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Nanostructured lipid carriers as delivery system for the phospholipase A_2 inhibitors PX-18 and PX-13 for dermal application

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PX-18 and PX-13 are secretory phospholipase A_2-IIA ($sPLA_2$-IIA) inhibitors. An increased expression of $sPLA_2$ in psoriatic skin has been reported. The selective inhibition of this enzyme is a new therapeutic approach. For dermal application PX-18 and PX-13 have been loaded to Nanostructured lipid carriers (NLC). The PX-18-loaded and PX-13-loaded NLC possessed an average particles size of about 250 nm, a narrow particle size distribution (PI < 0.2), a high entrapment efficiency as well as a good physical stability, as already indicated by their high zeta potential. Both NLC formulations have been incorporated into a hydroxyethyl cellulose gel and an o/w cream. In the gel and in the o/w cream PX-18-loaded and PX-13-loaded NLC showed a good physical stability. Neither aggregation nor dissolution of NLC took place.

1. Introduction

PX-18 and PX-13 are secretory phospholipase A_2-IIA ($sPLA_2$-IIA) inhibitors, which are poorly soluble in water. Fig. 1 shows the chemical structure of PX-18 and PX-13. PLA_2. Phospholipase reaction is the rate-limiting step for the metabolism of arachidonic acid by one of several enzymatic pathways for the production of lipid mediators (eicosanoids) (Pruzanski et al. 1997).

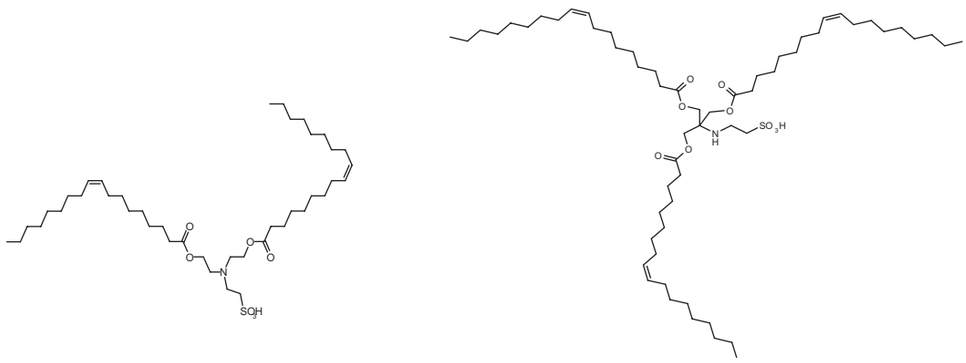
In healthy human skin limited amounts of $sPLA_2$-IIA are expressed in cells of the basal and spinous layers as well as in the uppermost cornified layer of the epidermis (Johansen et al. 1997). An increased expression of $sPLA_2$, especially $sPLA_2$-IIA, in psoriatic epidermis and dermis has been reported. Levels of $sPLA_2$ in involved and uninvolved psoriatic uppermost epidermal layers were increased compared to healthy skin Moreover, they found significantly higher amounts of $sPLA_2$ in psoriatic dermis than in healthy dermis (Andersen et al. 1994). The authors suggested that $sPLA_2$ detected in psoriatic skin is involved in eicosanoid overexpression in psoriatic tissue and potentiating cell activation, especially of T cells (Andersen et al. 1994). Haas et al. (2005) observed an upregulation of $sPLA_2$-IIA in the basal layer of psoriatic epidermis and in cells of psoriatic dermis. An overexpression of $sPLA_2$ gene in psoriatic skin compared to normal skin by an overall factor of about three was found by Johansen et al. (1997). They propose that the pathologic consequence of $sPLA_2$ overexpression and secretion from dermal fibroblasts is of importance in the activation of various inflammatory cells. Rys-Sikora et al. (2000) observed an upregulation of $sPLA_2$-IIA and $sPLA_2$–V in cultures of human primary keratinocytes after serum stimulation, suggesting a role of these enzymes in hyperproliferation. Grass et al. (1996) could show, that transgenic mice overexpressing $sPLA_2$-IIA develop chronic epidermal hyperplasia and hyperkeratosis supporting the possibility of a pathophysiological role of the enzyme. Therefore, selective inhibitors for PLA_2 enzymes might be useful for the therapy of various inflammatory syndromes, including epidermal hyperproliferation due to increased leukotriene production, related to eicosanoid production and cell activation, in both epidermal and dermal tissue of psoriatic skin (Sjursen et al. 2000). In this study PX-18 and PX-13 were loaded to nanostructured lipid carriers (NLC), a lipid nanocarrier system. NLC are a delivery system derived from o/w emulsions for parenteral nutrition. In NLC the oil of an o/w emulsion is replaced by a blend of a solid lipid and a liquid lipid being solid at body temperature.

Lipid nanoparticles have proven to have many advantages as a carrier system for dermal application, e.g., composition of well tolerated biodegradable lipids (Műller et al. 1997; Scholer et al. 2001), occlusive properties without glossy skin appearance (Wissing et al. 2001; Teeranachaideekul et al. 2008), enhancement of the chemical stability of active compounds sensitive to light, oxidation or hydrolysis (Jenning and Gohla 2001; Teeranachaideekul 2008), controlled release profiles (Müller et al. 2000; Wissing and Müller 2002; Souto et al. 2004a; Joshi and Patravale 2006), enhancement of penetration of active compounds into the skin (Santos Maia et al. 2000; Pardeike and Müller 2007) as well as drug targeting within the skin or even substructures of the skin improving the benefit-risk ratio of topical drug therapy (Santos Maia et al. 2002; Stecova et al. 2007). PX-18-loaded and PX-13-loaded NLC were prepared by hot high pressure homogenization, characterized and their stability was investigated. Furthermore, both NLC formulations were incorporated into a hydroxyethyl cellulose gel and an o/w cream.

2. Investigations, results and discussion

2.1. Preparation of NLC

Aqueous NLC dispersions composed of 20% lipid phase (liquid lipid, solid lipid and PX-18 or PX-13, respectively) were prepared by hot high pressure homogenization. Particles well in the



PX-18 PX-13

Fig. 1: Chemical structure of the $sPLA_2$-IIA inhibitors PX-18 and PX-13

nanometer range with a narrow size distribution were obtained by this method. PX-18-loaded NLC had an average particle size measured by PCS of 236 nm and a PI of 0.138 straight after production. The average particle size and the PI of the PX-13-loaded NLC were 251 nm and 0.161.

2.2. Encapsulation efficiency (E.E.)

Both carrier systems contained 2.5% drug calculated on the base of the lipid phase. The E.E. of PX-18 in NLC was 95.5%. For PX-13 an E.E. into the lipid particle matrix of 98.8% was found. The high E.E. of both drugs into the lipid particle matrix is caused by the high solubility of the actives in the oil and the solid lipid. In addition both drugs are practically insoluble in water. The saturation solubility of PX-18 and PX-13 is 24.4 μg/ml and 5 μg/ml, respectively. However, the free amount of PX-18 and PX-13 is higher than the saturation solubility in the aqueous phase being attributed to the presence of surfactant used to stabilize the systems.

2.3. Thermal properties

To gain information on the melting behaviour and the incooperation of PX-18 and PX-13 into NLC, the NLC under investigation as well as the according bulk materials used were analyzed by DSC measurements. Table 1 provides an overview of the onsets and the melting points of Softisan 154, a physical mixture of Softisan 154 and liquid paraffin in the ratio used for NLC production, PX-18-loaded and PX-13-loaded NLC as well as of the active compounds. Softisan 154 and the bulk mixture were heated up to 85 °C and kept at that temperature for 1 h to mimic the production conditions of the NLC. Due to mixing Softisan 154 with liquid paraffin the onset and the melting point were decreased. This indicates that liquid paraffin is dissolved in Softisan 154 and the crystalline structure of Softisan 154 is less pronounced (Saupe et al. 2005). PX-18-loaded and PX-13-loaded NLC showed one melting peak, with an onset and a melting point lower than that of the tempered mixture of Softisan 154 and liquid paraffin. Additionally to a possible effect of the active compounds and of the surfactant used to stabilize the NLC dispersion, this can be explained by the small particle size and the high specific surface area of the NLC according to Gibbs-Thomson equation (Saupe et al. 2005; Bunjes and Unruh 2007). Furthermore, the melting peaks of PX-18 and PX-13 were not found in the NLC formulations indicating that both active compounds are dissolved in the particle matrix and no recrystallization took place. The onset and melting point of PX-18-loaded and PX-13-loaded NLC were well above 32 °C, which is a prerequisite to preserve the solid particles after topical application and therefore the advantages associated with a topical application of lipid nanoparticles containing products.

Table 1: Onset and melting point of Softisan 154, lipid bulk mixture, PX-18-loaded NLC, PX-13-loaded NLC, PX-18 and PX-13

	Onset [°C]	Melting point [°C]
Softisan 154	55.1	58.3
Softisan 154: Liquid paraffin 4:1	51.3	54.4
PX-18-loaded NLC	48.5	53.7
PX-13-loaded NLC	48.1	53.4
PX-18	50	60
PX-13 I	42	51
II	59	65
III	84	86

2.4. Zeta potential and long term stability

The zeta potential, which is the electrical potential at the shear plane, is a useful tool to predict the physical stability of colloidal systems (Mehnert and Mäder 2001). The higher the zeta potential, the better is the physical stability of nanoparticles. The zeta potential values of PX-18-loaded and PX-13-loaded NLC were -41.7 ± 8.5 mV and -41.8 ± 7.5 mV, respectively. Both lipid nanoparticle dispersions were stabilized with TegoCare 450 and Pluronic F68. That means that the particles were not only electrostatically but also sterically stabilized. It has been reported that in a combined electrostatic and steric stabilization an absolute zeta potential of about 20 mV can be sufficient for physical long term stability (Jacobs and Müller 2002). Therefore, the zeta potential values measured for PX-18-loaded and PX-13-loaded NLC indicated good long term stability, i.e., avoidance of particle aggregation due to the electrostatic repulsion between the particles and the steric hindrance. This was confirmed by PCS and LD measurements over 90 days. As it can be seen in Figs. 2 and 3, no changes of PCS values and LD values took place over the observation period. PI values smaller than 0.2 were found for both NLC dispersions at all measuring time points indicating an unchanged narrow particle size distribution (Müller and Schuhmann 1996). A good physical stability of lipid nanoparticles up to three years has previously been reported (Freitas 1999).

2.5. NLC containing gel and o/w cream

Topically applied products containing NLC can be prepared by one of the following three techniques: (1) addition of a geling agent to the aqueous phase of NLC to obtain a gel, (2)

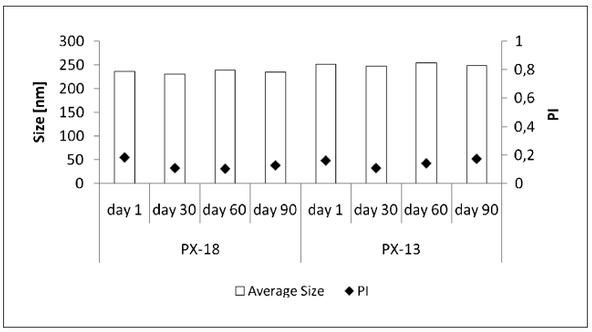


Fig. 2: Average particle size and PI of PX-18-loaded and PX-13-loaded NLC suspensions stored refrigerated for 90 days

reduction of the aqueous and lipid phase of a cream by the amount to NLC to be incorporated into the cream to obtain an o/w cream or a w/o cream and (3) production of a dermal product conataining only NLC in a one-step process (Müller et al. 2002; Pardeike et al. 2008). In this study a gel and an o/w cream containing PX-18-loaded and PX-13-loaded NLC were produced and investigated with regards to the physical stability of the NLC in this formulations. An instability that might occur if lipid nanoparticles are incorporated into gels and creams is aggregation of the nanoparticles. Furthermore, lipid nanoparticles might dissolve in the oil phase of a cream.

2.6. Particle size

Fig. 4 shows the LD volume distribution curves of PX-18-loaded NLC, PX-18-loaded NLC incooperated into a hydroxyethyl cellulose gel and an o/w cream after 90 days of storage. Fig. 5 shows the corresponding results obtained with PX-13-loaded NLC. Both NLC dispersions showed a monomodal particle size distribution located in the nanometer range. Drug-loaded NLC incorporated into gel and o/w cream showed bimodal particle size distributions, one particle size population in the nanometer range indicating the presence of NLC and one particle size population in the micrometer range indicating the presence of gelling agent or oil droplets of the o/w cream, respectively. The LD data suggest, that no aggregation of NLC took place after incorporation into the hydroxyethyl cellulose gel and the o/w cream (Shahgaldian et al. 2003; Souto et al. 2004b).

2.7. Thermal properties

The melting curves after 90 days of storage of drug-loaded NLC, o/w cream, o/w cream containing NLC, hydroxyethyl cellulose gel and hydroxyethyl cellulose gel containing NLC are shown

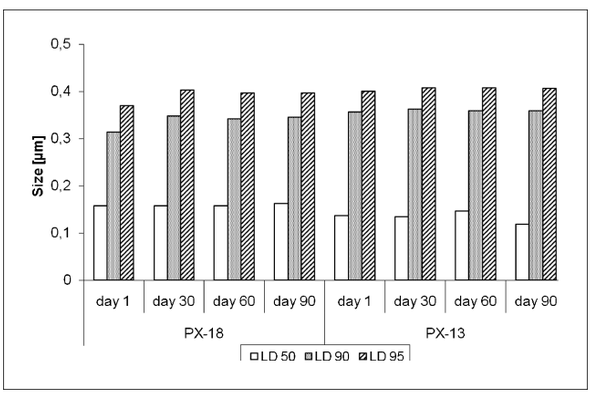


Fig. 3: Diameters LD 50, LD 90 and LD 95 of the PX-18-loaded and PX-13-loaded NLC suspensions stored refrigerated for 90 days

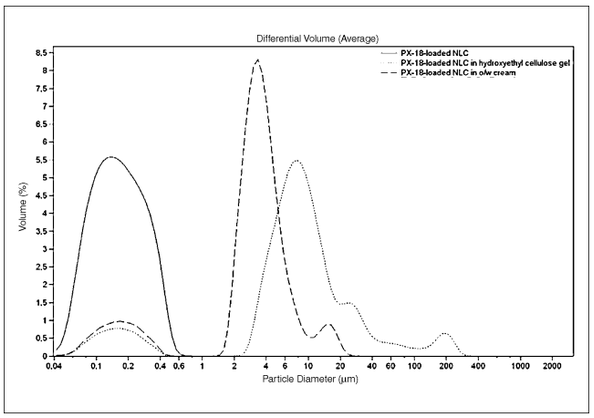


Fig. 4: LD volume distribution curves of PX-18-loaded NLC, PX-18-loaded NLC in hydroxyethyl cellulose gel and PX-18-loaded NLC in o/w cream after 90 days of storage

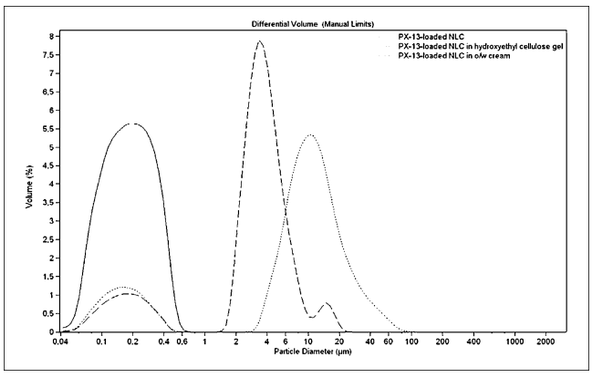


Fig. 5: LD volume distribution curves of PX-13-loaded NLC, PX-13-loaded NLC in hydroxyethyl cellulose gel and PX-13-loaded NLC in o/w cream after 90 days of storage

for PX-18 in Fig. 6 and for PX-13 in Fig. 7. The melting enthalpy of PX-18-loaded NLC dispersion was 28.6 J g^{-1} after 90 days of storage. For PX-13-loaded NLC dispersion a melting entahalpy of 29.0 J g^{-1} was obtained. Due to the absence of crystalline structures in the hydroxyethyl cellulose gel no endothermic event was observed. A melting peak of PX-18-loaded and PX-13-loaded NLC can be seen after incorporation into the gel, indicating the presence of the crystalline particle matrix. Due to the fact that 20% of the NLC dispersions were incorporated into the gels, a melting enthalpy which correspondences to $^1/_5$ of the one of the melting enthalpy of NLC dispersion

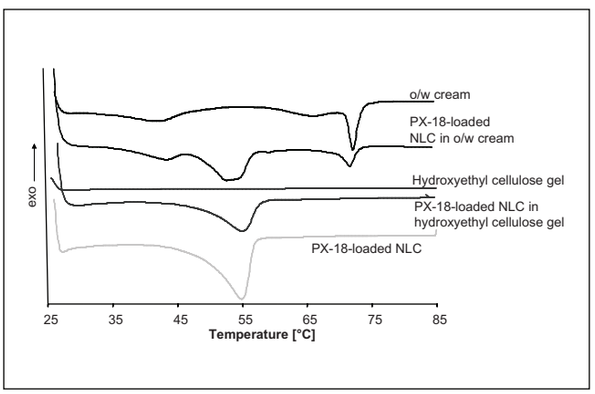


Fig. 6: Melting curves of PX-18-loaded NLC, hydroxyethyl cellulose gel, o/w cream as well as gel and cream containing PX-18-loaded NLC after 90 days of storage

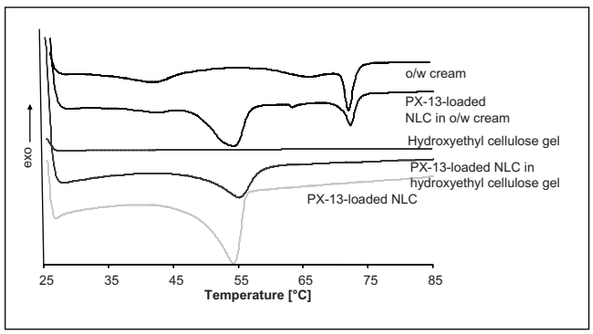


Fig. 7: Melting curves of PX-13-loaded NLC, hydroxyethyl cellulose gel, o/w cream as well as gel and cream containing PX-13-loaded NLC after 90 days of storage

indicates 100% presence of NLC in the gel after 90 days of storage. For the PX-18-loaded NLC and PX-13-loaded NLC in hydroxylethyl cellulose gel after 90 days of storage a melting enthalpy of 5.7 J g^{-1} and 5.8 J g^{-1}, respectively, was obtained. These values correlate well with the findings for the NLC dispersions. For the o/w cream three endothermic events, caused by the presence of partly crystalline structures of the incorporated surfactant and lipid, have been observed. After addition of drug-loaded NLC an additional melting peak for the lipid particle matrix was found for both PX-18-loaded and PX-13-loaded NLC, providing evidence for the presence of the solid particle matrix (Müller and Dingler 1998). The melting enthalpy of PX-18-loaded NLC and PX-13-loaded NLC in o/w cream after 90 days of storage was 5.4 J g^{-1} and 5.7 J g^{-1}, respectively. As 20% of the NLC dispersions were incorporated into the o/w creams, the melting enthalpies of PX-18-loaded NLC and PX-13-loaded NLC in o/w creams correlate well with the meting enthalpies of the NLC dispersions, indicating that no dissolution of NLC in the o/w creams took place. From the results obtained by LD and DSC measurements it can be concluded, that PX-18-loaded and PX-13-loaded NLC are physically stable in hydroxyethyl cellulose gel and the o/w cream under investigations.

In conclusion, the formulation of NLC containing the $sPLA_2$-IIA inhibitors PX-18 and PX-13 showed a good physical stability as indicated by the zeta potential values. The encapsulation efficiency of both drugs into NLC was high. A mean particle size of about 250 nm assessed by PCS and a narrow particle size distribution (PI < 0.2) was found for both NLC formulations. The stability of these carrier systems in hydroxyethyl cellulose gel and o/w cream was proven. Neither aggregation nor dissolution of NLC occurred in the dermal formulations. Therefore, an innovative gel and o/w cream formulation is available for PX-18 and PX-13, which provides the possibility for further investigations with regards to the treatment of inflammatory skin diseases such as psoriasis. The next studies to follow are penetration studies into pig ear skin, to assess to which extent the penetration is enhanced.

3. Experimental

3.1. Preparation of NLC, gel and o/w cream

NLC containing 0.5% (w/w) PX-18 or PX-13 (synthesized by the Department of Inorganic and Organic Chemistry of the Charles University, Hradec Kralove, Czech Republic), 3.9% (w/w) liquid paraffin (Caelo, Germany), 15.6% (w/w) Softisan 154 (Sasol, Germany), 1.8% (w/w) TegoCare 450 (Evonik Goldschmidt, Germany), 1.5% (w/w) Pluronic F68 (BASF, USA) and 76.5% (w/w) Milli-Q. water were prepared by hot high pressure homogenization (85 °C, 500 bar, 3 cycles) using a Micron LAB 40 (APV Homogeniser Systems, Germany).

20% NLC were incorporated into a gel containing 5% (w/w) hydroxyethyl cellulose 400 and an o/w cream, i.e. "Wasserhaltige Hydrophile Salbe".

3.2. Encapsulation efficiency (E.E.)

The E.E. of PX-18 and PX-13 in NLC was determined by ultracentrifugation technique. The amount of PX-18 and PX-13 in the NLC and the ultrafiltrate (free PX-18/PX-13) was analyzed by HPLC. The percentage of E.E. was calculated using the following equation:

$$E.E.[\%] = \left(\frac{Total\ amount\ of\ PX-18 - Free\ amount\ of\ PX-18}{Total\ amount\ of\ PX-18}\right) \times 100$$

3.3. Photon Correlation Spectroscopy (PCS)

The particle size of PX-18-loaded and PX-13-loaded NLC was determined by PCS using a Zetasizer Nano ZS (Malvern Instruments, UK). PCS yields the mean particle size and the polydispersity index (PI) as a measure of the width of the particle size distribution.

3.4. Laser diffractometry (LD)

An LS 230 (Beckman-Coulter, Germany) was used to detect aggregation of NLC as well as the particle size distribution in NLC-containing gel and o/w cream. LD data were evaluated using the Mie theory. Water with a RI of 1.33 was used as measurement medium. The real refractive index and the imaginary refractive index were set 1.456 and 0.01, respectively. Before LD measurements gels and o/w creams were diluted with Milli-Q water to obtain a homogeneous distribution of the formulation in the measurement cell. The diameter 50% (LD 50), 90% (LD 90) and 95% (LD 95), which means that 50%, 90% or 95% (volume distribution) of the measured particles are below this size, were evaluated.

3.5. Zeta potential measurements

The zeta potential of PX-18-loaded and PX-13-loaded NLC was measured in distilled water adjusted to a conductivity of 50 µS cm^{-1} using a Zetasizer Nano ZS (Malvern Instruments, UK). The measured electrophoretic mobility was converted to the zeta potential by applying the Helmholtz-Smoluchowski equation.

3.6. Differential Scanning Calorimetry (DSC)

The melting behaviour of the formulations was studied using a Mettler DSC 812e (Mettler Toledo, Germany). Appropriate amounts to obtain 2 to 4 mg calculated on the base of the solid particle mass of NLC were analysed in aluminium pans. Samples were heated from 25 °C to 85 °C with a heating rate of 5 K/min under constant flushing with nitrogen (80 ml/min).

3.7. Storage conditions

NLC, gels and o/w creams were stored refrigerated over 90 days to evaluate the physical stability.

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