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Research paper

Starch nanocapsules containing a novel neutrophil elastase inhibitor with improved pharmaceutical performance

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ABSTRACT

Psoriasis and atopic dermatitis patients show an excessive amount of elastase in peripheral blood neutrophils due to an imbalance between this proteolytic enzyme and its endogenous inhibitors, the search for new human neutrophil elastase (HNE) inhibitors are required. The HNE is an attractive therapeutic target and inhibitors with new molecular architectures have been extensively investigated. In this context a promising novel synthetic human neutrophil elastase inhibitor (ER143) was associated to a starch-based nanoparticulate system (StNC) with improved pharmaceutical performance, using a quality by design approach to support product development and optimization. The resulting formulation was characterized in terms of and *in vitro* release, permeation and retention studies in newborn pig skin, using Franz diffusion cells revealing the StNC have the ability to control the drug release rate and contribute to a high skin retention and/or permeation profiles. The anti-inflammatory activity accessed *in vivo* using the croton oil-induced ear inflammation model in mice showed that erythema and edema were attenuated in 98% following local application. These observations suggest the association of ER143 to the StNC promotes a deeper skin penetration and retention, also confirming StNC as a potential topical delivery system.

1. Introduction

Although topical drug delivery is one of the most promising routes of administration, the *stratum corneum* (SC) is still a major biological barrier that makes it a challenge. Topical formulations are selected due to their localized effects at the site of the application, taking advantage of drug permeation into the deeper layers of skin. However, approximately 40% of the novel promising molecules exhibit low or insignificant solubility, presenting problems often associated with low absorption and poor bioavailability [1,2]. Several formulation strategies have been suggested to overcome these bioavailability problems, including the use of polymeric and lipid-based nanoparticles, to improve percutaneous absorption due to their capacity to enhance the rate and extent of transport across the skin, as a result of their high specific surface area [3–6].

Nanoparticulate carriers can provide important advantages over the conventional drug delivery systems, namely the possibility to modulate the drug release by modifying some of its characteristics as well as the ability to deliver both hydrophilic and hydrophobic drugs. They carry drugs to the target in a controlled manner, offering further advantages such as reducing the dose frequency, increasing therapeutic control, reducing side effects, and, consequently, improving patient compliance [7]. Particularly, starch-based nanocapsules have attracted increasing interest as a nanobiomaterial for topical drug delivery [4,8]. These nanoparticulate carriers can enhance topical bioavailability due to their reduced particle size, increasing the rate of absorption, and by forming an occlusive layer on the skin surface that decreases water evaporation and creates wider diffusion channels [3,9]. Furthermore, starch is a natural polymer with an important role and safe application in the pharmaceutical industry, since it is biodegradable, non-toxic, renewable and sustainable [10]. It also allows simple and green processes for nanocapsule preparation, not requiring the use of hazardous organic solvents, while incorporating a wide range of drugs [8].

The human neutrophil elastase (HNE) is a proteolytic enzyme that plays a central role in several inflammatory diseases. An imbalance between HNE and its endogenous inhibitors lead to severe tissue injuries triggering various disease as for instance rheumatoid arthritis, chronic obstructive pulmonary disease, psoriasis or delayed wound healing [11,12]. The HNE is present inside the migrating neutrophils in the reticular dermis and dermal papillae, as well as outside the cells in

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micro-abscesses in psoriatic skin. Hence, psoriatic skin contains low concentrations of specific elastase tissue inhibitor, which results in an excessive *in vivo* hydrolytic activity of neutrophil elastase released from migrating cells [13].

Therefore, HNE is an attractive therapeutic target and the design of new HNE inhibitors is a demanding field that has been extensively investigated in order to provide inhibitors with new molecular architectures, including the potent oxo-β-lactam class [14]. In the present work, a coumarin-based oxo-β-lactam was designed as to be an irreversible HNE inhibitor that behaves as a turn-on probe as upon HNE inhibition releases the fluorescent hydroxy-coumarin, allowing appropriate signalization for skin permeation studies.

Based on our previous studies the purpose of this study was to develop starch nanocapsules for topical delivery of a novel HNE inhibitor (ER143) presenting low water solubility and putative anti-inflammatory action, in order to increase its bioavailability [8,14,15]. A quality by design (QbD) approach was performed to extract the maximum amount of information from the collected data, to establish the influence of several factors on the nanoformulation critical quality attributes. The *in vitro* release and permeation, and the *in vivo* studies allowed demonstrating the successful application of starch nanocapsules and highlighting their potential as a nanobiomaterial suitable for topical delivery of therapeutically relevant HNE inhibitors.

2. Materials and methods

2.1. Materials

Caprylic/capric triglycerides (Miglyol® 812) were a gift from Sasol Olefins & Surfactants GmbH (Hamburg, Germany). Cetrimonium bromide (Cetrimide) was a gift from DS Produtos Químicos (São Domingos de Rana, Portugal). Polysorbate 80 (Tween®80) was obtained from Merck (Kenilworth, USA). Ethanol was obtained from Carlo Erba Reagents (Cornaredo, Italy). Pregelatinized modified starch (Instant Pure-Cote® B793) was a gift from Grain Processing Corporation (Washington, USA). Purified water was obtained by reverse osmosis and electrodeionization (Millipore, Elix 3) being afterwards filtered (pore 0.22 μm).

2.2. Methods

2.2.1. HNE inhibitor synthesis

The chemical structure of the HNE inhibitor 3,3-diethyl-1-(4-(((2-oxo-2*H*-chromen-7-l)oxy)methyl)phenyl)azetidine-2,4-dione (ER143) is presented in Fig. 1. The synthesis of ER143 was performed as follows: to a solution of 6-hydroxycoumarin (100 mg, 0.616 mmol) in acetone (1 ml) was added potassium carbonate (94 mg, 0.677 mmol) and the resulted solution was subjected a stirring for 20 min. Then, a solution of the respective bromo precursor (193 mg, 0.622 mmol) in the same solvent (1 ml) was added dropwise, and the reaction was stirred for 6 h at 60 °C. The reaction mixture was diluted with water (5 volumes) and the product extracted with 3xEtOAc (5volumes). The organic layers were combined, dried with anhydrous Na_2SO_4, filtered and concentrated. The obtained residue was purified by flash chromatography (elution with Hexane-AcOEt gradient) to yield white solid (106.5 mg, 44%). m.p. 125–127 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 (m, 2H, H-Ar2-6), 7.64 (d, J = 9.5 Hz, 1H, H-Ar15), 7.48 (d, J = 8.6 Hz, 2H, H-Ar3-5), 7.39 (d, J = 8.6 Hz, 1H, H-Ar11), 6.91 (dd, J = 8.5, 2.4 Hz, 1H, H-Ar12), 6.86 (d, 1H, J = 2.5H, H-Ar9), 6.26 (dd, J = 9.5, 5.3 Hz, 1H, H-Ar14), 5.12 (s, 2H, H-7), 1.86 (q, J = 7.5 Hz, 4H, H-5), 1.07 (t, J = 7.5 Hz, 6H, H-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1 (C2,4), 161.6 (Ar7), 161.2 (Ar13), 155.8 (Ar10,9), 143.4 (Ar15), 134.3 (Ar4), 133.8 (Ar1), 128.9 (Ar11), 128.4 (Ar3,5), 119.5 (Ar2,6), 113.3 (Ar12), 112.9 (Ar14), 101.9 (Ar9), 72.3 (C3), 69.9 (C7), 23.9 (C5), 9.3 (C6). ESI-MS (+) m/z: 391 $[M+H]^+$. Anal. Calcd. ($C_{23}H_{21}NO_5$): C, 70.58; H, 5.41; N, 3.58. Found: C, 70.78; H, 5.79; N, 3.76%.

2.2.2. Biological assays

2.2.2.1. Enzymatic inhibition assay. Fluorometric assays for the HNE (Merck, Germany) inhibition activity were carried out in 200 μl assay buffer (0.1 M HEPES pH 7.5 at 25 °C) containing 20 μl of 0.17 μM HNE (stock solution 1.7 μM in 0.05 M acetate buffer, pH 5.5), 155 μl of assay buffer and 5 μl of each concentration of tested inhibitors. After 30 min of incubation at 25 °C, the reaction was initiated by the addition of 20 μl of fluorogenic substrate to a final concentration of 200 μM (MeO-Suc-Ala-Ala-Pro-Val-AMC, Merck, Germany). The Michaelis-Menten constant (K_m) of this substrate of HNE was previously determined to be 185 μM (data not shown). For all assays, saturated substrate concentration was used, throughout, in order to obtain linear fluorescence curves. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Sivelestat sodium salt hydrate, Sigma Aldrich, UK) [15].

2.2.3. *Preparation of neutrophil elastase inhibitor-loaded starch-based nanocapsules (StNC-ER143)*

The StNC-ER143 were prepared using a previously reported emulsion-solvent evaporation method [8]. Briefly, capric/caprylic triglycerides (CT) were used as the lipid component, Tween®80 and cetrimide as surfactants, pregelatinized modified starch as a polymer and ethanol as a co-solvent. According to solubility studies (results not shown), caprylic/capric triglycerides allowed maximal solubility for ER143 (30 μg/mg lipid). Thus, the drug ER143 was dissolved on capric/caprylic triglycerides and then added to the ethanolic phase, obtaining StNC-ER143 nanocapsules. The different concentrations of ER143 used in design of experiment (DoE) were selected based on preliminary solubility studies of ER143 in caprylic/capric triglycerides (see supplementary material). The nanoparticle dispersion was then kept under stirring at 25 ± 2 °C.

2.2.4. Particle size analysis and zeta potential measurements

Particle size distribution was determined using a Malvern Mastersizer 2000 (Malvern Instruments, UK) coupled with a Hydro S accessory and the procedure was performed according a previous published work [8].

The surface charge (zeta potential, ζ) was determined by electrophoretic light scattering in a Zetasizer Nano Z (Malvern Instruments, UK) at 25°C. Samples were diluted appropriately with filtered purified water (pH 5.6).

2.2.5. Encapsulation efficiency and drug loading

After preparation, non-incorporated ER143 was separated from the StNC dispersions by size exclusion chromatography on Sephadex G-25/PD-10 columns (Sigma Aldrich, UK). Drug incorporation in StNC was determined after dissolving the nanocapsules with acetonitrile, which promoted the precipitation of the lipid and polymeric phase, followed by centrifugation. The encapsulated ER143 remained in the supernatant and was quantified by fluorimetry at 360 nm (excitation) and 460 nm (emission) wavelengths using a fluorescence microplate reader (FLUOstar BMGLabtech, Germany). The supernatant of non-loaded nanocapsules was used as basic correction. The ER143 encapsulation efficiency (EE) and drug loading (DL) in StNC were calculated according to the following equations:

$$EE(\%) = \frac{W_{totaldrug} - W_{freedrug}}{W_{totaldrug}} \times 100 \quad (1)$$

$$DL(\%) = \frac{W_{totaldrug} - W_{freedrug}}{W_{lipid}} \times 100 \quad (2)$$

where $W_{total\ drug}$ is the weight of the drug used, $W_{free\ drug}$ is the weight of free drug detected in the supernatant after centrifugation of the aqueous dispersion and W_{lipid} represents the weight of the lipid vehicle.