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A Novel Quaternary Ammonium N-Propylamiodarone Bromide Provides Long-Lasting Analgesia Against Corneal Pain

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Purpose: Corneal pain is one of the most common eye symptoms caused by various types of epithelial injuries, including traumatic abrasion, chemical injury, ulcers, ultraviolet exposure, and infection. However, current therapeutic options for corneal pain are limited. In this study, we synthesized a novel quaternary ammonium compound, N-propylamiodarone bromide (NPA), and employed a rodent model of corneal injury to investigate whether NPA offers prolonged corneal analgesia through transient receptor potential vanilloid 1 (TRPV1) channel-mediated selective cellular entry, without hindering corneal epithelial recovery.

Methods: In the corneal injury model, 24 adult Wistar rats received a topical application of normal saline, oxybuprocaine, or NPA (n = 8 each), and corneal pain sensitivity was assessed using the von Frey technique. Another set of 32 rats with intact corneas received oxybuprocaine, capsaicin (a TRPV1 agonist), or NPA with or without capsaicin (n = 8 each), followed by a mechanical sensitivity evaluation. Potential adverse effects on normal epithelial recovery were evaluated using fluorescence and hematoxylin–eosin staining in an additional 8 rats with corneal injury.

Results: In the corneal injury model, NPA produced significantly longer-lasting analgesia than oxybuprocaine (duration of the maximum effect: 215 ± 11 vs 25 ± 2 min, $P < 0.001$). None of the animals presented any signs of eye irritability. In contrast to injured eyes, NPA alone did not significantly increase mechanical sensitivity in naïve eyes. However, the co-administration of NPA and capsaicin produced significantly longer-lasting corneal anesthesia than oxybuprocaine (duration of the maximum effect: 165 ± 15 vs 31 ± 2 min, $P < 0.001$). NPA did not hamper wound healing.

Conclusion: The novel quaternary ammonium NPA produced long-lasting analgesia against corneal injury without hampering corneal recovery, suggesting that it is a potential candidate for analgesic medicine targeting corneal pain.

Keywords: analgesics, capsaicin, corneal injury, pain, TRPV1 channel

Introduction

The cornea, being the most innervated organ in the human body, houses sensory neurons that are primarily associated with pain.[1–4] As a result, stimuli that are harmless to other parts of the body can cause corneal pain.[3–7] Therefore, corneal injuries, such as traumatic abrasion, chemical injury, ulcers, ultraviolet exposure, and infection, can result in intense pain.

Topical ophthalmic anesthetics, such as oxybuprocaine, efficiently alleviate corneal pain in emergency and general care settings. However, ophthalmic prescriptions for analgesic purposes often result in abuse,[8–10] as current ophthalmic anesthetics are short-acting with analgesic effects lasting only 10–30 min. The repetitive use of these anesthetics should be avoided because of concerns regarding corneal complications and delayed corneal healing.[8–12] Thus, the current therapeutic options for corneal pain are limited to non-steroidal anti-inflammatory drugs, acetaminophen, steroids, opioids, pressure patching, cooling, cycloplegics, and bandage contact lenses.[13,14] However, these options are often

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ineffective or insufficient to relieve corneal pain[13,15] and can produce harmful side effects, such as kidney injury and respiratory depression. Therefore, novel ophthalmic analgesics with long-lasting effects that do not hamper healing have been sought over the past few decades.

Traditional sodium channel blockers are tertiary amines that exist in equilibrium in charged and uncharged forms in the extracellular space.[16,17] The uncharged forms of the compounds permeate the nerve membrane and enter the intracellular space, where they become charged and bind to the target site of the sodium channels inside the cell.[16,17] Sodium channel blockers must be in their uncharged form to permeate the membrane and exert analgesic action. However, under specific conditions where open-state large-pore channels such as transient receptor potential vanilloid 1 (TRPV1) channels exist on the surface of target cells, even permanently charged quaternary ammonium cations can selectively enter the cells, blocking the sodium current.[18,19] Given that those large-pore transducer channels exist exclusively on nociceptors, this strategy renders selective pain blocking without affecting motor neurons or other cell types (cell-type selectivity). Another advantage of this strategy is the long-lasting analgesic effects. Charged compounds cannot enter off-target cells and are likely to remain in the extracellular space for longer than traditional local anesthetics.[16,20,21]

In this study, we synthesized permanently charged quaternary ammonium N-propylamiodarone bromide (NPA) for a novel ophthalmic analgesic that targets corneal pain. We hypothesized that NPA would provide long-lasting corneal analgesia via large-pore channel-mediated selective cellular entry without hindering the normal healing process.

Materials and Methods

All experiments adhered to ethical standards set forth by the National Institutes of Health, National Academy of Science, Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the International Association for the Study of Pain Guidelines for the Care and Use of Laboratory Animals. The experimental protocol was reviewed and approved by the University of Yamanashi Animal Care Committee (approval code: A4-6).

Animals

Adult Wistar rats (10–12 weeks old, males and females) were procured from Japan SLC (Tokyo, Japan). The rats were group-housed at 23 ± 2 °C with a 12-h light–dark cycle and had free access to food and water. All neurobehavioral experiments were conducted by a single trained observer blinded to the grouping between 9:00 and 18:00, and a temperature of 23 ± 2 °C was maintained under normal lighting conditions. Prior to the experiments, the animals underwent 1 h of acclimatization to the testing environment over two consecutive days.

Chemicals

N-[2-[4-(2-Butylbenzofuran-3-carbonyl)-2,6-diiodophenoxy]ethyl]-N,N-diethylpropan-1-aminium bromide or NPA was custom-synthesized according to the pathway described in Figure 1 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Briefly, amiodarone was alkylated with propyl trifluoromethanesulfonate in a 1,2-dichloroethane solution was combined and stirred at room temperature for 5.5 days. After reaction completion, the mixture was concentrated under reduced

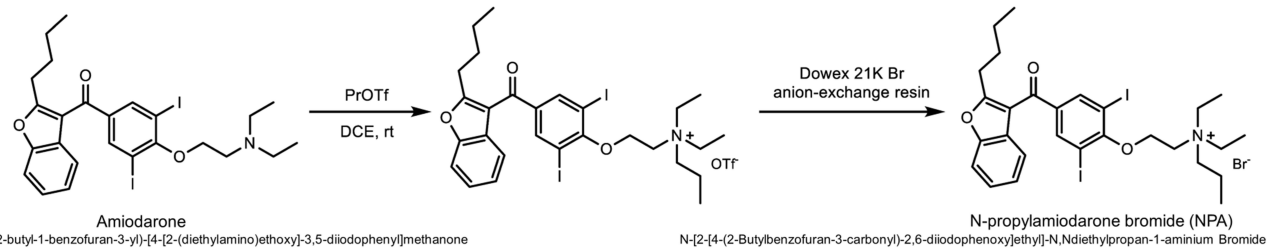

Figure 1 A novel quaternary ammonium N-propylamiodarone bromide was custom-synthesized using amiodarone as a parent compound.
Abbreviations: DCE, 1,2-dichlo roethane; PrOTf, propyl trifluoromethanesulfonate.

pressure. The residue was purified to yield intermediate N-[2-[4-(2-Butylbenzofuran-3-carbonyl)-2,6-diiodophenoxy] ethyl]-N,N-diethylpropan-1-aminium triflate, which was subsequently dissolved in methanol and charged onto an anion exchange resin (Dowex 21K, Sigma-Aldrich Japan, Tokyo, Japan). The aqueous fraction was concentrated under reduced pressure and dried to obtain the crude bromide salt of NPA. Methylene chloride was introduced, and the resulting precipitate was filtered as a solid. The final compound was obtained as a white powder. The molecular structure and purity exceeding 98% were verified by high-performance liquid chromatography-mass spectrometry (HPLC-MS) and nuclear magnetic resonance. NPA was dissolved in water at 80 °C, cooled to 20 °C, and then diluted with normal saline containing 5% dimethyl sulfoxide (DMSO) and 5% Tween20. The molecular stability of the compound at 80 °C was confirmed by HPLC-MS. Oxybuprocaine and capsaicin (Fujifilm Wako Pure Chemical, Osaka, Japan) and ruthenium red (Sigma-Aldrich Japan, Tokyo, Japan) were dissolved in 5% DMSO and 5% Tween20 in a normal saline solution. All drugs were freshly prepared before use. Dosing and preparation of drugs were performed by an individual different to the person conducting neurobehavioral experiments to ensure blinding.

Corneal Epithelial Injury Model and Pain Treatment

Corneal epithelial injury was induced following established procedures.[22] In brief, under 2% isoflurane anesthesia and 10× microscopic observation, a 4-mm trephine marked the left central cornea, and epithelial injury was created with a rotating burr (Ideal Microdrill; BioResearch, Nagoya, Japan) in 24 rats. After recovery from anesthesia, either normal saline, 12 mm oxybuprocaine, or 12 mm NPA was applied on the eye surface at a volume of 50 µL (n = 8 each, 4 females and 4 males). The drug concentration was determined based on the common clinical dose for oxybuprocaine (0.4% or approximately 12 mm).

Corneal Pain Sensitivity

Corneal pain sensitivity was evaluated using the von Frey technique[23] at baseline, and at intervals of 5, 10, 20, 30, 45 min and 1, 2, 3, 4, 5, and 6 h post-drug administration. The rats were placed in a plastic observation chamber, and their responses to mechanical stimuli were measured using a graded series of five von Frey filaments with bending forces of 0.04, 0.07, 0.16, 0.4, and 0.6 g. Starting from the lightest filament (0.04 g), each filament was applied perpendicular to the center of the cornea at the bending point for 1s. The pain sensitivity threshold was determined as the filament force that elicited a positive response—blinking, wiping, or escaping—indicating the animal's clear perception of a noxious stimulus. To avoid test-induced corneal epithelial injury, the threshold was recorded as 1.0 g if the animal did not respond to the heaviest filament (0.6 g).

Eye Irritability

To assess the potential eye irritability of the drugs, the number of wiping bouts during 1 min following topical administration was recorded. Additionally, any signs of eye irritability, such as hyperemia, corneal opacity, chemosis, or epiphora, were evaluated throughout the experiment and recorded as severe (2), mild (1), or absent (0).

Evaluation of Anesthetic Effects in the Naïve Corneas

In another set, 32 rats with naïve corneas were randomly assigned to 4 groups: 12 mm oxybuprocaine, 12 mm NPA with or without the TRPV1 agonist capsaicin (1%), and 1% capsaicin alone (n = 8 each, 4 females and 4 males). Similar to the previous experiment, corneal mechanical sensitivity was assessed using a graded series of five von Frey filaments at baseline, and at 5, 10, 20, 30, and 45 min and 1, 2, 3, 4, 5, and 6 h post-drug administration. Similar to the previous experiment, eye irritability was assessed in the oxybuprocaine and NPA groups.

Dose-Response Relationship and Corneal Pain Treatment at the Half Maximal Effective Concentration (EC_{50})

The dose-response relationship of NPA on corneal pain was assessed in comparison with oxybuprocaine. Similar to the previous experiments, another set of corneal injury model male rats were prepared, and various concentrations of NPA (0.12, 0.36, and 1.2 mm) or oxybuprocaine (0.36, 1.2, and 3.6 mm) were applied on the eye surface at a volume of 50 µL (n = 5/group). Another three male rats were used to obtain the data for vehicle alone (normal saline with 5% DMSO and

5% Tween20). The anesthetic/analgesic effects were assessed by applying a 0.6-g von Frey filament perpendicular to the center of the cornea at the bending point for 1s as described above. The drug at a certain concentration was considered effective when the animal did not show any painful responses (described above) to the stimuli. In addition, the data for 12 mm NPA and oxybuprocaine obtained from previous experiments were incorporated in this dose-response study. The EC_{50}, defined as the concentration that is needed to provide complete anesthesia/analgesia (no response to the 0.6-g von Frey filament) in 50% of animals, was calculated (refer to the Statistics section), and the analgesic effect of each drug at their EC_{50} against corneal pain was assessed over 360 min using another set of 12 male rats with corneal injury (6/group) and the same methods used in the previous experiment.

Influence of TRP Channel Blockade on the Analgesic Effect of NPA

To confirm the involvement of TRP channels in the mechanism underlying the analgesic effect of NPA against corneal pain, the effect of ruthenium red (a TRP channel blocker) was assessed using another set of rats with corneal injury (NPA alone vs ruthenium red pre-treatment plus NPA). After corneal epithelial injury as described earlier, rats with ruthenium red pre-treatment received ruthenium red solution (50 μL, 100 mm) on the eye surface 15s prior to NPA administration (50 μL, 1.0 mm = EC_{50}). To minimize the number of animals sacrificed, this experiment was performed as a part of the previous experiment (NPA treatment at its EC_{50}), and the data for the control group (1.0 mm NPA treatment alone) was reused.

Corneal Healing Model

Corneal Epithelial Injury and Drug Administration

Corneal epithelial injury was induced in both eyes of an additional 8 rats (4 females and 4 males). After recovering from isoflurane anesthesia, 50 μL of 12 mm NPA or normal saline was randomly applied on each eye. Corneal wound healing was assessed over 48 h post-injury using fluorescence and hematoxylin–eosin staining. As an additional experiment, the influence of multiple administrations of NPA on the corneal epithelial recovery was assessed using another set of 8 rats (4 females and 4 males). In this additional experiment, 50 μL of 12 mm NPA or normal saline was randomly applied on each eye at 0, 6, 12, 24, 30, and 36 h after injury, assuming thrice daily treatment.

Fluorescence Staining

Under isoflurane anesthesia, the corneal epithelial surface was stained with fluorescein and recorded using a camera under blue light and microscopy at 0, 24, and 48 h post-corneal injury. The defect area was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The relative defect area was calculated as the difference divided by the initial injury area.

Hematoxylin–Eosin Staining

Following fluorescein photography 48 h post-injury, the rats were euthanized under 5% isoflurane anesthesia, and the eyes were immediately enucleated and fixed with methanol and paraformaldehyde (Superfix KY-500, Kurabo Industries Ltd., Osaka, Japan) for 2 h at 23 °C, then overnight at 4 °C, followed by thorough washing with distilled water. The central corneas were dissected, trimmed, and stored in 70% ethanol at 4 °C until paraffin addition. Corneal sections were deparaffinized and stained with hematoxylin and eosin for 5 and 10 min, respectively. The epithelial layer thickness of the central cornea was observed and measured using a microscope.

Statistical Analysis

Statistical analyses utilized GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Mechanical nociceptive thresholds over 6 h and epithelial defect areas over 48 h underwent a repeated measures two-way analysis of variance (ANOVA) followed by Sidak or Dunnett multiple comparison tests as appropriate. The frequency of wiping bouts was analyzed using a two-tailed *t*-test or one-way ANOVA as appropriate. The duration of maximum effect (mechanical threshold = 1.0 g) between two groups was analyzed using a two-tailed *t*-test. Dose-response curves for drugs were analyzed and compared using a four-parameter logistic model. The statistical significance of the difference between the curves was assessed with an *F*-test. The Wilcoxon test was used to assess epithelial thickness. Experimental sample size

was determined to detect a difference in the responses of 15% while providing 80% power with an α level of 0.05 (G*Power 3.1.9.3). Data are presented as mean ± SEM. Statistical significance was set at $P < 0.05$.

Results

Analgesic Effect of NPA on Corneal Injury

In the corneal injury model, NPA significantly outperformed oxybuprocaine in prolonging analgesia, (duration of the maximum effect: 215 ± 11 vs 25 ± 2 min, n = 8 each, $P < 0.001$, Figure 2). No sex difference was observed in the analgesic effect of oxybuprocaine and NPA ($P = 0.837$ and 0.761 respectively, Figure 3C). The frequency of wiping bouts within 1 min post-administration did not vary significantly across groups (saline: 4.3 ± 0.7, oxybuprocaine: 5.1 ± 0.8, NPA: 5.25 ± 0.9, $F [2, 21] = 0.348$, n = 8 each, $P = 0.710$). None of the animals presented any signs of eye irritability (score 0 for all symptoms assessed in all rats).

Anesthetic Effect of NPA on Naïve Eyes

In contrast to the injured eyes, NPA alone did not significantly increase the mechanical threshold in naïve eyes (Figure 3A). However, when combined with capsaicin, it produced significantly longer-lasting corneal anesthesia than oxybuprocaine (duration of the maximum effect: 165 ± 15 vs 31 ± 2 min, n = 8 each, $P < 0.001$, Figure 3). No sex difference was observed in the analgesic effect of oxybuprocaine and NPA with capsaicin ($P = 0.381$ and 0.644 respectively, Figure 3C). Capsaicin by itself did not produce corneal anesthesia. The frequency of wiping bouts within 1 min post-administration did not vary significantly between the oxybuprocaine and NPA groups (3.4 ± 1.4 vs 4.3 ± 1.4, n = 8 each, $P = 0.658$). None of the animals in the oxybuprocaine and NPA groups presented any signs of eye irritability (score 0 for all symptoms assessed).

Dose-Response Relationship and Corneal Pain Treatment at EC_{50}

As shown in Figure 4, the potency of NPA was significantly greater than that of oxybuprocaine with the EC_{50} of 1.0 and 3.1 mm, respectively ($P = 0.002$). At their EC_{50}, NPA produced a significantly longer duration of analgesia against corneal pain than oxybuprocaine ($F [1, 10] = 9.840$, n = 6 each, $P = 0.011$, Figure 4B).

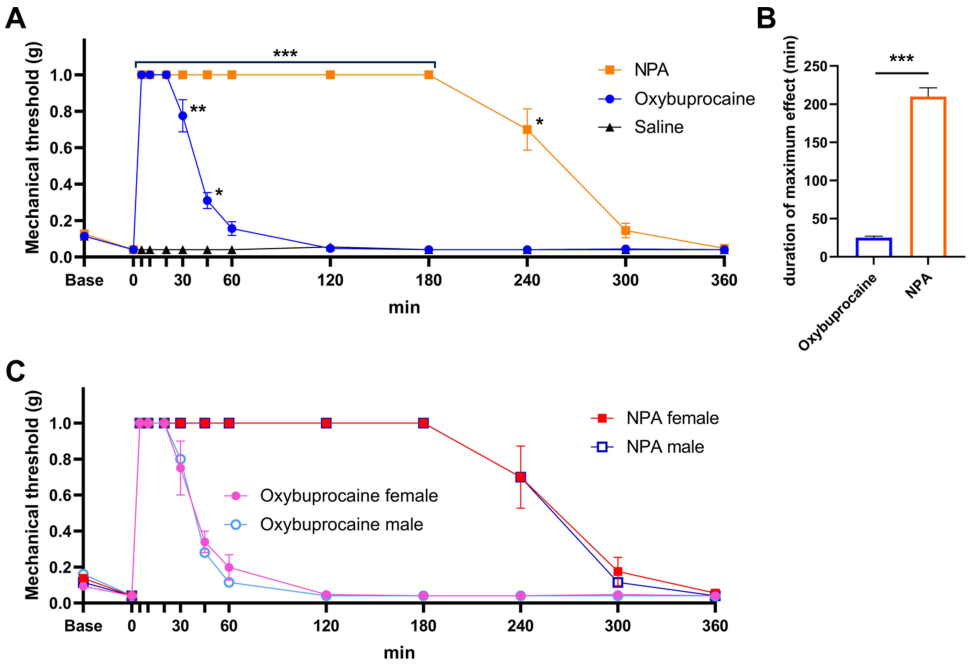

Figure 2 N-propylamiodarone produced long-lasting analgesia against corneal injury pain (n = 8/group). (**A**) Threshold for mechanical stimuli assessed using von Frey technique. (**B**) Duration of maximum effect (mechanical threshold = 1.0 g). (**C**) Sex difference in the threshold for mechanical stimuli assessed using von Frey technique.
Notes: *P < 0.05, **P < 0.01, ***P < 0.001 vs Saline. ***P < 0.001 vs Oxybuprocaine.
Abbreviation: NPA, N-propylamiodarone bromide.

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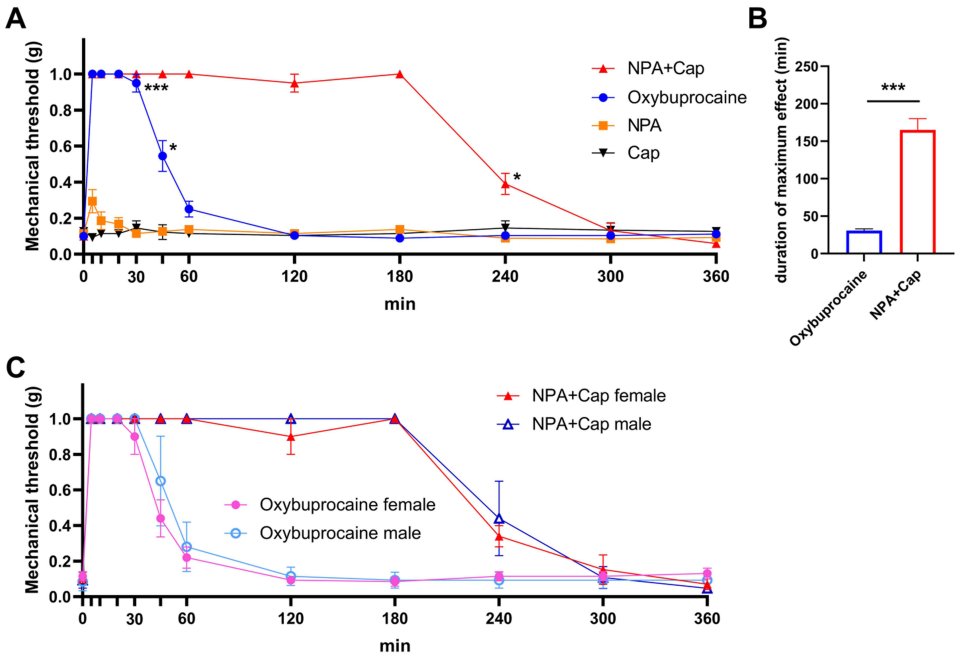

Figure 3 The co-administration of N-propylamiodarone bromide and capsaicin produced long-lasting anesthesia in naïve corneas (n = 8/group). (**A**) Threshold for mechanical stimuli assessed using von Frey technique. (**B**) Duration of maximum effect (mechanical threshold = 1.0 g). (**C**) Sex difference in the threshold for mechanical stimuli assessed using von Frey technique.
Notes: *P < 0.05, ***P < 0.001 vs baseline of the same group. ***P < 0.001 vs Oxybuprocaine.
Abbreviations: Cap, capsaicin; NPA, N-propylamiodarone bromide.

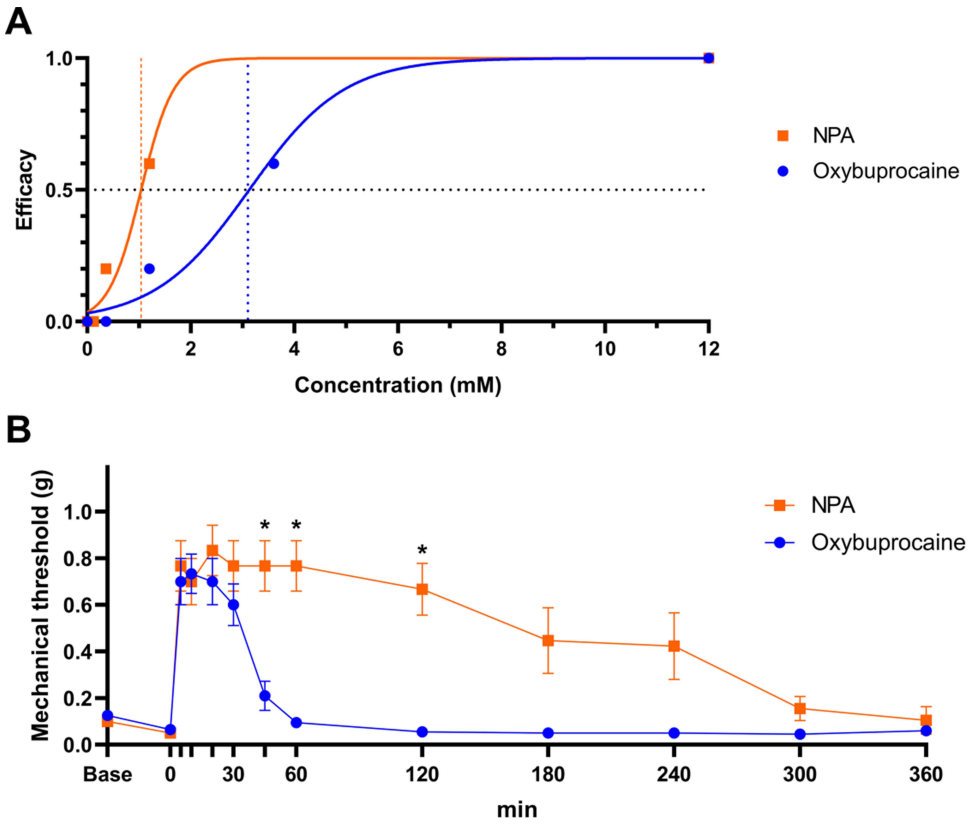

Figure 4 The potency of NPA was significantly greater than that of oxybuprocaine. (**A**) Dose-response relationship of NPA and oxybuprocaine on corneal pain. (**B**) Threshold for mechanical stimuli assessed using von Frey technique.
Note: *P < 0.05 vs Oxybuprocaine.
Abbreviation: NPA, N-propylamiodarone bromide.

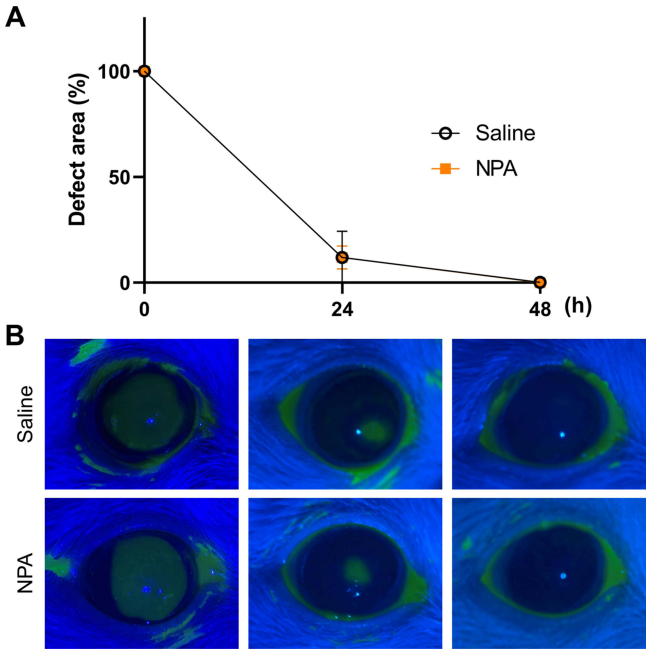

Figure 5 N-propylamiodarone did not hamper corneal wound healing (n = 8/group). (**A**) Corneal epithelial defect area after injury. (**B**) Representative images of corneal epithelial surface stained with fluorescein.
Abbreviation: NPA, N-propylamiodarone bromide.

Influence of TRP Channel Blockade on the Analgesic Effect of NPA

Pre-treatment with ruthenium red attenuated the analgesic effect of NPA ($F [1, 10] = 5.690$, n = 6 each, $P = 0.038$, Supplementary Figure 1).

Influence of NPA on Corneal Healing

As shown in Figure 5, NPA did not hamper wound healing. The epithelial defect area after injury was not significantly different between the NPA and normal saline groups (24 h: 11.2% ± 1.9% vs normal saline 11.2% ± 4.4%, $P = 0.461$, 48 h: 0.14% ± 0.14% vs 0% ± 0%, $P > 0.999$). In both the NPA- and normal saline-treated rats, the corneal surface was almost fully recovered by the epithelial layer 48 h after injury. In addition, the central epithelial thickness at 48 h was similar between groups (42.7 ± 2.9 mm vs 40.4 ± 3.3 mm, n = 8 each, $P = 0.637$, Figure 6). Additionally, there was no

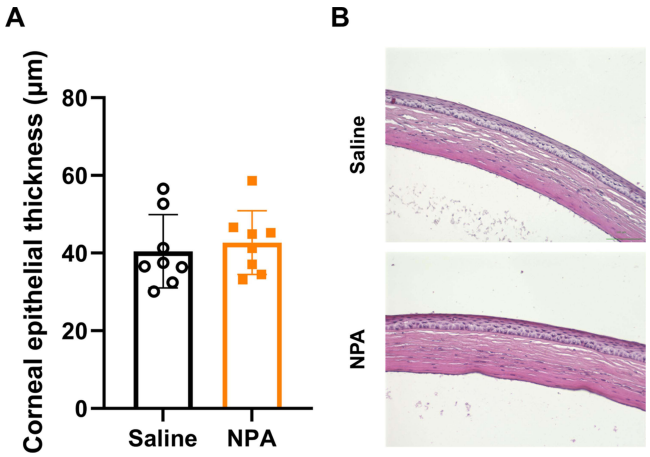

Figure 6 N-propylamiodarone did not influence corneal wound healing (n = 8/group). (**A**) Epithelial thickness of the central cornea at 48 h after injury (n = 8/group). (**B**) Representative images of the central cornea stained with hematoxylin and eosin.
Abbreviation: NPA, N-propylamiodarone bromide.

significant difference in corneal recovery between the multiple administrations of NPA and normal saline (24 h: 17.0% ± 3.9% vs normal saline 16.2% ± 2.1%, P = 0.742, 48 h: 0.59% ± 0.21% vs 0.36% ± 0.30%, n = 8 each, P = 0.563) (Supplementary Figure 2).

Discussion

Several efforts have been made to develop analgesics that utilize nociceptor-selective cellular entry via open-state large-pore channels, such as TRPV1 channels.[19,24,25] QX314, a quaternary derivative of lidocaine, has shown extended effects when co-administered with a TRPV1 channel agonist.[20] However, because TRPV1 channel activation itself evokes intense pain, the requirement of TRPV1 agonist co-administration has been the barrier for this strategy to be applied to clinical settings. Interestingly, large-pore channels are in an open-state in injured or irritating tissues.[26,27] In this situation, permanently charged sodium channel blockers can penetrate nociceptors through these large-pore channels and provide analgesic effects.[19,21] Consistent with these previous findings, NPA alone produced long-lasting analgesia without the need for capsaicin co-administration in the injured cornea in the current study.

NPA was synthesized as a quaternary derivative of amiodarone, but it shares the core molecular structure with the parent compound to retain its basic chemical and pharmacological characteristics, except for the electrostatic state. While amiodarone is not typically categorized as a sodium channel blocker, it demonstrates inhibitory activity against voltage-gated sodium channels similar to that of lidocaine.[28] In addition, with its high protein binding ratio (96%) and long half-life (up to 80 h),[29,30] amiodarone reportedly has the potential to be a lead compound for potent and long-acting analgesics.[31–33] However, amiodarone can exert pharmacological effects on off-target cells in addition to nociceptors. Furthermore, amiodarone causes irritability when injected directly into local tissues.[34] However, this is not a barrier for topical or ophthalmic applications, because the cornea is abundantly innervated and has numerous nerve endings in its shallow layer, and drugs do not need to be injected into the tissue. Additionally, the cell-type selectivity and positive charge of NPA prevents it from affecting other cell types, which is considered beneficial for minimizing possible adverse off-target effects. Therefore, topical application of NPA to the cornea can provide long-acting analgesic effects without causing tissue irritability. Indeed, the present results confirmed the efficacy of NPA in providing long-lasting pain relief without worsening corneal injury or affecting epithelial recovery. The analgesic effect of NPA lasted for at least 4 h, which was remarkably longer than that of oxybuprocaine at the same concentration. Our results indicated that NPA is a potential novel long-acting analgesic for the treatment of corneal pain. NPA would have advantages over other analgesics, such as non-steroidal anti-inflammatory drugs, acetaminophen, and steroids that are often insufficient to relieve pain or have systemic side effects. In addition to corneal injury pain, NPA might also be applicable to other pathologies including post-operative pain, dry eye, and allergic conditions, as large-pore transducer channels are involved in these pathologies.[2,35,36] On the other hand, since NPA requires open-state large-pore channels to permeate the cell membrane, NPA cannot be used as a local anesthetic for medical procedures.

This study has several limitations. Although we demonstrated the involvement of TRP channels underlying the analgesic effect of NPA against corneal injury pain, the possible involvement of other pathways that NPA might have utilized to permeate cells could not be excluded. Future extensive studies that use advanced techniques such as patch-clamp electrophysiology or high-resolution imaging are needed to verify the detailed mechanism. In addition, although NPA did not cause eye irritability in this study, the potential adverse effects should be carefully evaluated before clinical translation.

Conclusions

The novel quaternary ammonium NPA delivered long-lasting analgesia against corneal injury without hindering healing. The present results suggest that NPA is a potential candidate for ophthalmic analgesics targeting corneal pain.

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Disclosure

Masakazu Kotoda is named as the inventor on a patent pending covering the design and use of NPA (application number: 2023-146529). The other authors declare that no competing interests exist.

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