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Long-acting liposomal corneal anesthetics

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ABSTRACT

Eye drops producing long-acting ocular anesthesia would be desirable for corneal pain management. Here we present liposome-based formulations to achieve very long ocular anesthetic effect after a single eye drop instillation. The liposomes were functionalized with succinyl-Concanavalin A (sConA-Lip), which can bind corneal glycan moieties, to significantly prolong the dwell time of liposomes on the cornea. sConA-Lip were loaded with tetrodotoxin and dexmedetomidine (sConA-Lip/TD), and provided sustained release for both. A single topical instillation of sConA-Lip/TD on the cornea could achieve 105 min of complete analgesia and 608 min of partial analgesia, which was significantly longer than analgesia with proparacaine, tetrodotoxin/dexmedetomidine solution or unmodified liposomes containing tetrodotoxin and dexmedetomidine. sConA-Lip/TD were not cytotoxic in vitro to human corneal limbal epithelial cells or corneal keratocytes. Topical administration of sConA-Lip/TD provided prolonged corneal anesthesia without delaying corneal wound healing. Such a formulation may be useful for the management of acute surgical and nonsurgical corneal pain, or for treatment of other ocular surface diseases.

1. Introduction

Conventional amino-ester and amino-amide local anesthetics are commonly used to reduce ocular pain related to corneal injury [1,2] and ophthalmic surgery [3–5]. However, these agents only produce short periods of analgesia (15–20 min) after a single topical instillation [6]. Thus, continuous analgesia would require frequently repeated administration, which could cause anterior segment inflammation, corneal ulceration, and delay epithelial healing [7,8]. Long-acting ocular anesthetics with minimal toxicity are clinically desirable, particularly during longer surgical procedures and for outpatient management of minor corneal injury during the period when ocular pain is most intense.

Tetrodotoxin (TTX) is a naturally occurring toxin found in several organisms, whose mechanism of action is unimolecular blockade of site 1 on the extracellular surface of sodium channels on nerves [9,10]. TTX is an extremely potent local anesthetic [11,12]. Unlike commercially available amino-amide and amino-ester local anesthetics, tissue toxicity from TTX after injection at peripheral nerves can be minimal [13], even when delivered for prolonged periods [14]. Co-administration of TTX with adjuvant agents can enhance anesthetic effect. For example, the combination of TTX and the α_2-adrenergic agonist dexmedetomidine can significantly prolong the duration of corneal local anesthesia over that from TTX alone [8].

Liposomes are a class of versatile carriers for encapsulating both hydrophilic and hydrophobic drugs [15–17]. Liposomes are biocompatible and biodegradable, and have been widely investigated for delivery of a variety of therapeutic agents for ocular diseases [18–20]. Liposome-based eye drops can improve the bioavailability of encapsulated drugs [21]. TTX and DMED can be co-encapsulated in liposomes, exhibiting sustained release profiles and generating prolonged local anesthetic effects on peripheral nerves [22–24].

Concanavalin A (ConA) is a lectin (carbohydrate-binding protein) that binds specifically to glycoproteins and glycopeptides [25]. ConA has been previously reported to bind corneal epithelial cells at low concentrations in vitro, without apparent toxicity [26,27]. sConA is the succinylated ConA [28], which exhibits comparable binding affinity to glycan but less effects on the migration of corneal epithelial cells [29]. We hypothesize that sConA modified liposomes (sConA-Lip) would remain on the cornea for longer periods than unmodified ones, enabling sustained release of TTX/DMED on the cornea and long-acting ocular anesthesia.

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Here, we report liposome-based formulations encapsulating TTX and DMED for long-acting topical ocular anesthesia, and compare them with TTX/DMED solution and the widely used amino-ester ocular anesthetic, 0.5% (wt/vol) proparacaine. We also characterize the cytotoxicity of those formulations to corneal cells in vitro and their effect on corneal healing in vivo.

2. Materials and methods

2.1. Materials

Tetrodotoxin (TTX) was obtained from Abcam PLC (Cambridge, MA); dexmedetomidine hydrochloride (DMED) was from R&D systems, Inc. (Minneapolis, MN), and dexmedetomidine free base was prepared by alkaline precipitation. Lipids (DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol)) were acquired from CordenPharma International (Plankstadt, Germany). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (mal-PEG-DSPE) was purchased from Laysan Bio, Inc. (Arab, AL). Succinyl-Concanavalin A (sConA), Traut's reagent and cholesterol were obtained from Sigma (St, Louis, MO). Tetrodotoxin ELISA kits were from Reagen LLC (Moorestown, NJ).

2.2. Animal care

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) 6–8 weeks in age were cared for in accordance with protocols approved by the Animal Care and Use Committee at Children's Hospital, and the Guide for the Care and Use of Laboratory Animals of the U.S. National Research Council. They were housed in groups, in a 7 a.m. to 7 p.m. light-dark cycle.

2.3. Preparation of liposomal formulations

Liposomes were synthesized by hydrating a thin lipid film, using DOPC, DOPG and cholesterol [22,30]. The molar ratios between DOPC and DOPG varied from 16:0 to 12:4 (Table 1). The content of cholesterol was fixed at a 6:16 M ratio to the total lipids (DOPC + DOPG) in all liposomal formulations. 160 mg lipids (DOPC + DOPG), 30 mg cholesterol and 200 μg dexmedetomidine free base were dissolved in a chloroform: methanol (v/v, 9:1) mixture and the thin lipid film formed under reduced pressure, followed by hydration at 60 °C with 2 mL TTX solution (1 mg/mL) or rhodamine 6G (R6G) in phosphate buffered saline (PBS). The formed liposomes were further homogenized at 10,000 × g with a 3/8″ MiniMicro workhead on a L4RT-A Silverson Laboratory Mixer (East Longmeadow, MA) for 10 min to narrow the size distribution of liposomes and drug loading was increased after 10 freeze-thaw cycles [14]. For the determination of drug loading, unencapsulated drugs were removed by dialysis bag with MWCO 50 kDa (Spectrum Laboratories, Inc., Rancho Dominguez, CA) at 4 °C. Size and zeta-potential of liposomes were analyzed by a particle analyzer (Delsa Nano C, Beckman Counter). To assess the release kinetics from TTX and DMED, liposomes (0.4 mL) were placed in dialysis membranes (molecular weight cutoff 10 kDa) and dialyzed against PBS. At each predetermined time point, the buffer was changed with fresh PBS buffer. TTX and DMED contents were determined using a TTX ELISA kit (Reagen LLC, Moorestown, NJ) and analytic HPLC (Wonda-Cract ODS-2 column, 5 μm, 4.6 × 150 mm, mobile phase: Water/Acetonitrile = 4/6, UV absorbance at 254 nm), respectively. Lipid concentration in the final formulations were measured using the Bartlett assay [14,31].

sConA was thiolated (sConA-SH) with Traut's reagent according to the manufacturer's specifications, and the final concentration of sConA-SH in PBS was determined by UV absorbance at 280 nm [32]. Liposomes for this application were prepared with mal-PEG-DSPE (1 mol% of the total lipid), to provide a maleimide for the sConA-SH to react with, mixed with lipids (DOPC, DOPG and cholesterol at a molar ratio of 15:1:6) to form a thin film. Hydration and drug loading were carried out as above. To prepare sConA decorated liposomes, sConA-SH (with a final concentration of 25 μg/mL) was incubated with maleimide-bearing overnight at 4 °C. The conjugation efficiency of sConA was measured using the UV spectroscopic assay at 280 nm and the BCA protein assay according to the manufacturer's instruction.

2.4. Cell viability assay

Cell viability assay was conducted as reported [33]. Immortalized human corneal limbal epithelial cells (HCLE) were cultured in keratinocyte serum-free (KSF) medium (Invitrogen, Carlsbad, CA) supplemented with epidermal growth factor (EGF) and bovine pituitary extract, until cells reached 50% confluence. Culturing medium was switched to a 1:1 mixture of KSF medium and a combination of 1:1 unsupplemented low-calcium Dulbecco's minimum essential medium (DMEM) and F12 Ham's nutrient mixture (Invitrogen). For differentiation and stratification, HCLE cells were exposed to 1:1 DMEM/F12 medium (Mediatech, Manassas, VA) supplemented with newborn calf serum and EGF. Human corneal keratocytes (HCK) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37 °C in a 5% CO_2 environment.

Corneal keratocytes and differentiated HCLE cells were exposed to media containing liposomes (50 mM lipids), and cellular viability was assessed at 4, 8, 16, and 24 h, using the MTS colorimetric assay (CellTiter96 Proliferation Assay; Promega, Fitchburg, WI). Results are presented as percent viability from 4 separate experiments, normalized to cultured cells that were not exposed to test compounds.

Table 1
Characterization of liposomal formulations.

	PC/PG	Size/nm	PDI	Zeta potential/mV	EE[a]/%		LC[b]/%	
					DMED	TTX	DMED	TTX
LipA	16:0	2010	0.62	- 1 ± 1	58 ± 7	26 ± 5	0.07 ± 0.01	0.3 ± 0.1
LipB	15:1	446	0.33	- 12 ± 1	65 ± 5	44 ± 4***	0.08 ± 0.01	0.5 ± 0.1
LipC	14:2	502	0.33	- 25 ± 1	72 ± 3†††	42 ± 3***	0.08 ± 0	0.5 ± 0.1
LipD	12:4	449	0.32	- 29 ± 1	73 ± 6††	52 ± 3***	0.09 ± 0	0.6 ± 0.1
sConA-LipB	15:1	508	0.32	- 14 ± 1	62 ± 3	43 ± 5	0.07 ± 0.01	0.5 ± 0.1

Data are means ± SD (n = 3). Groups were compared using 2-way analysis of variance with Bonferroni post hoc test. ***p < 0.001 compared with TTX encapsulation efficiency in LipA; ††p < 0.01 and †††p < 0.001 compared with DMED encapsulation efficiency in LipA. Encapsulation efficiency of both drugs in LipB was not statistically significantly different from that in sConA-LipB.

[a] EE = Encapsulation efficiency, EE %= $\frac{\text{Measured drug loading}}{\text{Theoretical drug loading}} \times 100\%$.

[b] LC = Loading capacity, LC %= $\frac{\text{Weight of encapsulated drug}}{\text{Weight of liposome}} \times 100\%$.