International Journal of Pharmaceutics 502 (2016) 161–169

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Biopharmaceutical evaluation of epigallocatechin gallate-loaded cationic lipid nanoparticles (EGCG-LNs): *In vivo*,*in vitro* and *ex vivo* studies

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ARTICLE INFO

Article history:
Received 27 January 2016
Received in revised form 21 February 2016
Accepted 22 February 2016
Available online 24 February 2016

Keywords:
Draize test
HET-CAM test
Epigallocatechin gallate
Lipid nanoparticles
Ocular delivery

ABSTRACT

Cationic lipid nanoparticles (LNs) have been tested for sustained release and site-specific targeting of epigallocatechin gallate (EGCG), a potential polyphenol with improved pharmacological profile for the treatment of ocular pathologies, such as age-related macular edema, diabetic retinopathy, and inflammatory disorders. Cationic EGCG-LNs were produced by double-emulsion technique; the *in vitro* release study was performed in a dialysis bag, followed by the drug assay using a previously validated RP-HPLC method. *In vitro* HET-CAM study was carried out using chicken embryos to determine the potential risk of irritation of the developed formulations. *Ex vivo* permeation profile was assessed using rabbit cornea and sclera isolated and mounted in Franz diffusion cells. The results show that the use of cationic LNs provides a prolonged EGCG release, following a Boltzmann sigmoidal profile. In addition, EGCG was successfully quantified in both tested ocular tissues, demonstrating the ability of these formulations to reach both anterior and posterior segment of the eye. The pharmacokinetic study of the corneal permeation showed a first order kinetics for both cationic formulations, while EGCG-cetyltrimethylammonium bromide (CTAB) LNs followed a Boltzmann sigmoidal profile and EGCG-dimethyldioctadecylammonium bromide (DDAB) LNs a first order profile. Our studies also proved the safety and non-irritant nature of the developed LNs. Thus, loading EGCG in cationic LNs is recognised as a promising strategy for the treatment of ocular diseases related to anti-oxidant and anti-inflammatory pathways.

1. Introduction

The anatomical and physiological barriers that ocular globe offers against the entrance of drugs are a challenge that could be overcome by the use of nanoparticles (Abrego et al., 2015; Fangueiro et al., 2015, 2016). The main barriers encountered in ocular drug delivery are the physical barriers (cornea, sclera and retina), the blood aqueous and blood-retinal barriers, and the physiological barriers (blood flow, lymphatic clearance, enzymatic degradation, protein binding and the tear dilution and renovation), compromising the delivery of drugs specially targeted to the posterior segment of the eye (Fangueiro et al., 2016).

Topical application of drugs in the ocular mucosa is usually used to reach the anterior and/or posterior segments of the eye (Araujo et al., 2011). The anterior segment of the eye is anatomically composed of the cornea, conjunctiva, sclera and anterior uvea.

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http://dx.doi.org/10.1016/j.ijpharm.2016.02.039
0378-5173/

Usually, the most commonly applied formulations to reach these structures are the eye drops, which are rapidly drained from the ocular surface reducing drastically drug residence time (Urtti, 2006). The posterior segment of the eye is composed of retina, vitreous and choroid (Thrimawithana et al., 2011). Because of their anatomic location, the pathologies affecting these structures are difficult to treat. To reach them, invasive and painful approaches (*e.g.* intravitreal injection) are usually required for the delivery of high drug doses. However, intravitreal injection can cause complications, such as endophthalmitis and, because of the administered high doses, systemic absorption may be compromised (Kim et al., 2014). These pathologies include age-related macular degeneration (AMD), retinitis pigmentosa, diabetic retinopathies (DR), and neural consequences caused by glaucoma (Kim et al., 2014; Urtti, 2006).

Designing non-invasive and sustained delivery nanoparticles for targeting drugs to the anterior and posterior segment of the eye is an ultimate objective of our work. Cationic lipid nanoparticles (LNs) for ocular delivery have been reported to be safe, and long-term stable (Fangueiro et al., 2014a,b). The use of physiological lipids is being documented as a safe, biocompatible and biodegradable approach (Souto et al., 2013). However, the main concern is the toxicity related to the surfactants and, in this particularly case, to the use of cationic molecules. We have recently produced biocompatible and biodegradable cationic epigallocatechin gallate LNs (EGCG-LNs) (Fangueiro et al., 2014a,b), composed of natural and physiological lipids, cationic lipid, surfactants, EGCG and water. LNs have been widely exploited for targeted drug delivery because of their nanometer size, long-term physicochemical stability, biocompatibility and biodegradability, controlled release of drugs, low cytotoxicity and easily scale-up production (Mishra et al., 2010; Müller et al., 2000; Sinha et al., 2011; Souto and Muller, 2010; Souto and Müller, 2007; Souto et al., 2010; Vazzana et al., 2015). It is therefore anticipated that LNs could overcome some of the disadvantages of ocular drug delivery and could provide a modified release profile (controlled/prolonged), keeping the drug concentration within the therapeutic levels, over an extended time (Araujo et al., 2009).

The topical administration of drugs provides a short period of residence time in the mucosa (usually 1–2 min), and these are efficiently removed *via* the lacrimal fluid renovation (Araujo et al., 2009). Cationic LNs seem to offer high capacity to increase the bioavailability of topical drugs when administered onto the ocular mucosa since they promote electrostatic interactions between the surface of the cationic particles and the anionic ocular mucosa, with a considerable improvement of the drug residence time (Fangueiro et al., 2014a).

The polyphenol EGCG with anti-oxidant properties has been successfully formulated in cationic LNs (Fangueiro et al., 2014b). Currently, there are no studies involving the topical use of this drug to treat ocular diseases such as DR, diabetic macular edema (DME), glaucoma or AMD. Some of the biological mechanisms involved in the development of these ocular pathologies are related to the increased oxidative stress due to the inability of the organism to suppress the production of reactive oxygen species (ROS) (Kowluru and Chan, 2007; Madsen-Bouterse and Kowluru, 2008; Wakamatsu et al., 2008). It is well-known that polyphenols have potential human health benefits (Heim et al., 2002), and recently EGCG is being reported as one of the most potent polyphenols (Du et al., 2012; Yamauchi et al., 2009). Our group stabilized EGCG in biological medium for further characterization of the physicochemical stability and release/permeation profile under physiological conditions (Fangueiro et al., 2014c).

Our efforts have been driven towards the development of cationic LN formulations that should maximize the EGCG ocular drug absorption *via* prolonged drug residence time in the cornea and conjunctival sac, as well as to enhance the transcorneal and transscleral drug penetration. In the present paper, the *ex vivo* transcorneal and transscleral permeation profiles of the EGCG-LNs have been evaluated, in parallel with the *in vitro* release profile of EGCG from the LNs. Pharmacokinetic parameters for each tissue were determined to establish the mechanism for transcorneal and transscleral permeation of the EGCG. In order to assess the local LNs toxicity and irritancy, an *in vitro* test (HET-CAM test) and an *in vivo* test (Draize test) have been carried out.

2. Materials and methods

2.1. Materials

Epigallocatechin gallate (EGCG, 98% purity, molecular weight 458.7 g/mol and pKa 7.59–7.75) was purchased from CapotChem (Hangzhou, China). Softisan®100 (S100) was a free sample from Sasol Germany GmbH (Witten, Germany), Lipoid®S75, 75% soybean phosphatidylcholine was purchased from Lipoid GmbH (Ludwigshafen, Germany), Lutrol®F68 or Poloxamer 188 (P188) was a free sample from BASF (Ludwigshafen, Germany). Transcutol®P was a gift from Gattefossé (Barcelona, Spain). Acetic acid (glacial, AR grade), acetonitrile (HPLC gradient), Cetyltrimethylammonium bromide (CTAB), sodium lauryl sulfate and ascorbic acid were acquired from Sigma-Aldrich (Sintra, Portugal). Dimethyldioctadecylammonium bromide (DDAB) was acquired from Avanti Polar Lipids (Alabama, USA). Anhydrous glycerol was purchased from Acopharma (Barcelona, Spain). Ethanol absolute (AR grade) was acquired from Panreac (Barcelona, Spain). The water used for sample preparation and for HPLC analysis was produced with a Super Purity Water System (Purite Ltd, England) with a resistivity over 17.5 MΩ cm. Ultra-purified water was obtained from a MiliQ Plus system (Milipore, Germany). All reagents were used without further treatment.

2.2. Cationic LNs production

Cationic LN dispersions were prepared based on a previously developed multiple emulsion (w/o/w) technique described by Fangueiro et al. (2014a,b). The composition is shown in Table 1. Briefly, EGCG and ascorbic acid were dissolved in ultra-purified water, which was added to the lipid phase at same temperature (5–10 °C above the melting point of lipid S100) and homogenized 60s with a sonication probe (6 mm diameter) by means of an Ultrasonic processor VCX500 (Sonics, Switzerland). A power output with amplitude of 40% was applied. The poloxamer

Table 1
Composition of the developed cationic LNs dispersions in wt%.

LN dispersion	S100	Glycerol	Lipoid® S75	CTAB	DDAB	P 188	A.A.	EGCG
CTAB-LN drug free	4.5	37.5	0.5	0.5	–	1.0	0.25	–
EGCG-CTAB LN	4.5	37.5	0.5	0.5	–	1.0	0.25	0.075
DDAB-LN drug free	4.5	37.5	0.5	–	0.5	1.0	0.25	–
EGCG-DDAB LN	4.5	37.5	0.5	–	0.5	1.0	0.25	0.075

S100: Softisan®100; p188: Poloxamer 188; A.A.: Ascorbic acid.