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 α2-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); dexamethasone hydrochloride (DMED) was from R&D systems, Inc. (Minneapolis, MN), and dexamethasone 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-distearyl-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 dexamethasone 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 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 Barlett assay [14,31].

sConA was thioldated (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. Hydrolysis 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-SH 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% CO₂ 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.

<table>
<thead>
<tr>
<th>Liposome</th>
<th>PC/PG</th>
<th>Size/nm</th>
<th>PDI</th>
<th>Zeta potential/mV</th>
<th>EE/%</th>
<th>LC%</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipA</td>
<td>16:0</td>
<td>2010</td>
<td>0.62</td>
<td>-1 ± 1</td>
<td>58 ± 7 × 100%</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>LipB</td>
<td>15:1</td>
<td>446</td>
<td>0.32</td>
<td>-12 ± 1</td>
<td>65 ± 5 × 100%</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>LipC</td>
<td>14:2</td>
<td>502</td>
<td>0.33</td>
<td>-25 ± 1</td>
<td>72 ± 3 × 100%</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>LipD</td>
<td>12:4</td>
<td>449</td>
<td>0.32</td>
<td>-29 ± 1</td>
<td>73 ± 6 × 100%</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>sConA-LipB</td>
<td>15:1</td>
<td>508</td>
<td>0.32</td>
<td>-14 ± 1</td>
<td>62 ± 3 × 100%</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

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.

EE = Encapsulation efficiency, EE % = Weight of encapsulated drugs / Measured drug loading × 100%

LC = Loading capacity, LC % = Weight of encapsulated drugs / Weight of liposome × 100%
2.5. Ocular analgesia testing

Towel-restrained rats received formulations to the left eye, while the right eye served as an untreated control. All test formulations were administered in 30 μL. Corneal tactile sensitivity was tested using a Cochet–Bonnet esthesiometer (Luneau Ophthalmologie, Chartres, France) [33], which consists of a retractable nylon monofilament that exerts pressure inversely proportional to its length. At full extension, the monofilament is 6 cm. Testing began by gently placing the tip of the fully extended filament on the cornea, followed by application of sufficient force to slightly buckle the filament. A reflexive blink was considered a positive response. In the absence of blink response, the filament length was reduced by 0.5 cm. The process of filament length reduction and retesting was repeated until a blink was elicited. Animals were tested every 15 min after the administration of a test substance until animals had a positive response to filament length of 6.0 cm. The duration of nonresponsiveness to filament length of 0.5 cm (block0.5) was defined as the duration of complete corneal block. Similarly, nonresponsiveness to 2.0 cm (block2) and 6.0 cm (block6) were considered the duration of dense and partial corneal block, respectively.

2.6. Ocular persistence study

Towel-restrained rats received 30 μL fluorescently labeled formulations or R6G solution (2 mg/mL) to the left eye, with care taken to avoid spillage of dye from the eye. Imaging of the left eye with an IVIS in vivo imaging system (PerkinElmer, Waltham, MA) was conducted at different time points up to 24 h after administration. Imaging was done under isoflurane in oxygen anesthesia. Fluorescence on the eye was quantitated with the IVIS image software. The fluorescence at 5 min was set as 100%, and that at following time points were normalized accordingly.

2.7. Assessment for sedation

Rats were assessed for sedation from systemically absorbed DMED immediately after application of liposomes and every 15 min thereafter until ocular sensation returned to baseline. The sedation rating scale [34] is a 6-point scale ranging from 0 (asleep, eye fully closed, loss of right reflex) to 5 (fully awake, eyes wide open, grooming, feeding and ambulating).

2.8. Corneal debridement studies

To assess the effect of formulations on the rate of corneal epithelialization, rats were anesthetized with isoflurane in oxygen. A corneal trephine (3-mm diameter) was placed on the left cornea and filled with 50% ethanol for 90 s. This resulted in loosening and detachment of the ethanol-soaked cornea within the trephine. Care was taken to ensure no ethanol leaked outside the trephine. The eye was flushed with warm saline, and the detached cornea was gently scraped away using a sterile surgical blade. Immediately after the lesion was made, a single dose of 30 mL of the formulation was placed in the wounded eye. Photographs were taken after fluorescein instillation (FUL-GLO strips; Akorn, Inc, Lake forest, IL), using a Nikon D60 camera, every 4 h until complete reepithelialization. All images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) for corneal wound area estimation. The rate of corneal epithelialization was calculated as previously reported [33].

2.9. Statistical analysis

Data are presented as means ± SDs for in vitro data, and medians with 25th and 75th quartiles for in vivo data unless noted otherwise. Statistical differences in mean corneal block duration and average rate of corneal wound healing between the experimental groups were compared using 1-way analysis of variance with Bonferroni post hoc test. Data from in vitro analysis at various time points were compared using 2-way analysis of variance with Bonferroni post hoc test; P < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Characterization of liposomal formulations

Liposomal formulations were synthesized by hydrating thin lipid films (containing DMED free base) with TTX solution (in PBS, 1 mg/mL). Since these liposomes were intended to stay on the cornea for a relatively short period (days) the low-transition temperature lipids DOPC and DOPG were used, as they would provide relatively rapid drug release [35]. Cholesterol content was fixed at a 6:16 M ratio to total lipids (DOPC + DOPG) in all liposomal formulations. Since the negatively charged lipid DOPG could enhance the loading capacity and slow the release of TTX (a positively charged small molecule) [14], we prepared four populations of liposomes containing different DOPC/DOPG molar ratios, and studied their effect on drug loading and release (Table 1, Fig. 1A and B). LipA was formulated with DOPC (without DOPG) and cholesterol, demonstrating a neutral zeta potential and relatively low encapsulation efficiency for TTX (26.3%). LipA was 2010 nm in diameter with a high polydispersity (0.62). Incorporation of DOPG decreased the size of liposomes to around 500 nm. Increasing the DOPG content did not significantly affect the size and polydispersity of liposomes, but did decrease the zeta potential significantly. DOPG also greatly increased the efficiency of encapsulation of TTX. DOPG had a modest but statistically significant effect on the encapsulation of the hydrophobic DMED free base.

3.2. Drug release in vitro

The release profiles of TTX and DMED could be crucial for achieving long-acting corneal anesthesia. Liposomes (0.4 mL, 80 mg/mL of lipids) were placed in a dialysis bag (MWCO 50 KDa) against PBS at 37 °C. The release of TTX and DMED were determined by ELISA and HPLC, respectively. Release of DMED was substantially similar between all the formulations (no statistically significant differences, Fig. 1B). TTX release from LipB/TD was slightly higher than from LipC/TD and LipD/TD over the desired time frame of effect in vivo, indicating that
negative charge (imparted by DOPG) could extend TTX release to some extent. Given that the duration of local anesthesia in vivo depends on the release of local anesthetics above a threshold (unknown a priori) level [36] - i.e. higher release is more likely to be effective - LipB/TD was used in all subsequent in vitro and in vivo experiments (LipA/TD was not used due to its low encapsulation efficiency of TTX, see Table 1).

sConA was covalently conjugated on the surface of LipB (sConA-LipB). The unconjugated sConA was separated in the supernatant by centrifugation and quantified spectroscopically by UV measurements at 280 nm, calculated according to a published algorithm [37], showing that 73.5% of sConA was conjugated on the surface of liposomes. In another independent test, we detected 64.8% of sConA conjugating on liposome surface by using the BCA protein assay kit (ThermoFisher, Waltham, MA), which was close to the result of the aforementioned assay. As shown in Table 1 and Fig. 1C, sConA conjugation did not significantly alter the size, zeta potential or release profiles of LipB.

3.3. Cytotoxicity in vitro

To determine the cytotoxicity of LipB/TD and sConA-LipB/TD, human corneal keratocytes and human corneal limbal epithelial (HCLE) cells were incubated with media containing liposomes (40 μg/mL lipids) (Fig. 2). The viability of human corneal keratocytes and HCLE cells, as measured by the MTS assay was not reduced over 24 h incubation with LipB/TD or sConA-LipB/TD.

3.4. Ocular anesthesia after treatment with drug solutions and liposomal formulations

Animals received a single 30-μL eye drop of proparacaine (PPC, 5 mg/mL; a local anesthetic commonly used in ophthalmic practice [38]), TTX/DMED solution (1 mg/mL TTX and 100 μg/mL DMED), LipB/TD and sConA-LipB/TD (1 mg/mL TTX and 100 μg/mL DMED; ~60% DMED and ~40% TTX were encapsulated in liposomes, see Table 1). The liposomal formulations included encapsulated and free drugs; the latter were not removed after preparation of drug loaded liposomes so as to achieve immediate deep anesthetic effect. We used a validated rat model to measure corneal sensitivity to touch after anesthetic solutions were applied (Fig. 3, Table 2).

Animals treated with PPC achieved complete corneal block (block0.5, t = 0 min), extended block2 to 45 min, and block6 to 129 min. LipB/TD also did not significantly alter the duration of block0.5, but prolonged block2 to 2.3-fold of that of the TTX/DMED group (p < 0.05), and block6 to 2.3-fold of that of TTX/DMED solution group (p < 0.001). Topical application of 30 μL of sConA-LipB/TD greatly prolonged the duration of corneal anesthesia in comparison to the unmodified liposomal formulation, yielding 3.9-fold prolongation of complete block0.5, 282 ± 152, 608 ± 72, and 300 ± 60% of TTX/DMED solution, LipB/TD, and sConA-LipB/TD, respectively (p < 0.001) when sConA-LipB/TD was compared with LipB/TD.

Table 2

<table>
<thead>
<tr>
<th>Duration of corneal anesthesia achieved by various agents.</th>
<th>Block0.5 (min)</th>
<th>Block2 (min)</th>
<th>Block6 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td>9 ± 8</td>
<td>18 ± 7</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>TTX/DMED Solution</td>
<td>18 ± 13</td>
<td>45 ± 0</td>
<td>129 ± 13</td>
</tr>
<tr>
<td>LipB/TD</td>
<td>27 ± 25</td>
<td>105 ± 7514</td>
<td>300 ± 6014†††</td>
</tr>
<tr>
<td>sConA-LipB/TD</td>
<td>105 ± 5013†</td>
<td>282 ± 152†§§§</td>
<td>608 ± 72†§§§</td>
</tr>
</tbody>
</table>

Data are means ± SD (n = 5), and are compared by a one-way ANOVA with Bonferroni post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001 when sConA-LipB/TD was compared with PPC. †P < 0.05 and †††P < 0.001 when sConA-LipB/TD was compared with TTX/DMED solution. §P < 0.05 and §§§P < 0.001 when sConA-LipB/TD was compared with LipB/TD. ||||P < 0.001 when LipB/TD was compared with PPC. |||P < 0.05, ||||P < 0.001 when LipB/TD was compared with TTX/DMED solution.

Comparison to LipB/TD group). LipB or sConA-LipB formulations (without drug loading) did not achieve any effective corneal nerve block.

The Sedation Rating Scale (see Methods) was used to measure potential sedation from dexmedetomidine. All groups, regardless of treatment, received scores of 5 ± 0 (i.e., no sedation was observed).

3.5. Liposome persistence on the cornea

Liposomes were loaded with the fluorescent dye rhodamine 6G (R6G; loaded particles referred to as LipB/R6G and sConA-LipB/R6G; see Methods) to track their persistence on the cornea. R6G in saline, LipB/R6G and sConA-Lip-R6G were topically administered on the cornea, at a concentration of 2 mg/mL R6G (n = 5). Eyes were imaged by an in vivo imaging system (IVIS) 5 min after administration and the fluorescence was quantitated (Fig. 4A and C). Fluorescence at subsequent time points were normalized to the fluorescence at that first time point.

With free R6G solution, 45% of fluorescence was eliminated from the eye after 30 min. With LipB/R6G, 50% of dye was still detectable on the eye after 4 h, and fluorescence returned to baseline after more than 8 h. With sConA-modified liposomes, 57% of fluorescence was still retained on the eye after 12 h, and returned to baseline after more than 24 h. Those results were consistent with the durations of corneal nerve block observed with the various formulations (Fig. 4B). A large fraction of eye drops are lost from the ocular surface by tearing and nasolacrimal drainage, although some can persist in conjunctiva [39,40]. Irrespective of exact location on the ocular surface, it is clear that sConA greatly prolonged liposomal presence (Fig. 4). It is unlikely that liposomes of this size would be able to cross the corneal/conjunctiva [41], but the encapsulated drugs probably could [42], depending on their loading and physicochemical properties.
3.6. Effect on the rate of corneal healing

The combination of TTX and DMED has been reported to have no effect on corneal re-epithelialization [8]. However, ConA has been reported to delay the migration of corneal epithelial cells at high concentration in vitro [27], which could cause delayed corneal healing. To assess whether sConA-LipB/TD altered corneal healing, a 2 mm² area of the corneal epithelium was debrided, then exposed to a 2 mm-diameter circular area of corneal epithelium that was debrided, then exposed to a 2 mm² area of corneal epithelium. Data are means ± SD, n = 5.

4. Conclusion

Unmodified liposomes in the present study persisted on the cornea longer than R6G solution, which is consistent with previous studies [43]. Modification with ConA greatly prolonged liposomal persistence on the corneal surface and the resulting duration of corneal anesthesia. The longer period of intense analgesia (block0.5 and block2) afforded by the ConA-modified liposomes, a median of 5 h, could be of practical value in prolonging the time frame for performing surgery under topical anesthesia. The longer timeframe for lesser degrees of analgesia (block2), a median of 9 h, could be of benefit in postoperative care or in the management of some painful non-surgical conditions. Moreover, the modified liposomes loaded with local anesthetics did not have any effect on corneal wound healing, suggesting adequate biocompatibility. This approach to topical corneal drug delivery could be applied to other situations where drugs have to be delivered frequently to the eye [44,45].

Data availability

The data that support the findings of this study are available within the paper. All other data are available from the authors upon reasonable request.

Acknowledgement

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References


