstrate that formulations from PC group can be considered safer for topical use compared to SB formulation, which is consistent with the choice of components described to preserve skin integrity.

4. Discussion

The phase diagrams demonstrated the diversity of mesophases that can be obtained varying the composition and aqueous content of SB and PC formulations. A very similar behavior in terms of phase formation as a function of water content was observed for PC and SB formulations when the surfactant:oil phase ratio was 6:4 (w/w), suggesting that the inclusion of shea butter and phytosphingosine to aid the integrity of the cutaneous barrier did not disrupt phase behavior. This is corroborated by previous studies demonstrating that formulations containing phytosphingosine and phytoceramides derived from sunflower oil and shea butter enable formation of lamellar phases (Oh et al., 2017). The presence of PC in both groups of formulations might be responsible for the similar phase behavior, as its ability to form lamellar phases upon self-aggregation in water depending on its concentration and of other components is supported by the extensive literature employing this phospholipid as structure-forming agent (Apolinário et al., 2021; Gosenca et al., 2013; Mojeiko et al., 2022; Salata et al., 2021).

As expected, the aqueous content affected the structural organization: while bulk lamellar phases were observed at 20 % of water, an emulsified system containing droplets (in the nanorange) that coexist with lamellar phases were obtained with 70 % of water. Previous studies support the possibility of obtaining hybrid systems in which the lamellar phase is formed in the aqueous continuous phase of emulsified systems by self-aggregation of phosphatidylcholine (Alshorafa et al., 2012; Devaux et al., 2012). Our group, for example, has previously demonstrated that PC can be combined with shea butter, oleic and caprylic acid to obtain nanoemulsions stabilized with lamellar liquid crystalline phases similar to SB-70 and PC-70, except that they were able to disrupt the barrier function of the skin, increasing TEWL in a significant manner as a function of the concentration of the fatty acid (Mojeiko et al., 2022). As a result of this unique organization, PC-70 and SB-70 present several advantages for topical delivery: they contain (i) droplets in the nanorange, which may improve drug transfer to the skin and penetration, (ii) high content of water, which has been associated with lower irritation potential, and (iii) additional regions for incorporation of lipophilic compounds like Dexa-TBZ despite its high content of water due to the formation of the lamellar structure in the continuous phase (Apolinário et al., 2022; Pepe et al., 2013; Zhang and Michniak-Kohn, 2011).

In spite of the similar organization and phases formed, there were differences in specific characteristics of the formulations. For example, PC-based formulations were more viscous compared to their SB counterpart containing the same aqueous content. A possible reason is the presence of shea butter, which is solid at room temperature. Although both SB-70 and PC-70 contained droplets in the nanorange, their diameter was influenced by the choice of components. Smaller droplets (1.6–2.7-fold) were observed in SB-70 in comparison to PC-70, which can be justified by the ability of polysorbate 80 and glycerol to lower surface tension and reduce droplet size (Shah et al., 2018). Additionally, compared to water, the bioadhesive properties of PC-based formulations (measured as the force necessary to detach the formulations form the cutaneous surface) were more pronounced, especially PC-20, despite the absence of poloxamer. It is worth mentioning that the bioadhesive properties of poloxamer-based formulations have been described at higher concentrations of the polymer than employed here (Fabri et al., 2011). Although bioadhesive properties may derive from multiple mechanisms that are often independent on viscosity, none of the components of the PC formulations have been recognized as mucoadhesive. The exception might be phytosphingosine, since cationic components can bind non-specifically to negatively charged surfaces (Park and Robinson, 1984). Nevertheless, it is reasonable to suggest that the bulk viscosity of the formulation might have affected the detachment of the formulation in the absence of specific adhesive components.

Despite the smaller droplets and presence of oleic acid (a known penetration enhancer) in SB formulations, those from the PC group had a more pronounced penetration-enhancing effect. The reasons for this are not completely clear, but we can speculate based on concepts learned from liposomes. Penetration of liposomes into/across the dermis has been suggested by earlier studies, but this topic and the relationship with composition has been extensively debated (Apolinário et al., 2021; du Plessis et al., 1994; Elsayed et al., 2007; Foldvari et al., 1990; Elsayed et al., 2007). Observations that classic PC liposomes might break and

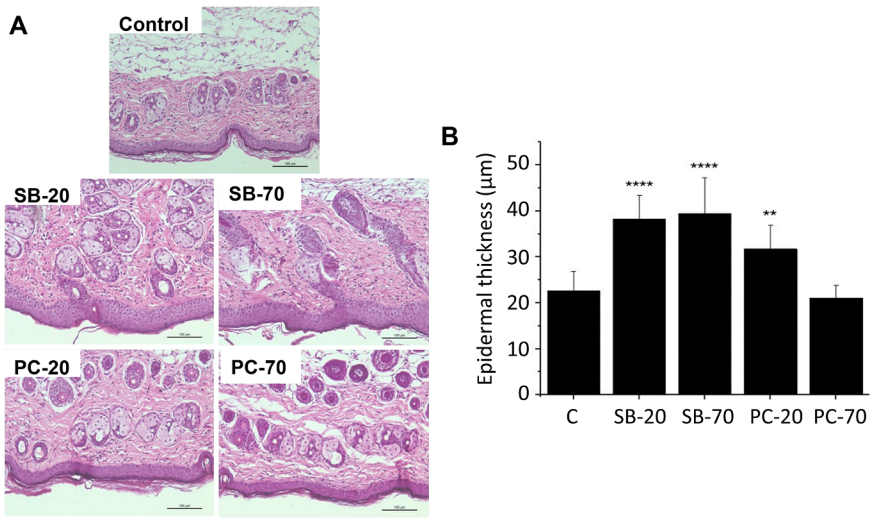

Fig. 6. Effect of selected formulations on the integrity and histological characteristics of mice dorsal skin after treatment during 5 days. A: Cross-section of mice dorsal skin after treatment with the formulations to demonstrate changes on the epidermis thickness and tissue integrity. B: Changes on the epidermal thickness. ****$p < 0.001$ and **$p < 0.01$ compared to the control (vaseline). Bar: 100 µm.

remain largely confined within cutaneous superficial layers (Cevc et al., 2002) help to justify Dexa-TBZ increased localization in SC when PC-based formulations were employed. Other studies also suggested the ability of liposome lipids to adhere to the cutaneous surface, and destabilize, fuse or mix with the lipid matrix, acting as penetration enhancers mainly for drugs encapsulated or concomitantly applied (du Plessis et al., 1994; Foldvari et al., 1990; Kirjavainen et al., 1996). Lecithin has been demonstrated to increase skin permeability by fluidization, but skin barrier destabilization was demonstrated to depend on the lipid composition of liposomes, with addition of fusogenic lipids (like DOPE) contributing more pronouncedly to the effect (Kirjavainen et al., 1996; Paolino et al., 2002). Our results did not indicate that PC-20 and PC-70 induced extensive barrier function disruption as no increase on TEWL was observed, although longer applications of PC-20 might affect the epidermis as demonstrated *in vivo*. Changes on TEWL are useful to assess whether variations in composition affects the formulation's ability to disrupt the cutaneous barrier function as a possible mechanism of penetration enhancement (Elmahjoubi et al., 2009; Thomas et al., 2014). The lack of changes on this parameter compared to the control solution (contrary to SB-20) suggests that changes on cutaneous barrier may be subtler (and not detectable by the technique) at 8 h and/or that other mechanisms might contribute to the penetration-enhancing effect of PC-based formulations (Fini et al., 2008).

Another hypothesis for the superiority of PC- over SB-based formulations relates to differences of bioadhesive properties and viscosity. Increasing viscosity may increase residence time and promote stronger interactions between formulation and skin, benefiting penetration (Biruss and Valenta, 2008). For example, silicon dioxide and Pemulen TR1 increased viscosity of microemulsions and improved progesterone permeation (Biruss and Valenta, 2008). Increases in bioadhesive properties were also previously related to an increased skin penetration (Costa-Fernandez et al., 2021). It is also worth mentioning that, contrary to previous reports, we did not observe a pronounced difference in the penetration of Dexa-TBZ as the aqueous content increased in both formulation groups (Cichewicz et al., 2013; Pepe et al., 2013). A possible reason for this finding is the presence of the lamellar phase formed in the continuous phase of the emulsified system, which might delay drug diffusion and release even in the formulation containing 70 % of water (Otto et al., 2009; Santos et al., 2020).

Finally, since the surfactant blend is distinct in SB and PC-based formulations, it is relevant to consider its contribution to the formulation performance. The influence of the surfactant type on drug release and, consequently, skin penetration, is complex and may affect drug solubility, diffusion coefficient and formulation properties (Montenegro et al., 2011; Nornoo et al., 2009). Very distinct results have been reported depending on the type of drug and delivery system. In microemulsions, one of the most studied types of delivery system when it comes to surfactant effects, octylmethoxycinnamate release was influenced by the surfactant lipophilicity and ability to interfere with the interfacial layer packing (Montenegro et al., 2011). Surfactants that increased drug solubility and reduced formulation viscosity were reported to increase aceclofenac release (Todosijevic et al., 2015). We did not observe differences in Dexa-TBZ solubility comparing SB and PC-based formulations, but drug content increased in two-fold increments. Lower increments might be useful to detect small differences in solubility. In addition, PC-based formulations were more viscous and more efficient at improving drug penetration. Maulvi et al. (2017)) demonstrated that an increase in the length of the acyl chain of the surfactant led to a higher amount and longer duration of cyclosporine release. Despite the presence of PC in both formulations, those from the SB group also contain glycerol, which is shorter. Nevertheless, because the surfactants can also be released from the formulation and interact with the skin (affecting its permeability) (Hathout et al., 2010), their influence on drug penetration goes beyond the effects on drug release, making it difficult to establish general guidelines on how they can be changed to match a desired pharmacokinetic profile.

Considering that the hybrid molecule should release H_2S in aqueous environments, its content in the precursor formulations was preliminarily assessed for 28 days. Even though it contained no water, we observed a ~20 % reduction on the content of the hybrid molecule within the first week, which subsequently remained unchanged. Although the mechanism was not investigated, this result suggests some initial degradation and H_2S release in the presence of surfactants/oil phase, which has been described for other hybrid molecules and prodrugs. The degradation of penethamate, an ester prodrug of benzyl penicillin, was rapid in oily solutions and dependent on moisture content, with less than 10 % remaining after 7–15 days (Jain et al., 2015). Thumma et al. (2008) reported that degradation of an ester prodrug of Δ^9-Tetrahydrocannabinol in polyethylene oxide (PEO) polymeric matrices increased in the presence of selected pH modifier and antioxidants. These reports support the relevance of controlling moisture in the formulations and flasks, which was not accounted for in the present study, as well as the importance of incorporating additives to increase

stability.

In formulations containing water, the degradation of the hybrid molecules varied slightly with the formulation composition within 24 h, which is in accordance with a previous study reporting that degradation of a lipoic acid-tocopherol co-drug within the first hours was independent on the aqueous fraction of microemulsions (Thomas et al., 2014). At 8 h and longer, degradation was higher in SB-70, although not significantly different at 24 h. This result suggests that Dexa-TBZ should remain intact in PC-20, SB-20 and PC-70 during a daily application.

Although we did not investigate systemic toxicity in this study, formulation-induced alterations on the integrity and histological characteristics of murine dorsal skin were assessed, and can provide insights on the local irritation potential of the formulations. Compared to the control, SB-based formulations increased more pronouncedly the epidermis thickness than PC-based systems, which suggests a more pronounced potential of the former to cause irritation, and is consistent with the biocompatibility described for delivery systems based on PC (Elnaggar et al., 2016; Paolino et al., 2002; Salata et al., 2021; Salata and Lopes, 2022).

In conclusion, our results demonstrate the feasibility to obtain nanostructured delivery systems based on lamellar phases for topical delivery of hybrid molecules that combine dexamethasone and a H_2S donor. We demonstrated that replacing polysorbate, glycerol and oleic acid with higher contents of PC, phytosphingosine and shea butter enabled protection of the cutaneous barrier function, especially as aqueous content reached 70 % (when acanthosis was also avoided), suggesting no formulation-related local damage to the tissue after a longer application period. Our results support the potential applicability of PC-70 for topical delivery of Dexa-TBZ, as no skin damage was observed while penetration was improved.

CRediT authorship contribution statement

Daniel A.G. Miranda: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Anderson R.A. Cerqueira:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Marcelo N. Muscará:** Writing – review & editing, Formal analysis. **Beatrice Severino:** Writing – review & editing, Methodology. **Giuseppe Caliendo:** Writing – review & editing, Methodology. **Angela Corvino:** Writing – review & editing, Methodology. **Giorgia Andreozzi:** Writing – review & editing, Methodology. **Antonia Scognamiglio:** Methodology. **Marlus Chorilli:** Writing – review & editing, Methodology, Investigation. **Francesco Frecentese:** Formal analysis, Methodology. **Soraia K.P. Costa:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Luciana B. Lopes:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Moacir F. de Brito and Simone Teixeira for their support. This study was supported by São Paulo Research Foundation (FAPESP, grant# 2018/13877–1, 2016/06146–5) and CAPES (finance code 001). ARA Cerqueira and D Miranda received fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico scholarship - CNPq. Fellowships CNPq (grant #306866/2020–0, 307928/2023–3, 306294/2019–2 and 312514/2019–0) to L.B. Lopes, SKP Costa and MN Muscará, respectively, are greatly appreciated. SKPC was a recipient of a Royal Society 2016/R1 Newton Grant- eGAP SZ50730.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106925.

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