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Inhibition of skin inflammation by baicalin ultradeformable vesicles

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ABSTRACT

The topical efficacy of baicalin, a natural flavonoid isolated from *Scutellaria baicalensis Georgi*, which has several beneficial properties, such as antioxidative, antiviral, anti-inflammatory and antiproliferative, is hindered by its poor aqueous solubility and low skin permeability. Therefore, its incorporation into appropriate phospholipid vesicles could be a useful tool to improve its local activity. To this purpose, baicalin at increasing concentrations up to saturation, was incorporated in ultradeformable vesicles, which were small in size (~67 nm), monodispersed (PI < 0.19) and biocompatible, regardless of the concentration of baicalin, as confirmed by *in vitro* studies using fibroblasts. On the other hand, transdermal flux through human epidermis was concentration dependent. The *in vivo* results showed the significant anti-inflammatory activity of baicalin loaded nanovesicles irrespective of the concentration used, as they were able to reduce the skin damage induced by the phorbol ester (TPA) application, even in comparison with dexamethasone, a synthetic drug with anti-inflammatory properties. Overall results indicate that ultradeformable vesicles are promising nanosystems for the improvement of cutaneous delivery of baicalin in the treatment of skin inflammation.

1. Introduction

Baicalin (7-glucuronic acid 5, 6-dihydroxyflavone), is one of the most abundant flavonoid in the root of *Scutellaria baicalensis Georgi*, used as medical agent in traditional Chinese medicine thanks to its multiple therapeutic benefits. Indeed, it has shown strong anti-inflammatory (Chen et al., 2001) and anti-oxidant properties (Shi et al., 2015), as well as antimicrobial and antifungal activities (Shi et al., 2014) and a great potential to prevent and inhibit tumor (Chen et al., 2016). Further, baicalin is able to protect the skin from damages caused by exposure to solar ultraviolet (UV) radiations. Considering all the protective and beneficial effects and its low toxicity, baicalin can be considered a suitable molecule for eliminating causes and effects of skin aging and injuries, including UV radiation damages, wounds and burns (Zhang et al., 2014a,b). Despite these promising properties, baicalin efficacy and actual use in topical formulations are hampered by its lipophilic nature and consequent low water solubility, which account for a poor bioavailability (Xing et al., 2005). To overcome this problem, baicalin has been loaded in different nanocarriers (Shi-Ying et al., 2014; Zhang et al., 2014a,b) for systemic, brain, corneal delivery and tumor targeting (Chen et al., 2016). In other studies, nanocarriers have been used to achieve effective transdermal delivery. Unfortunately, the barrier nature of the stratum corneum represents an important obstacle to drug accumulation into and passage through the skin, not only for conventional dosage forms, but also for some innovative dosage systems. To this purpose, several techniques or systems have been tested and the most widely studied approach in the last decades is the use of lipid nanocarriers, such as phospholipid vesicles and derived systems: ethosomes, glycerosomes and hyalurosomes (Manca et al., 2015; Castangia et al., 2013). Their use for skin delivery can offer some advantages over classical topical dosage forms, and some specially designed vesicular carriers can be able to efficiently increase drug penetration and permeation through the skin. Although there are

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several studies on the incorporation of baicalin in liposomes (Wei et al., 2014), only a few studies specifically addressed the enhancement of baicalin efficacy following application on the skin. Hence, alternative phospholipid vesicles can result in a greater improvement of its therapeutic index and effectiveness in skin protection.

In light of this, in the present study, baicalin was incorporated in ultradeformable liposomes made with a mixture of soy lecithin and polysorbate 80, used as edge activator to improve bilayer fluidity. Thanks to this property, vesicles can squeeze themselves between the cells in the stratum corneum driven by the hydration gradient and reaching deeper skin strata. In addition, considering that drug saturation may improve its thermodynamic activity, maximizing the flux through biological membranes, irrespective of the selected vehicle and the drug solubility, baicalin was incorporated into ultradeformable vesicles at increasing amounts up to the maximum possible concentration (2.5, 5 and 10 mg/mL) which is supposed to saturate the system. Vesicle morphology, size distribution, zeta potential, and entrapment efficiency were evaluated, as well as the ability of the vesicles to promote *in vitro* baicalin skin delivery. The cytotoxic effect of empty and baicalin loaded vesicles was evaluated in 3T3 mouse fibroblasts. The drug and carrier performances and their ability to reduce oxidative inflammation and neutrophil infiltration induced by TPA in mice were studied, as well. To detect the damages induced by TPA on the skin and the effect provided by the tested formulations, a histological evaluation was also carried out.

2. Materials and methods

2.1. Materials

Lipoid® S75, a mixture of soybean lecithin containing phosphatidylcholine (70%) and phosphatidylethanolamine (10%), lysophosphatidylcholine (3% maximum), triglycerides (3% maximum), fatty acids (0.5% maximum), tocopherol (0.1–0.2%) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 and disodium phosphate were purchased from Scharlab S.L. (Barcelona, Spain). Monosodium phosphate was purchased from Panreac quimica S.A. (Barcelona, Spain). Dexamethasone 21-sodium phosphate (DEX) was from Acofarma S.A. (Madrid, Spain) and the DEX solution (2 μg/mL) was prepared in buffer solution (pH 7.4). Baicalin (BA) was purchased by Cymit química S.L. (Barcelona, Spain) and saturated solution was prepared.

2.2. Vesicle preparation

Samples were prepared weighing Lipoid® S75 (180 mg/mL), polysorbate 80 (2.5 mg/mL) and BA (2.5, 5 and 10 mg/mL) in a glass vial and hydrating them overnight with a buffer solution (pH 7.4) composed by a mixture of monosodium phosphate (0.3%) and disodium phosphate (2.9%). Then, the dispersion was sonicated for 3 min with a CY-500 ultrasonic disintegrator (Optic Ivymen system, Barcelona, Spain) to obtain clear opalescent dispersions. To achieve a uniform particle size distribution, the liposomal suspension was extruded with an Avanti® Mini-Extruder (Avanti Polar Lipids, Alabaster, Alabama) through a 200 nm membrane (Whatman, GE Healthcare, Fairfield, Connecticut, US) (Manconi et al., 2003). Empty liposomes were prepared as reference.

2.3. Analytical method

The BA content was quantified using a PerkinElmer® Series 200 HPLC equipped with a UV detector and a column Teknokroma® Brisa "LC2" C18, 5.0 μm (150 cm × 4.6 mm). The mobile phase consisted of a mixture of water and methanol (30:70), delivered at a flow rate of 1 mL/min. BA content was measured at 278 nm. The limit of detection and quantification for the BA was 0.45 μg/mL and 1.36 μg/mL, respectively.

2.4. Vesicle characterization

The formation and morphology of ultradeformable liposomes were checked by transmission electron microscopy (TEM) using a JEM-1010 microscope (Jeol Europe, Croissy-sur-Seine, France), equipped with a digital camera MegaView III at an accelerating voltage of 80 kV. Vesicles were examined using a negative staining technique: non-diluted dispersions were stained with 1% phosphotungstic acid on a carbon grid and examined/visualized.

The average diameter and polydispersity index (PI) of non-diluted samples were determined by Photon Correlation Spectroscopy using a Zetasizer nano-S (Malvern Instruments, Worcestershire, United Kingdom). Zeta potential was estimated using the Zetasizer nano-S by electrophoretic light scattering, which measures the particle electrophoretic mobility in a thermostated cell. Vesicles were purified from non-incorporated BA by dialysis. Each sample (1 mL) was loaded into Spectra/Por® tubing (12–14 kDa MW cut-off; Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialyzed against buffer (1 L) for 2 h, at room temperature. Both non-dialyzed and dialyzed samples were disrupted with methanol (1:100) and assayed by HPLC to quantify the BA content in the vesicular systems. The drug entrapment efficiency (EE%) of the three systems was calculated as follows (Eq. (1)):

$$EE(\%) = \left(\frac{actualBA}{initialBA}\right) \times 100 \quad (1)$$

where *actual BA* is the amount of BA in vesicles after dialysis, and *initial BA* is the amount of drug before dialysis, as calculated by HPLC.

2.5. Cell culture

3T3 mouse fibroblasts (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich, Spain), supplemented with 10% (v/v) foetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Sigma Aldrich, Spain) in 5% CO_2 incubator at 37 °C to maintain exponential cell growth.

2.6. Cell viability studies

3T3 cells were seeded in 96-well plates with cell density being approximately 5×10^5 cells/well, at passages 11–12. After 24 h of incubation, 3T3 cells were treated for 2, 4, 8 and 24 h with ultradeformable vesicles, empty or loaded with BA at different concentrations: 2.5 BA (2.5 mg/mL), 5 BA (5 mg/mL) and 10 BA (10 mg/mL) or BA saturated solution (25 μL of formulation in 250 μL of medium). Cell viability was determined by the MTT [3 (4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] colorimetric assay. Briefly, 200 μL of MTT reagent (0.5 mg/mL in PBS) was added to each well and after 2 h the formed formazan crystals were dissolved in DMSO. The reaction was spectrophotometrically measured at 570 nm with a microplate reader (Multiskan EX, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, United States). All experiments were repeated at least three times, each time in triplicate. Results are shown as percentage of cell viability in comparison with non-treated control cells (100% viability) (Eq. (2)):

$$CellViability(\%) = \left(\frac{Absorbance_{sample}}{Absorbance_{control}}\right) \times 100 \quad (2)$$