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(54) Title: HALOGENATED XANTHENE-CONTAINING TOPICAL ANTI-GRAM-POSITIVE BACTERIAL OPHTHALMIC COMPOSITION AND METHOD

(57) Abstract: The present invention contemplates a topical ophthalmic system for treating a Gram-positive bacterially-infected mammalian eye. The system comprises an ophthalmic composition containing a halogenated fluorescein or a pharmaceutically acceptable salt or ester thereof dissolved or dispersed in an aqueous ophthalmic carrier and present in an anti-bacterial keratitis-treating effective concentration of about 0.2 µg/mL to about 50 µg/mL. The ophthalmic composition has a pH value of about 6.5 to about 7.6, a viscosity of about 10 to about 300 cps and an osmolality of about 270 mOsm/ kg to about 340 mOsm/ kg. The ophthalmic composition is present in a vessel opaque to actinic light. Also contemplated is a method of treating a mammal having a Gram-positive bacterial infection of an eye by administering the ophthalmic composition to the infected eye and maintaining the treated eye in the substantial absence of actinic light for about 3 hours to about 12 hours.

HALOGENATED XANTHENE-CONTAINING TOPICAL ANTI-GRAM-POSITIVE BACTERIAL OPHTHALMIC COMPOSTION AND METHOD

Description

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to US application Serial No. 63/294252, filed on December 28, 2021, whose disclosures are incorporated herein by reference.

BACKGROUND ART

Corneal disease is a leading cause of monocular blindness worldwide, especially affecting marginalized populations. Corneal opacities, which are largely caused by infectious keratitis, are the fourth leading cause of blindness globally and are responsible for 10% of avoidable visual impairment in the world's least resourced countries. About two million people develop a corneal ulcer every year in India alone. In the United States, infectious keratitis is often associated with contact lens wear, but in under-resourced countries it is more commonly caused by ocular trauma sustained during agricultural work. [Austin et al., *Ophthalmology* **124(11):**1678-1689 (2017).]

A corneal ulcer, keratitis, is an open sore on the cornea and is a common human eye condition. It can be caused by trauma, particularly with vegetable matter, as well as chemical injury, contact lenses and infections. Other eye conditions can cause corneal ulcers, such as entropion,

distichiasis, corneal dystrophy, and keratoconjunctivitis sicca (dry eye).

Many micro-organisms cause infective corneal ulcer. Among them are bacteria, fungi, viruses, protozoa, and chlamydia.

Bacterial keratitis can be caused by a number of bacteria, including *Staphylococcus aureus*, *Streptococcus viridans*, *Escherichia coli*, *Enterococci*, *Pseudomonas*, *Nocardia*, *N. gonorrhoea* and many other bacteria. The increasing emergence of multidrug-resistant (MDR) Gram-positive bacteria is a major public health threat [Bassetti et al., *Annal. Clinic. Microbiol. Antimicrob.* **12**:1-15 (2013); Butler et al., *J. Antibiot.* **66**:571-591 (2013); and Woodford et al., *J. Infect.* **59**:S4-16 (2009)]. Particularly, MDR strains of *Staphylococcus*, *Enterococcus*, and *Streptococcus spp.* have a significant impact on morbidity and mortality [Dupont et al., *J. Antimicrob. Chemother.* **66**:2379-2385 (2011)].

Fungal keratitis causes deep and severe corneal ulceration. It is typically caused by *Aspergillus* sp., *Fusarium* sp., *Candida* sp., as also *Rhizopus*, *Mucor*, and other fungi. The typical feature of fungal keratitis is slow onset and gradual progression, where signs are much more than the symptoms. Small satellite lesions around the ulcer are a common feature of fungal keratitis and a condition involving inflammatory cells in the anterior chamber of the eye (hypopyon) is observed.

Viral keratitis causes corneal ulceration. It is caused most commonly by herpes simplex, herpes zoster, and adenoviruses. It can also be caused by coronaviruses and many other viruses.

Herpes virus causes a dendritic ulcer, which can recur and relapse over the lifetime of an individual. Herpes simplex virus (HSV) keratitis affects an estimated 500,000 people in the United States and an estimated 1.5 million globally. It is the most common cause of unilateral infectious corneal blindness in much of the developed world. [Austin et al., *Ophthalmology* **124(11):**1678-1689 (2017).]

Viral keratitis differs from bacterial and fungal keratitis in that it can become chronic and recurrent. Besides being a painful, sight-threatening infection, HSV keratitis has been shown to significantly impact quality of life even when patients are not experiencing an active infection. Less common forms of viral keratitis include varicella-zoster virus (VZV) keratitis, and cytomegalovirus (CMV) keratitis. [Austin et al., *Ophthalmology* **124(11):**1678-1689 (2017).]

Protozoa infection such as *Acanthamoeba* keratitis is characterized by severe pain and is associated with contact lens users swimming in pools.

Superficial ulcers involve a loss of part of the epithelium. Deep ulcers extend into or through the stroma and can result in severe scarring and corneal perforation. Descemetoceles occur when the ulcer extends through the stroma. This type of ulcer is especially dangerous and can rapidly result in corneal perforation, if not treated in time.

The location of the ulcer depends somewhat on the cause. Central ulcers are typically caused by trauma, dry eye, or exposure from facial nerve paralysis or exophthalmos. Entropion, severe dry

eye, and trichiasis (inturning of eyelashes) may cause ulceration of the peripheral cornea. Immune-mediated eye disease can cause ulcers at the border of the cornea and sclera. These include Rheumatoid arthritis, rosacea, and systemic sclerosis, which lead to a special type of corneal ulcer called Mooren's ulcer. Mooren's ulcer has a circumferential crater like depression of the cornea, just inside the limbus, usually with an overhanging edge.

Proper diagnosis is essential for optimal treatment. The cause of the ulcer is to be decided; whether infective or non-infective.

Bacterial corneal ulcer normally requires intensive fortified antibiotic therapy to treat the infection. Fungal corneal ulcers require intensive application of topical anti-fungal agents. Viral corneal ulceration caused by herpes virus may respond to anti-viral agents like topical acyclovir ointment instilled at least five times a day. Alongside, supportive therapy like pain medications are typically administered, including topical cycloplegics like atropine or homatropine to dilate the pupil and thereby stop spasms of the ciliary muscle.

Superficial ulcers may heal in less than a week. Deep ulcers and descemetoceles may require conjunctival grafts or conjunctival flaps, soft contact lenses, or corneal transplant. Proper nutrition, including protein intake and vitamin C are usually advised. In cases of keratomalacia, where the corneal ulceration is due to a deficiency of vitamin A, supplementation of the vitamin A by oral or intramuscular route is given. Drugs that

are usually contraindicated in corneal ulcer are topical corticosteroids [Alhassan et al., *Cochrane Database Syst Rev.* **1** (1):CD006131 (2014)] and anesthetics—these should not be used on any type of corneal ulcer because they prevent healing, may lead to superinfection with fungi and other bacteria, and will often make the condition much worse.

'Red eye', 'conjunctivitis' and 'corneal ulcer/keratitis' were among the problems commonly referred to. Conjunctivitis is a common condition that causes dilation of the conjunctival blood vessels and results in inflammation. Both viral and bacterial conjunctivitis present with a red eye and are highly contagious. Assessment should include checking visual acuity and examination with a torch or slit lamp. Fluorescein drops should be instilled in the conjunctival sac and the eye viewed with the cobalt blue light of the slit lamp or funduscope, to rule out any signs of corneal ulceration or infection.

Viral conjunctivitis is the most common cause of infectious conjunctivitis. This infection is more common in adults than in children. Most cases are caused by adenovirus. Occasionally, herpes simplex or zoster virus is responsible. Patients can generally be advised that viral conjunctivitis is self-limiting, as there are no specific treatments.

Bacterial conjunctivitis, although a less frequent cause of conjunctivitis, is more common in children. The most common bacteria are *Haemophilus influenza*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. The use of antibiotic eye drops is associated with improved rates of clinical

and microbiological remission. A broad-spectrum topical antibiotic is often recommended.

Infection of the cornea (microbial keratitis) is an ophthalmic emergency requiring immediate attention as it can progress rapidly. It is one of the most common causes of visual impairment in working age adults. In the USA, about 30,000 cases of microbial keratitis are reported annually [Sharma et al., *Ocul Surf* **15**:670-679 (2017)].

Bacterial infection is the most common cause of infectious keratitis. Common causal bacteria include *S. aureus*, coagulase-negative staphylococci, *S. pneumoniae* and *Pseudomonas aeruginosa* [Teweldmedhin et al., *BMC Ophthalmol* **17**:212 (2017)]. *P. aeruginosa* is the most common microorganism implicated in bacterial keratitis among contact lens wearers.

Although bacterial ulcers are usually responsive to treatment with available topical antibiotic drops as discussed below, an increase in the rates of antibiotic resistant infections such as methicillin resistant *Staphylococcus aureus* (MRSA) in North America has caused concern. The United States Center for Disease Control (CDC) estimates that 2 million people are infected with drug resistant microbes each year. Approximately 80% of ocular isolates of MRSA in the US have been reported to be resistant to the most commonly prescribed antibiotic class, the fluoroquinolones. [Austin et al., *Ophthalmology* **124(11)**:1678-1689 (2017).]

Topical antibiotics are the current mainstay of treatment and options include monotherapy with fluoroquinolones (ciprofloxacin

0.3% or ofloxacin 0.3% 1-2 drops hourly for 48 hours, then every 4 hours until healed) or fortified aminoglycoside/cephalosporin combinations (fortified cefalotin 5% plus gentamicin 0.9% 1-2 drops hourly for 48 hours, then reduce frequency according to treatment response). These regimens have similar effectiveness, but fluoroquinolones reduce the risk of chemical conjunctivitis and ocular discomfort. Compared to ofloxacin, ciprofloxacin increases the risk of white corneal precipitates. Occasionally, corneal grafting may be needed to eradicate the organism or repair damage. Although apparently effective, hourly treatment with eye drops is a difficult regimen to follow for many patients.

Chloramphenicol is the most common first-line antibiotic prescribed for red eye. Several commercial products are available, both as eye drops and as an ophthalmic ointment. Illustratively, an ophthalmic solution containing chloramphenicol at 0.25% and 0.5% and an ophthalmic ointment containing chloramphenicol at 1% are available under the trade name ^{Pr}PENTAMYCETIN® from Sandoz Canada Inc.

The dosage and administration instructions for the ophthalmic solution state only "2 drops of the ophthalmic solution to the affected eye(s) every 3 hours or more often if necessary". The dosage and administration instructions for the ophthalmic ointment state: "A small amount of ointment to the affected eye(s) every 3 hours or more often if necessary." Again, frequently-repeated administration is required. [*Prescribing Information, ^{Pr}PENTAMYCETIN®, ^{Pr}PENTAMYCETIN/HC®* Sandoz Canada Inc., Date of Revision: June 14, 2918.]

Naturally occurring and synthetic dyes have been applied as antibacterial or antiprotozoal agents [Zheng et al., *BMC Microbiol* **20** (2020]. For example, methylene blue and clofazimine are still considered to be important orphan drugs [Ginimuge et al., *J. Anaesthesiol. Clin. Pharmacol*. **26**:517-520 (2010); and Ammerman et al., *J. Antimicrob. Chemother*. **72**:455-461 (2017)].

Rose bengal (RB) is a bright rose-red xanthene derivative compound that was first synthesized in the 19th century as a wool dye and subsequently used as a food dye in Japan (food red no. 105) [Mizutani et al., *J. Environ. Public Health*. **2009**, 953952]. More particularly, RB is a derivative of the xanthene compound fluorescein. Compared to fluorescein, RB has two types of additional halogens: four chloride and four iodide substituents.

The use of RB for the visual diagnosis of human ocular surface damage (via ocular instillation) was first described in 1914 [Feenstra et al., *Ophthalmology* **99**:606-617 (1992)]. RB was later introduced as an intravenously-administered, relatively rapid, diagnostic aid to evaluate the functional capacity of a human liver after a single 100 mg dose [Wachter et ala., *Lasers Surg Med*. **32**:101-110 (2003)]. In 1971, ^{131}I RB (Robengatope®, rose bengal sodium ^{131}I injection USP) was approved by the U.S. Food and Drug Administration (FDA) for use as a diagnostic aid in determining liver function [Baroyan et al., *Eksperimental'naya Meditsina* (Riga) **20**:74-78 (1985); and Mincev et al., *Folia Medica* (Plovdiv) **16**:35-41 (1974)].

In 2009, Robengatope's manufacturer Bracco Diagnostics Inc. formally withdrew the RB diagnostic product from the U.S. market because of the emergence of newer methods of liver imaging, such as computed tomography. In 1974, Barnes-Hind Pharmaceuticals Inc. (Barnes-Hind) introduced a medical device product of 1% RB in an aqueous solution for the disclosure of corneal injury, the diagnosis of keratitis, keratoconjunctivitis, and sicca, and the detection of foreign bodies in the eye [Gilger et al., *Vet. Ophthalmol.* **16**:192-197 (2013)]. In 1981, Barnes-Hind introduced ophthalmic strips of the same concentration for the same indications. Although both diagnostic products were accepted by the U.S. Food and Drug Administration (FDA) for marketing, neither the solution and strip devices nor their respective claims were approved because their introductions predated formal FDA review and approval.

Commercial-grade RB, with marketed dye contents that can vary between 80% and 95% RB, including gross contaminants and substance-related impurities, is manufactured using an historical process developed by Gnehm in the 1880s. It is thought that RB used in diagnostic applications is a commercial-grade RB that contains some impurities [Paczkowski et al., *Free Radic. Biol. Med.* **1**:341-351 (1985)]. The United States Pharmacopeia (USP) previously listed RB as an analytical standard. RB was removed from the USP in 2019. Thus, commercial-grade RB lacks relevance in the context from modern diagnostic and therapeutic settings. Therefore, it poses significant regulatory challenges to validate

RB for application to the treatment of human diseases.

U.S. Patents No. 8,530,675, No. 9,273,022 and No. 9,422,260 to Singer et al. describe and claim the synthesis of highly purified rose bengal, as well as similarly purified similar compounds containing different halogen substituents and different numbers of those halogen substituents, as well as their lactone forms. Those compounds are collectively referred to herein as "halogenated xanthenes".

Rose bengal (RB) dye (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) has been clinically investigated for the treatment of melanoma and the other solid cancers [Maker et al., *J. Clin. Cell. Immunol.* **6**:343-349 (2015; Liu et al., *Oncotarget*. **7**:37893-37905 (2016); Patel et al., *J. Clin. Oncol.* **38**:3143 (2020); Kim et al., *J. Control. Release*. **156**:315-322 (2011); and Qin et al., *Cell Death Dis*. **8**:e2584 (2017)]. Photodynamic antibacterial properties of RB have been sporadically reported [Pérez-Laguna et al., *Photodiagnosis Photodyn. Ther*. **21**:211-216 (2018); Uekubo et al., *Laser Ther*. **25**:299-308 (2016); Anju et al., *Photodiagnosis Photodyn. Ther*. **24**:300-310 (2018); Gavara et al., *Front. Med*. **8**:494 (2021); Joanna et al., *Front. Microbiol*. **9**:1949 (2018); Hirose et al., *Arch. Oral Biol*. **122**:105024 (2021); Dai et al., *Photodiag. Photodyn. Ther*. **6**:170-188 (2009); Ghorbani et al., *Laser Ther*. **27**:293-302 (2018); Kim et al., *J. Food Sci*. **73**:C540-545 (2008); Manoi et al., *J. Photochem. Photobiol. B*. **162**:258-265 (2016); Nakonieczna et al., *Front. Microbiol*. **9**:1949 (2018); Sabbhi et al., *Appl. Water Sci*. **8**:56

(2018); Santos et al., *Antibiotics* **8**:211 (2019): and Worzella et al., In *Cancer Cell Culture*; Humana Press: Totowa, NJ, USA, 285–291. (2011)].

For example, Dees et al. U.S. Patent No. 8,974,363 teaches use of a topical formulation of RB at 10-100 μM (i.e., 10 μg/mL to 100 μg/mL) in conjunction with green light irradiation in the 500-600 nm wavelength band against Gram-positive and Gram-negative antibiotic-resistant bacteria without specifics as to the light source, its intensity or duration of irradiation.

Naranjo et al., *Am J Ophthalmol* **208**:387-396 (2019), reported the use of rose bengal photodynamic antimicrobial therapy (RB-PDAT) on the eyes of 18 patients with progressive infectious keratitis that were unresponsive to standard therapy. Compositions containing 0.1 or 0.2 % rose bengal (RB) were applied to de-epithelized corneas for 30 minutes followed by irradiation with 5.4 J/cm^2 of green light from a LED source for 15 minutes. *Acanthamoeba* (an amoeba) was the most frequently found microbe (10/17; 59%), followed *by Fusarium spp.* (a fungus; 4/17; 24%), *Pseudomonas aeruginosa* (a Gram-negative bacterium; 2/17; 12%) *and Curvularia spp.* (a fungus; 1/17; 6%) and one patient had no confirmed microbiologic diagnosis. Successful RB-PDAT (avoidance of therapeutic keratoplasty) was reported achieved in 72% of the cases, with an average time to clinical resolution (decreased pain and inflammation with reepithelization and infiltrate resolution) of 46.9 ± 26.4 days after RB-PDAT.

Amescua et al., *Cornea* **36(9)**:1141-1144 (2017), teach that rose bengal (RB) at about 0.1 %

that was photoactivated using 15 minutes of irradiation at 375 nm or 518 nm could inhibit the fungal growth of multidrug-resistant *Fusarium keratoplasticum in vitro*. Following multiple successive anti-fungal medication treatments, a study was conducted using an above concentration of RB dripped onto a *Fusarium keratoplasticum*-infected, debrided cornea epithelium of a human patient for 30 minutes followed by irradiation using light at 518 nm to provide a total energy of 0.9 J/cm^2. Resolution of pain was noted 36 hours post irradiation, and the corneal infiltrate shrank over the next two weeks. Four days after treatment, the infiltrate was approximately 1 mm smaller in all directions.

At post irradiation treatment day 13, the infiltrate was almost resolved, and a second session of rose bengal RB-irradiation treatment (1.8 J/cm^2) was performed. After topical corticosteroid treatment, by day 246 after the first RB treatment, the patient demonstrated a healthy and quiet ocular surface of the treated eye and a clear cornea. A follow-up study of the treated patient's eye is reported in Martinez et al., *Cornea* **37(10)**:e46-e48 (2018).

Halili et al., *Am J Ophthalmol* **166**:194-202 (2016), reported *in vitro* studies of patient-isolated methicillin-resistant *Staphylococcus aureus* (MRSA) on agar using RB dissolved in high-purity water at 0.1 and 0.03% in the dark, under ambient room light for 30 minutes and under LED green light irradiation for 34 minutes to provide 5.4 J/ cm^2. Samples were thereafter grown in a light-protected box for 72 hours in an incubator. Complete growth

inhibition of both MRSA strains was demonstrated (1) for both rose bengal concentrations under ambient and green LED irradiation, and (2) for the 0.1% rose bengal in the dark. The 0.03% rose bengal in dark conditions showed complete inhibition of strain 2 but incomplete inhibition of strain 1.

Arboleda et al., *Am J Ophthalmol* **158(1)**:64-70 (2014), reported, *inter alia*, on the *in vitro* growth inhibition of fungal keratitis patient isolates using RB in irradiation (518 nm; 5.4 J/cm^2) and dark conditions followed by three days of incubation. Isolates of *Fusarium solani*, *Aspergillus fumigatus* and *Candida albicans* were studied. Data from that study are summarized in the table below.

	Day 3 Growth Inhibition Percentage			
Organism	**RB + 518 nm Irradiation**	**RB Only**	**Only 518 nm Irradiation**	**Fungus only**
F. solani	78.2±2.1	7.3±1.1	6.8±9.3	9.8±4.7
A. fumigattus	79.8±9	6.6±0.	0.6±0.2	0
C. albicans	95.6±3	42.2±3.7	35.6±6.7	24.2±2.6

As disclosed hereinafter, the present invention provides a gentle and more easily-used treatment as is needed. In addition, the treatment contemplates use in the substantial absence of light that produces an identifiable or measurable change when it interacts with matter (actinic light), as when the treated subject is asleep with her/his eyes closed.

BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention contemplates an ophthalmic system of an actinic

light-opaque vessel that contains a topical ophthalmic composition of a halogenated xanthene (fluorescein) such as rose bengal or a pharmaceutically acceptable salt or ester thereof dissolved or dispersed in an aqueous ophthalmic carrier and present in a bacterial keratitis-treating effective concentration of about 0.2 μg/mL to about 50 μg/mL (or about 0.00002 to about 0.005 wt. %) in the composition. In preferred practice, the composition has a pH value of about 6.5 to about 7.6 and preferably about 7.0 to about 7.4, the pH value of normal tears. A contemplated composition preferably further includes thickening agent and an electrolyte. More preferably, hyaluronic acid is also present.

In another aspect of the invention, a mammalian subject in need is treated with an above topical ophthalmic composition by placing a keratitis-treating effective amount of that composition, such as one or two drops per eye, onto the corneal surface of the subject's microbially-infected eye in the substantial absence of actinic light, and the so-treated subject is thereafter maintained in the absence of actinic light for a period of about 3 to about 12 hours. The treatment is typically administered just prior to the mammalian subject retiring to sleep. This treatment regimen is typically repeated a plurality of times until the microbial infection has been overcome.

As used herein, the phrase "actinic light" denotes light that can cause a photochemical reaction of one or more of the ingredients of topical ophthalmic composition. In accordance with this definition, the actinic light is of a

wavelength that is absorbed by a recipient molecule of the composition and there is a sufficient flux of photons of the absorbed wavelength to cause a detectable chemical reaction induced in or by the absorbing recipient halogenated fluorescein molecule. Illustrative chemical reactions include decomposition, and photosensitization.

The term "substantial absence of actinic light" is used herein to mean that, after application to the infected eye, the composition is subjected to ambient light for a time period of about two minutes or less. Thus, after application, the eye lid is closed, a light-blocking patch is placed over the treated eye, ambient light is shut off, or the like. Similarly, there is an insufficient flux of photons of an absorbed wavelength to cause a detectable chemical reaction induced in or by the absorbing recipient halogenated fluorescein molecule.

The microbial source of infection can bacterial, viral, fungal or amoebic. Preferably, the source of infection is bacterial, and an infecting bacterium is preferably a Gram-positive bacterium.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention, in one aspect, contemplates an ophthalmic system of a vessel opaque to actinic light that contains a topical ophthalmic composition of a halogenated xanthene (fluorescein) such as rose bengal or a pharmaceutically acceptable salt or ester thereof dissolved or dispersed in an aqueous ophthalmic carrier and present in a keratitis-treating effective concentration of about

0.2 μg/mL to about 50 μg/mL (or about 0.00002 to about 0.005 wt. %) in the topical ophthalmic composition. Preferably, the concentration of halogenated fluorescein (xanthene) is about 0.5 μg/mL to about 20 μg/mL. In preferred practice, the composition has a pH value of about 6.5 to about 7.6, and more preferably about 7.0 to about 7.4, the pH value of normal tears. A contemplated composition preferably further includes a thickening agent and an electrolyte.

It is noted that a contemplated composition contains about one-tenth to about one thousandth the concentration of halogenated xanthene compared to that of the rose bengal used in the before-discussed Naranjo et al., Amescua et al., Martinez et al., Halili et al., and Arboleda et al. papers.

More preferably, hyaluronic acid, a polymer of disaccharides composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked via alternating β-(1→4) and β-(1→3) glycosidic bonds is also present. Hyaluronic acid provides some thickening, buffering and tonicity to the composition, and helps maintain water of the applied composition in contact with the surface of the eye.

In another aspect of the invention, a mammalian subject in need is treated with an above topical ophthalmic composition by placing a keratitis-treating effective amount of that composition, such as one or two drops per eye, onto the surface of the subject's microbially-infected eye in the absence of additionally-provided actinic light, and the so-treated subject is thereafter maintained in the absence of actinic light for a

period of about 3 to about 12 hours. The treatment is typically administered just prior to the mammalian subject retiring to sleep. The use of an actinic light-opaque eye patch to cover the treated eye following administration can also be followed when the treated subject is or is expected to be in the presence of actinic light after administration. This treatment regimen is typically repeated a plurality of times until the microbial infection has been overcome.

Topical Ophthalmic Pharmaceutical Composition

Aqueous Ophthalmic Carrier

A contemplated composition is primarily (by weight) an aqueous ophthalmic carrier in which other ingredients are dissolved or dispersed. The principal component of the composition is sterile water, such as sterile water for injection USP.

A contemplated composition has at least one builder or thickener present at a level sufficient to provide a viscosity of about 10 to about 300 cps, and preferably about 30 to about to 120 cps, and more preferably about 50 to about 80 cps. Typical thickeners are polymers although solvents such as glycerin and propylene glycol can also provide some thickening action to the composition.

Glycerin and propylene glycol alone or together can comprise zero to about 2.5 percent by weight of the composition. More preferably one or both together is present at about 0.4 to about 2 percent by weight. Each of glycerin and propylene glycol is miscible with water.

A contemplated topical ophthalmic composition contains one or more dissolved solutes sufficient to provide the composition with an osmolality (Osm) of the medicament composition of about 270 mOsm/kg to about 340 mOsm/kg osmolality is provided, and preferably about 300 mOsm/kg to 325 mOsm/kg, which is about the range of osmolalities exhibited by human tears.

At least some of that osmolality can be obtained by the presence of water-soluble sodium, potassium, calcium and magnesium chlorides, phosphates, and nitrates. Sodium, such as in the form of sodium chloride, is a preferred embodiment as the electrolyte due to its inherent physiologic compatibility. It is preferable that such an electrolyte be present in the composition at a concentration of about 0.1 to about 2 percent, and more preferably at a concentration of about 0.5 to about 1.5 percent, more preferably at a concentration of about 0.8 about 1.2 percent, and most preferably at a concentration of approximately 0.9 percent. The osmolality of aqueous 0.9 N sodium chloride is 308 mOsmol/liter.

When the subject in need of treatment presents with swelling of the cornea, a hypertonic composition containing about 2 percent to about 5 percent sodium chloride can be utilized to lessen the corneal swelling while treating the infection. The osmolality of eye-treating compositions containing 2 and 5 percents sodium chloride were calculated to be 684 and 1711 mOsm, respectively. [Yorek et al., *Invest Ophthalmol Vis Sci*. **57**:2412-2419 (2016)]. Those illustrative products are sold

under the trademark Muro 128® by Bausch & Lomb (Bridgewater, New Jersey).

A low molecular weight poly(ethylene glycol) (PEG) such as PEG 400 or PEG 1000 can provide lubrication and some relief from dry eye symptoms to a composition. These polymers also add a relatively small amount of osmolality due to their lack of ionic charge. Depending upon the other solutes present in a contemplated composition, a low molecular weight PEG can be absent or present at about 1 to about 10 mg/mL.

Dextran, a complex branched, non-ionically charged poly-α-d-glucoside of microbial origin having glycosidic bonds that are predominantly C-1→C-6, with branches from α-1,3 linkages. Dextran chains are of varying lengths (from about 3 to about 2000 kilodaltons). Two commonly used dextrans are referred to as dextran 40 and dextran 70 in view of their molecular weights of about 40,000 and 70,000 kDa, respectively.

Dextrin is a water-soluble, straight chain polyglucose tethered by α-1,4 or α-1,6 linkages. Dextrins are mixtures produced by the hydrolysis of starch and glycogen and are free of ionic charge. The characteristic branching distinguishes a dextran from a dextrin, which is a straight chain glucose polymer tethered by α-1,4 or α-1,6 linkages.

Like the PEGs, being uncharged, dextrans and dextrins present in a contemplated composition do not tend to greatly change the composition's osmolality. Dextrans can provide some lubrication to the eye and can be absent of present at about 0.5 to about 2 mg/mL.

Poly(vinylpyrrolidone) that is often sold commercially as povidone is another non-ionically charged polymer that, like the dextrans, has found use as a blood extender for intravenous use, in ophthalmic medicaments, and does not appreciably alter a contemplated composition's osmolality when present. Povidone when present is present at about 1 to about 2 mg/mL, and more preferably at about 7 to about 15 mg/mL.

A still further useful non-ionic polymer is hydroxypropyl methylcellulose (HPMC) also sold commercially as hypromellose. U. S. Patent No. 5,679,713 teaches the use of a dry eye-treating active agent cholinergic compound along with hypromellose, methyl cellulose, poly(vinyl alcohol), or hyaluronic acid could be used in a pharmaceutical composition. The above patent's results-free example utilized methyl cellulose at 0.5-1 percent by weight and poly(vinyl alcohol) at 1.4 percent by weight.

Another water-soluble non-ionic polymeric thickener is hydroxyethyl cellulose (HEC). This material is available in several molecular weights that provide different amounts of thickening per wt % present. One source of HEC sold under the name Natrosol™ 250 is Ashland Specialty Chemicals of Covington, KY.

The commercially available dry eye medication sold under the tradename Refresh® Tears® and Refresh® Relieva™ both contain anionic carboxymethyl-cellulose sodium at 0.5 percent as an active ingredient, with the Refresh® Relieva™ product also containing glycerin as an active ingredient at 0.9 percent. The active ingredients

are present along with a number of inactive ingredients dissolved in purified water. These products are sold by Allergan, Inc. an AbbVie company (North Chicago, IL).

Hyaluronic acid (HA) is an anionic, non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It is polymer of repeating units composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked via alternating β-(1→4) and β-(1→3) glycosidic bonds. It is unique among glycosaminoglycans as it is non-sulfated and can have molecular weight of several million Daltons. Because it is anionic and typically contains several carboxyl groups per molecule, HA can have a relatively large effect on the osmolality of a contemplated composition.

Another anionic thickening polymer is partially neutralized water-dispersible carboxymethyl cellulose. This material is available from many suppliers in differing molecular weights to provide different viscosities per given weight percentage in water.

The phrase "partially neutralized" is used in conjunction with carboxyl group-containing polymers whose carboxyl functionalities are reacted with a base to form anionically charged carboxylate groups and aid in providing the polymer with water-solubility or dispersibility.

Additional anionic thickening agents can also be used such as the partially neutralized cross-linked acrylic acid, methacrylic acid and co-polymers such as the water-soluble cross-linked acrylate polymers of one or more of U.S. Patents No.

3,074,852, No. 3,330,729 and No. 4,226,848 and exemplified by the material sold under the trademark CARBOLOL® 934P NF or 974P NF by Lubrizol Corp., Wickliffe, Ohio. These materials are acrylic acid homopolymers cross-linked with a polyalkenyl polyether such as allyl sucrose or allyl pentaerythritol, with each cross-linker containing an average of at least three allyl groups per molecule. Both polymers are reportedly water-soluble and provide viscosities at a pH value of 7.5 when present at 0.5 weight percent. The 934P NF is polymerized in benzene, whereas the 974P NF is polymerized in ethyl acetate as solvents.

Another useful class of thickener is a water-swellable but water-insoluble cross-linked acrylic polymer that is described in U.S. Patents No. 3,202,577 and No. 4,615,697 and is often referred to in the literature as polycarbophil and as a bioadhesive. These polymers can be defined as a reaction product of the copolymerization of at least 80 weight percent monoethylenically unsaturated carboxy-functional monomer and about 0.05 to about 1.5 weight percent of a cross-linking agent that is 3,4-dihydroxy-1,5-hexadiene, or 2,5-dimethyl-1,5-hexadiene. The carboxyl groups of this polymer are also at least partially neutralized at the p value of the composition.

These bioadhesive polymers are free of polyalkenyl polyether cross-linkers discussed before. The remaining monomers that can be present to constitute 100 percent by weight of the monomers are discussed below.

In addition to the above two ingredients, a bioadhesive polymer can also include polymerized

monoethylenically unsaturated repeating units such as C_1-C_6 alkyl esters of one or more of the above-described acids such as hexyl acrylate, butyl methacrylate and methyl crotonate; hydroxyalkylene-functional esters of the above-described acids that contain a per molecule average of 1 to about 4 oxyalkylene groups containing 2-3 carbon atoms such as hydroxyethyl methacrylate, hydroxypropyl acrylate and tetraethylene glycol monoacrylate; methacrylamide, acrylamide and their C_1-C_6 mono- and di-alkyl derivatives such as N-methyl acrylamide, N-butyl methacrylamide and N,N-dimethyl acrylamide; styrene; and the like as are known in the art as being copolymerizable with the above described carboxyl functionality-containing monomers and cross-linking agents. The bioadhesive polymers most preferably are prepared from only the monoethylenically unsaturated carboxy-functional monomer and the cross-linking agent.

A bioadhesive useful herein can be prepared by conventional free radical polymerization techniques utilizing initiators such as benzoyl peroxide, azobisisobutyronitrile, and the like, and can also be polymerized in an aqueous medium, and are not agglomerated by steam action. Exemplary preparations of useful bioadhesives are provided in the two patents cited immediately above, whose disclosures are incorporated herein by reference.

As noted previously, the bioadhesives can be polymerized in an aqueous medium. In preferred practice that aqueous medium is a saturated solution of an alkaline earth metal salt such as magnesium sulfate.

The alkaline earth metal salt serves at least two functions. First, it increases the density of polymerization medium so that the polymerized bioadhesive floats on the surface of the aqueous medium and can be easily removed therefrom. Second, the use of magnesium sulfate, in particular, reduces the swelling of the bioadhesive in the aqueous medium so that polymerization and recovery are facilitated. Bioadhesives typically contain about 0.5 to about 1 percent of the alkaline earth metal ion after several water rinses of the polymer.

U.S. Patent No. 5,221,722 teaches the preparation of what may be called an enhanced polycarbophil. This polymer is prepared in a solvent selected from acetone and alkyl acetates containing 1 to 6 carbon atoms in the alkyl group in the presence of a suitable initiator and divinyl glycol crosslinker such as 3,4-dihydroxy-1,5-hexadiene. Polycarbophil prepared in this manner is said to have the attributes of bioadhesion, as well as having small particle size without grinding and viscosity of above 20,000 cPs when 1% by weight mucilages thereof are measured in water.

To prevent gelling of the polymer and to promote discrete particle formation during polymerization, at least a part of the carboxyl groups are taught to be neutralized with a group I-A metal compound in the form of a hydroxide, oxide, or carbonate, and the like. Examples of these include sodium, potassium, and the like, as well as reaction with ammonia and certain amines including morpholine; mono, di, and triethanolamine; mono-propanolamine; and other amines where the partial polymeric salt is less soluble in the reaction

medium. Preferably, greater than 0.1 percent by weight of the carboxyl groups on the monomer are neutralized or formed into a salt of the above listed materials. More preferably, greater than 1 percent by weight and up to about 10 percent by weight of the carboxyl groups are neutralized or converted to the equivalent salt prior to polymerization, especially less than about 5 percent.

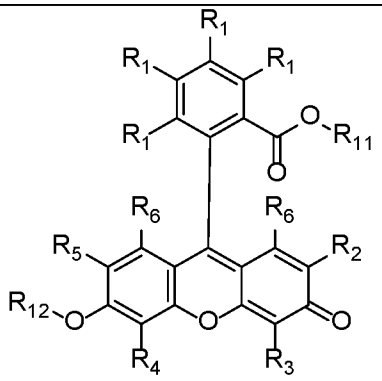
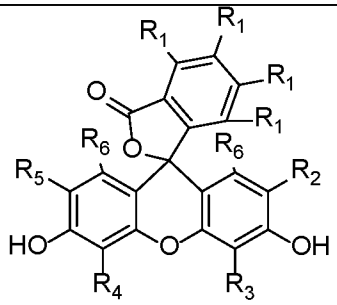
The above-described synthesis is reported to have provided polymer with an average particle size of less than 10 μ, such that the particles will pass through a Mesh No. 635 (ASTM-E11). One commercial supplier is The Lubrizol Corporation, above, which sells the polymer for medical uses under the name Noveon® AA-1, Polycarbophil, USP.

The specific amount of any above polymer is not as relevant as is the effect of the polymer as a thickening agent and or bioadhesive. Thus, the amounts of the polymers used typically differ among themselves because they have differing thickening and bioadhesive abilities. Generally speaking, a thickeningly-effective and/or a bioadhesively-effective amount is used.

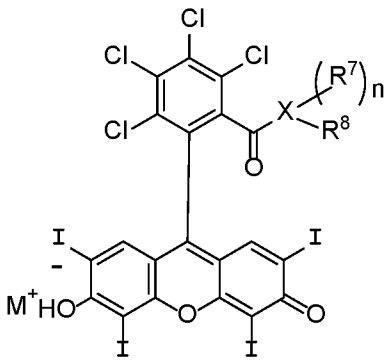
Halogenated Xanthene (Fluorescein)

A halogenated xanthene (fluorescein) compound of a contemplated topical ophthalmic composition can be a compound of **Formula 1**, below, in which R_1 is independently F, Cl, Br, I, H or C_1-C_4 alkyl; R_2, R_3, R_4, and R_5 are independently Cl, H or I with at least one substituent selected from R_2, R_3, R_4, R_5 being I; and R_6 is independently H or C_1-C_4 alkyl; R^{11} is H or C_1-C_4 alkyl; R^{12} is H or C_1-C_7 acyl;

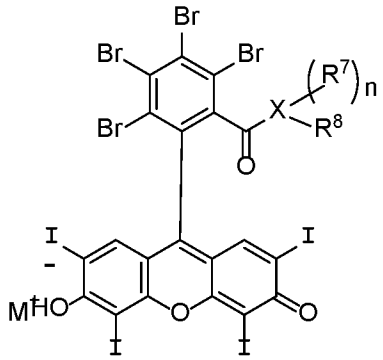
and all (a) tautomeric forms; (b) atropisomers, (c) closed lactone forms as depicted in **Formula 2** (below), (d) enantiomers of lactone forms depicted in **Formula 2**, and (e) pharmaceutically acceptable salts thereof.

	
FORMULA 1	**FORMULA 2**

A contemplated halogenated xanthene is more believed to be more accurately referred to as a halogenated fluorescein inasmuch as fluorescein is a derivative of xanthene. In preferred practice, each R_1 of a halogenated fluorescein of **Formulas 1** and **2**, above, is either a chloro or a bromo substituent, whereas each of R_2, R_3, R_4, and R_5 of those formulas is an iodo substituent as are present in Formulas **I** and **II**, below. In those formulas below, X is oxygen or nitrogen, "n" is zero or 1 such that when X is



Formula I



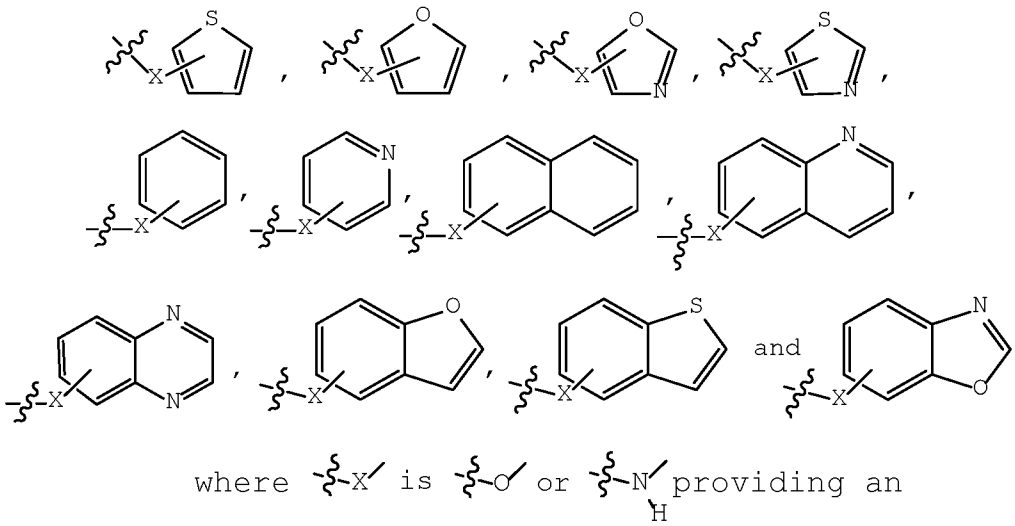
Formula II

oxygen, n is zero and R^7 is absent, whereas when X is nitrogen, n is 1 and R^7 is present. When X is oxygen, R^7 is selected from the group consisting of hydrogen (H), M^+ that is a pharmaceutically acceptable cation, C_1-C_4 alkyl, and an aromatic ring-containing substituent as defined herein after. When X is nitrogen, R^7 and R^8 are the same or different and are selected from the group consisting of hydrogen, C_1-C_4 alkyl, an aromatic ring-containing substituent or together with amido nitrogen atom R^7 and R^8 form a 5- or 6-membered ring, and an aromatic ring-containing substituent. The aromatic ring-containing substituent is a single ring containing 5- or 6-members, or a 5,6- or 6,6-fused aromatic ring system that contains 0, 1 or 2 hetero ring atoms that are independently nitrogen, oxygen or sulfur.

For ease of description, an aromatic ester or aromatic amide are collectively referred to as an aromatic derivative. As such, those derivatives are formed from an alcohol or amine, preferably monosubstituted, having a single 5- or 6-membered aromatic ring, or a 5,6- or 6,6-fused aromatic ring

system that contains 0, 1 or 2 hetero ring atoms that are independently nitrogen, oxygen or sulfur.

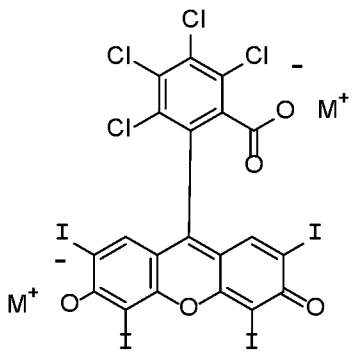
Structural formulas of exemplary aromatic ring substituents are set out below:



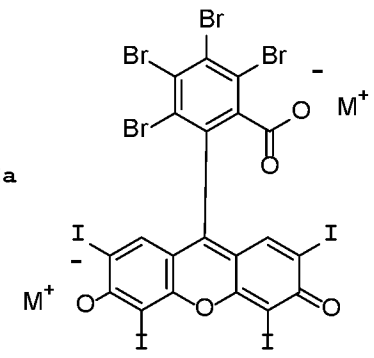
where is or providing an ester or a monosubstituted amide, respectively.

Rose bengal (RB) is a preferred compound for use herein, and its disodium salt, rose bengal disodium (RBD), is the most preferred RB compound. These compounds are used illustratively herein for the group of RB compounds.

The chemical name for rose bengal is 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodo-fluorescein. A preferred form, rose bengal disodium (RBD), has the following structural Formula **Ia**:



Ia



IIb

Preparation of highly purified halogenated fluorescein molecules such as rose bengal (Formula

Ia above) and the tetra-bromo-tetra-iodo analog (Formula **IIb**, above) are discussed in U.S. Patents No. 8,530,675, No. 9,273,022 and No. 9,422,260. The use of RBD in photochemically-induced treatments is disclosed in U.S. Patents No. 5,998,597, No. 6,331,286, No. 6,493,570, No. 7,390,668 and No. 8,974,363. Preferably, the cation, M^+, in the above structural formulas is sodium (Na^+) or potassium (K^+).

The terms "physiologically acceptable salt" and "pharmaceutically acceptable salt" in their various grammatical forms refer to any non-toxic cation such as an alkali metal, alkaline earth metal, and ammonium salt commonly used in the pharmaceutical industry, including the sodium, potassium, lithium, calcium, magnesium, barium, ammonium and protamine zinc salts, which can be prepared by methods known in the art. Such salts are usefully prepared by neutralization of an acid form of a contemplated halogenated fluorescein using an alkali metal, alkaline earth metal, or ammonium hydroxide or by admixture with a hydroxide form of an ion exchange resin. A cation halogenated fluorescein salt so formed is preferably soluble in an ultimately formulated topical ophthalmic composition.

A contemplated cation provides a composition-soluble xanthene (fluorescein or fluorescienate) salt. Preferably, the salts are sodium, potassium, calcium and ammonium in either the mono or dibasic salt form. The reader is directed to Berge, *J. Pharm. Sci.* 1977 **68(1)**:1-19 for lists of commonly used physiologically (or pharmaceutically) acceptable acids and bases that

form physiologically acceptable salts with pharmaceutical compounds.

A halogenated fluorescein is present in the composition in a keratitis-treating effective concentration. In usual practice, a keratitis-treating effective concentration is about 50 μg/mL to about 3,000 μg/mL, preferably about 100 μg/mL to about 2,000 μg/mL, and more preferably about 500 μg/mL to about 1,500 μg/mL, in the topical ophthalmic composition, measured as DRB.

Thus, if a halogenated fluorescein other than DRB is used, the amount used is based upon the molecular weight of DRB, which is 1017.6 g/Mole. For example, if the four chloro substituents are replaced by four bromo substituents, the molecular weight would be 1195.2 g/Mole. The molecular weight difference is about 17.45 percent. An equivalent amount of the tetra-bromo-tetra-iodo fluorescein to use compared to a given amount of DRB would therefore be about 17.45 percent greater.

A contemplated halogenated fluorescein compound such as rose bengal is dibasic, having pK_a values of 2.52 and 1.81. pK_a value determinations for several contemplated halogenated fluoresceins can be found in Batsitela et al., *Spectrochim Acta Part A* **79(5)**:889-897 (Sept. 2011).

The pH value of the halogenated xanthene (fluorescein) pharmaceutical composition can be regulated or adjusted by any suitable means known to those of skill in the art. The composition can be buffered or the pH value adjusted by addition of acid or base or the like. As the halogenated xanthenes (fluoresceins), or physiologically

acceptable salts thereof, are weak acids, depending upon halogenated xanthene (fluorescein) concentration and/or electrolyte concentration, the pH value of the composition may not require the use of a buffer and/or pH value-modifying agent. It is especially preferred, however, that the composition not be buffered, permitting it to conform to the biological environment, e.g., tear fluid, once administered.

Abelson et al., *Arch Ophthalmol* **99(2):**301 (1981) reported measuring the pH value of the tear fluid in the inferior cul-de-sac from 44 normal subjects and found that pH value to be about 6.5 to about 7.7, with a mean value of about 7.0. Thus, a contemplated composition preferably has a pH value of about 6.5 to about 7.6, and preferably about 7.0 to about 7.4.

It is also preferred that the pharmaceutical composition not include any preservatives, many of which can deleteriously interfere with the pharmaceutical composition or formulation thereof, or may complex or otherwise interact with or interfere with the delivery of the halogenated xanthene composition active component. To the extent that a preservative is used, imidurea is a preferred preservative as it does not interact with halogenated xanthenes, either in the pharmaceutical composition or upon administration.

A contemplated treatment method is utilized to treat a mammalian subject in need thereof. A "mammal in need" is a mammal that presents with an eye infection, such as a bacterial, viral, fungal or amoebic infection. Such infections tend to usually be a bacterial infection, and the

infecting bacteria are typically Gram-positive bacteria.

Illustrative treated Gram-positive bacteria include one or more of drug-susceptible and drug-resistant *S. aureus, S. epidermis, E. faecalis* and *E. faecium,* as well as one or more of *Bacillus subtilis, Bacillus cereus,* and *Streptococcus salivarius*. Gram-positive bacteria are present within or on mammalian cells of the eye when an infected eye is treated (the cells are contacted).

Some Gram-positive bacterial strains are referred to herein as being "resistant" or "drug resistant" or other grammatical variants of resistance. The "resistance" meant here is of the bacteria to treatment with one or more antibacterial pharmaceutical products that are usually deemed to be bactericidal at known concentrations and bacterial cell densities to that type of bacteria. There are thus, both "drug-susceptible" and "drug-resistant" strains of bacteria such as *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis (S. epidermis*), *Enterococcus faecalis* (*E. faecalis*), and *Enterococcus faecium (E. faecium)*. Illustrative drugs to which several common bacteria strains have become resistant include vancomycin, methicillin and gentamicin.

A similar method is also contemplated for treating Gram-negative bacteria that is preferably one or more of *Burkholderia, Salmonella*, and *Proteus*. Here, an above RB compound is present dissolved or dispersed in an aqueous pharmaceutical composition at a concentration of about 10 μg/mL to about 100 μg/mL, and preferably at about 20 μg/mL to about 50 μg/mL.

As used herein, the word "administration" is used to mean the beginning of a treatment regimen in which the composition is applied to the outer surface of the infected eye. Thus, administration typically entails placing a dose of an ophthalmic composition described herein onto the surface of the eye or into the pocket or pouch formed by pulling down the lower eyelid, and then closing the eyelid to distribute the ophthalmic composition over the external surface of the treated eyeball.

The Treatment Method

A contemplated treatment method comprises contacting the corneal surface of the subject in need with a composition containing an anti-Gram-positive bacterial amount of a halogenated fluorescein compound. Using rose bengal as illustrative of such compounds, an anti-Gram-positive bactericidal effective amount of RB is administered to the mammalian subject in need and can be formulated using a thickened aqueous liquid, gel, or other formats as discussed previously.

The treated eye is thereafter maintained in the substantial absence of actinic light for a time period of about 3 to about 12 hours. Thus, for example, after application, the eye lid is closed, a light-blocking patch is placed over the treated eye, ambient light is shut off, or the like. Perhaps most easily, the eye is treated just prior to the recipient retiring to sleep for the evening.

In one embodiment, the Gram-positive bacterial cells are present on or in (e.g., infecting) a subject mammal. Illustratively, a subject mammal can have a dermatological Gram-

positive bacterial infection such as that of *Streptococcus pyogenes*, or particularly in the case of a topical treatment of an open wound, as a treatment for a present infection and as a preventative from subsequent Gram-positive bacterial infection.

A treated subject mammal can be a primate such as a human, an ape such as a chimpanzee or gorilla, a monkey such as a cynomolgus monkey or a macaque, a laboratory animal such as a rat, mouse or rabbit, a companion animal such as a dog, cat, horse, or a food animal such as a cow or steer, sheep, lamb, pig, goat, llama or the like.

Each contemplated composition administration is typically repeated until the treated bacterial disease (infection) is diminished to a desired extent, such as not being detectable. Thus, the administration to a mammalian subject in need can occur a plurality of times within one day, daily, weekly, monthly or over a period of several months to several years as directed by the treating physician.

As directed by medical personnel involved with a treatment, the corneal epithelium in an area surrounding the infection, e.g. a corneal ulcer, can be debrided prior to administration of a contemplated composition to obtain a de-epithelized area to increase rose bengal absorption.

Results

Pharmaceutical grade of rose bengal (RB)

Provectus Biopharmaceuticals, Inc. of Knoxville, TN, (Provectus) used commercial-grade RB

and sought a manufacturing process to prepare a pure form of RB as a drug substance. However, several challenges in the purification of RB were found due to commercial-grade reactions occurring during the dye manufacturing process, and the other by-products which lost one or more of the iodide substituents.

It was concluded that commercial-grade RB is not capable of efficiently yielding a pharmaceutical-grade material in sufficient quantity to support clinical development and registration by the FDA and other global drug regulatory agencies. A novel multi-step approach for synthesizing and manufacturing RB was established by Provectus [Singer et al., U.S. Patents No. 8,530,675, No 9,273,022 and 9,422,260. A key element of that work was the ability to eliminate the conditions that lead to the formation of the key historical impurities.

Those synthesis and purification methods have been applied to a good manufacturing practice (GMP), producing RB with a pharmaceutical grade. This RB, PV-10®, is sterile 10% solution of rose bengal disodium (RBD) in 0.9% aqueous saline available from Provectus that is manufactured under the guidelines of The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), and designed to apply as an injectable pharmaceutical. The pharmaceutical grade RBD is also present in a differently-formulated topical medication [U.S. Patent No. 9,422,260; Innamarato et al., *BMC Cancer* **21**:756 (2021); Thompson et al., *Melanoma Res.* **31**:232-241 (2021); and Swift et al., *Onco Targets Ther.* **12**:1293-1307 (2019)] referred to as PH-10®.

Both preparations utilize rose bengal disodium (RBD), whose structural formula was shown previously, as the active ingredient. Because many soluble salts of rose bengal can be utilized in addition to the disodium salt, "RB" is used herein as the designation for any pharmaceutically acceptable salt of rose bengal, whereas the designation "RBD" refers specifically to rose bengal disodium.

Microorganisms

The microbial source of infection can bacterial, viral, fungal or amoebic. In usual practice, the source of infection is bacterial, and an infecting bacterium is preferably a Gram-positive bacterium.

The antibacterial activity of RB via photodynamic approaches has been studied in several research groups [See, Kurosu et al., *Molecules* **27**:322 (2022) and the citations therein]. Applications of photodynamic therapy of RB are not limited to skin infections, including cellulitis, erysipelas, impetigo, folliculitis, and furuncles and carbuncles. However, spectrum of activity, rate of killing, and biofilm eradication activity of RB have been reported sporadically.

The bactericidal activity of pharmaceutical-grade of RB against a battery of microbes including Gram-positive, Gram-negative aerobic bacteria, including *Mycobacterium* spp. Under different light sources (fluorescence, LED, and sun light) and in a dark condition has been reinvestigated by Kurosu et al., above. Interestingly, although the irradiation with actinic

light exhibited greater immediate potency, RB also exhibited anti-bacterial activity in the dark.

The present invention utilizes that anti-bacterial activity in the darkness in the present invention. Thus, the mammalian subject in need of treatment, such as a human with a bacterial eye infection, applies an anti-bacterial effective amount of a contemplated pharmaceutical composition within zero to about five minutes before retiring and closing her/his/their eyes.

Minimum inhibitory concentrations (MICs, in μg/mL) obtained via broth dilution and agar dilution methods for a time period of 24 hours (h) in a dark room, using 96-well plates covered with aluminum foil from the dark condition of the above Kurosu et al. paper are summarized in Table 1, below. RB used in Table 1 is a diluted form of PV-10® that was provided by Provectus Biopharmaceuticals, Inc. (Knoxville, TN, USA) [Singer et al., U.S. Patents No. 8,530,675, No 9,273,022 and 9,422,260.].

Table 1

MIC of a series of fungus, Gram-positive and Gram-negative bacteria via broth dilution method

Entry Number	Microbe	MIC (μg/mL)
1	*Bacillus subtilis* ATCC6051	50
2	*Bacillus cereus* NRRL B-569	50
3	*Bacillus cereus* 13061™	50
4	*Staphylococcus aureus* 6538™	50.0

5	*Staphylococcus aureus* subsp. *Aureus* BAA-1683 (a MRSA strain)	50
6	*Staphylococcus aureus* subsp. *Aureus* BAA-41™ (a MRSA strain)	25.0
7	*Staphylococcus aureus* subsp. *Aureus* BAA-42™ (a MRSA strain)	50.0
8	*Staphylococcus aureus* subsp. *Aureus* BAA-44™ (a MRSA strain)	50.0
9	*Staphylococcus aureus* subsp. *Aureus* BAA-2094™ (a MRSA strain)	25.0
10	*Staphylococcus aureus* BAA-2313™ (a MRSA strain)	25.0
11	*Staphylococcus aureus* subsp. *Aureus* 33592™ (a methicillin and gentamicin resistant strain)	50.0
12	*Staphylococcus aureus* AIS2006032 (a vancomycin-resistant strain)	50.0
13	*Staphylococcus aureus* BR 5 (A methicillin and vancomycin resistant strain)	25.0
14	*Staphylococcus aureus* strain AIS 1000505 (VRS10, a vancomycin-resistant strain)	25.0
15	*Staphylococcus aureus* USA100 strains 71080 (VRS8, a vancomycin-resistant strain)	25.0
16	*Staphylococcus epidermidis* 35984™	25.0
17	*Enterococcus faecalis* 19433™	25.0
18	*Enterococcus faecium* 349™	50.0
19	*Enterococcus faecium* BAA-2320	25.0
20	*Enterococcus faecium* NR-32065 (a vancomycin- resistant strain)	50.0
21	*Enterococcus faecium* patient #3-1, NR-31912 (a vancomycin-resistant strain)	50
22	*Enterococcus faecium* UAA714 (a vancomycin-resistant strain)	50
23	*Streptococcus salivarius* subsp. *Salivarius* 7073™	25.0
24	*Streptococcus pneumoniae* 6301™	>100
25	*Mycobacterium smagmatis* 607™	25.0
26	*Mycobacterium avium* subsp. *Avium* 2285	50.0
27	*Mycobacterium kansasii* 824™	50.0

28	*Mycobacterium bovis* 35734™ (BCG)	50.0
29	*Mycobacteroides abscessus* 19977™	50.0
30	*Saccharomyces cerevisiae* BY4743	125

RB's bactericidal activity was examined against a panel of 7 methicillin-resistant *S. aureus* (MRSA) with different SCCmec types (entries 5-11) of Table 1 [Zuo et al., *Sci. Rep.* **11:Article** 5447 (2021)]. All MRSA strains tested in Table 1 were killed by RB at 0.78-3.1 μg/mL concentrations under the fluorescent or LED light. RB was further examined against 4 vancomycin-resistant *S. aureus* strains (entries 12-16); all vancomycin-resistant strains were killed at below 1.0 μg/mL concentrations under either lighting condition.

Staphylococcus epidermidis is an anaerobic bacterium, but grows well under aerobic conditions. It was effectively killed by RB under an aerobic condition (entry 16). Drug-susceptible and drug-resistant *Enterococcus faecalis* including vancomycin-resistant strains were killed by RB (entries 17-21). *Streptococcus salivarius* was susceptible to RB (entry 23), whereas *Streptococcus pneumoniae* showed resistance to RB (entry 24). The basis for that resistance is presently unknown.

Thus, under the dark condition, RB showed antibacterial activity against all Gram-positive bacteria listed in entries 1-23 (Table 1) at 25.0-100 μg/mL concentrations. Under the dark condition RB is known to display antibacterial activity at high concentrations. All Gram-negative bacteria assayed in Table 1 were not susceptible to RB under

the dark condition. These data support the idea that RB has one or more unknown mechanisms to inhibit the growth of bacteria other than via the excitation mechanism of triplet oxygen, generating cytotoxic reactive oxygen species.

Antibacterial activity of RB against 5 *Mycobacterium* spp. Was examined (entries 25-29). Interestingly, under the dark condition, these *Mycobacteria* were killed at similar MIC to those observed under illumination conditions. RB inhibited growth of *Saccharomyces cerevisiae* at higher concentrations than the bacteria under the dark condition (entry 30).

The MIC values of RB determined by the agar dilution method differed from those determined by the broth dilution method (Table 1). Selected examples of the MIC values determined by the agar dilution method are summarized in Table 2 shown by renumbering the assayed microbes.

Table 2

MIC of a series of Gram-positive bacteria and Mycobacteria via agar dilution method

Entry Number	Microbe	MIC (μg/mL)
1	*Bacillus subtilis* ATCC6051	125
2	*Bacillus cereus* 13061™	125
3	*Staphylococcus aureus* 6538™	25.0
4	*Staphylococcus aureus* subsp. *Aureus* BAA-44™ (a MRSA strain)	50.0
5	*Mycobacterium smegmatis* 607 ™	50.0

The growth of Gram-positive bacteria such as *B. subtilis*, *B. cereus*, and *S. aureus* were inhibited at about 25 to about 125 μg/mL concentrations (entries 1-4 in Table 2), which are greater than the MIC values determined by the broth dilution method. *M. smegmatis* (ATCC607™) was killed at lower concentration on the drug-containing agar plates (or wells) than in those in broth (entry 29, Table 1; entry 5, Table 2) [Lelovic et al., *J. Antibiot* **73**:780-789 (2020)].

Under the dark condition, the MICs of RB determined via the agar dilution method displayed good agreement with the values measured in the broth dilution method. Minimum bactericidal concentrations (MBCs) of RB against the selected bacteria are also summarized in Table 2, above.

Exemplary Gram-positive bacteria for treatment and killing include one or more of drug-susceptible and drug-resistant *S. aureus, S. epidermis, E. faecalis* and *E. faecium*, as are one or more *Bacillus subtilis, Bacillus cereus,* and *Streptococcus salivarius*.

Anti-biofilm activity of Pharmaceutical Grade RB (HP-RBf)

The antibacterial character of RB observed in the previous section encouraged evaluation of antibiofilm efficacy of RB in Gram-positive bacteria. The data summarized above indicate that RB possesses a significant drug affinity or permeability onto (or into) Gram-positive bacteria.

Antimicrobial and antifungal photodynamic therapy have been studied with the photosensitizers under biofilm conditions; however, a limited number

of bacterial biofilms have been examined with RB [Pérez-Laguna et al., *Photodiagnosis Photodyn. Ther.* **21**:211-216 (2018)].

Here, efficacy of RB against biofilms of a drug-susceptible *S. aureus* 6508™, drug-resistant *S. aureus* 71080 (VRS8) and *E. faecium* NR-32065 was studied under florescent light and dark conditions. Linezolid is not an effective drug in eradicating biofilms of Gram-positive bacteria, but has a beneficial effect in prevention of biofilm formations [Reiter et al., *J. Med. Microbiol.* **62**:394-399 (2013)], and was used as a control.

Linezolid was applied as a positive control at very high concentration of 600 μg/mL (>100xMIC for the planktonic cells) in biofilm assays reported by Kurosu et al., *Molecules* **27**:322 (2022). Those authors reported that they confirmed that all strains tested form strong biofilms on the polystyrene well plates. Under the fluorescent light, RB could eradicate the biofilms of *S. aureus* 6508™ with a 7-log reduction at 30.0 μg/mL (38xMIC) concentration, which demonstrated the same level of efficacy as observed for linezolid (at 600 μg/mL) (Kurosu et al., above, Figure 3).

RB showed a biofilm eradication activity in a dose-dependent manner in both the under light and in the dark conditions. Although requiring higher concentrations, RB showed a biofilm eradication activity under the dark condition. At 50.0 μg/mL (2xMIC under dark) concentration, RB significantly reduced the number of viable bacteria. At 500 μg/mL(20xMIC) concentration, only about 110 to about 150 CFU/mL were observed. No viable

bacteria (N.D.) were appeared at 1,000 μg/mL concentration. Similar trends were observed in the biofilms of the drug-resistant *S. aureus* 71080 (VRS8) and *E. faecium* NR-32065 with much lower RB concentrations.

Antibacterial mechanisms of RB

Antibacterial photodynamic therapy of RB has been reported in several articles. Permeation of RB through bacterial cell walls and binding to cell membranes followed by production of reactive oxygen species are likely bactericidal mechanisms in illumination conditions [Nsubuga et al., *Nanoscale Adv.* **3**:1375 (2021); and Lambert et al., *Photochem. Photobiol.* **66**:15-25 (1997)]. Although relatively high concentrations are required, RB kills a majority of Gram-positive bacteria including *Mycobacterium* spp. In dark conditions. It also effectively eradicates biofilms of Gram-positive bacteria (above). Antibacterial activity of RB in dark conditions remains far from completely understood [Nakonechny et al., *Int. J. Mol. Sci.* **29**:3196 (2019); and Kim et al., *J. Enzyme. Inhibit. Med. Chem.* **35**:1414-1421 (2020)].

RB kills *Mycobacterial* spp. At 12.5-25.0 mg/mL in illumination conditions, and at 25.0-50.0 mg/mL in dark conditions (Table 1). In both conditions, RB kills five *Mycobacterial* spp. At a slower rate than that of Gram-positive bacteria. RB seems to have low-permeability of mycobacterial cell walls. Due to rapid bactericidal effect of RB against Gram-positive bacteria even in the dark condition, generation of RB-resistant mutants of

Gram-positive bacteria is an extremely difficult task.

We successfully generated RB-resistant mutants of *M. smegmatis*, which had the MIC value of 200 mg/mL [Lelovic et al., *J. Antibiot.* **73**:780-789 (2020)]. The RB-resistant strain was susceptible to most of TB drugs [amikacin, capreomycin, rifampicin, aminouridyl phenoxypiperidinylbenzyl butanamide (APPB), and ethionamide] (Table 3, below).

Table 3

MICs of RB and anti-mycobacterial agents against *M. smegmatis* and its PV-10-resistant strain[a]

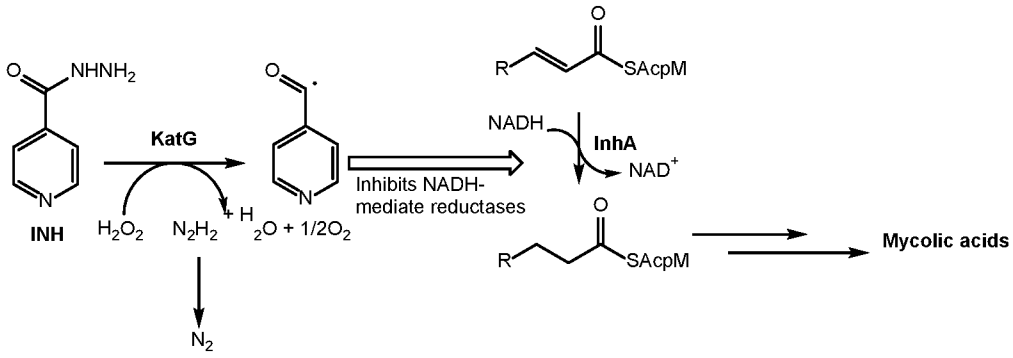
Drug	MIC (μg/mL) against *M. smegmatis* 607™ (wild-type)	MIC (μg/mL) against *M. smegmatis* 607™ (RB mutant)
Rose Bengal	12.5	**200**
Isoniazid (INH)[b]	1.56	**25-50**
Ethionamide (ETH)	12.5	12.5
Amikacin	0.78	0.78
Capreomycin	3.13	3.13
Rifampicin	1.56	1.56
APPB	0.20	0.20

[a]The MIC values were determined in dark condition. All studies were triplicated. [b]An inhibitor of mycolic acid synthesis. [c] APPB = aminouridyl phenoxypiperidinylbenzyl butanamide, a MraY/WecA inhibitor.

However, it showed a cross-resistance to Isoniazid (INH). INH is a prodrug that requires oxidative activation by the enzyme KatG, which belongs to catalase-peroxidase families. KatG oxidizes INH to form an electrophilic species, an isonicotinoyl radical molecule, which reacts with the NADH-dependent enoyl-ACP (acyl carrier protein) reductase, an enzyme involved in the biosynthesis of mycolic acids of mycobacteria (Scheme 1, below) [Vilchèze et al., *Microbiol. Spectr.* **2**: MGM2-0014-

2013 (2014); and Morlock et al., *Antimicrob. Agents Chemother* **47**:3799-3805 (2003)].

Scheme 1



The RB-resistant *M. smegmatis* strain acquired medium-INH resistance but did not show resistance to ethionamide (ETH). The major mechanism of INH resistance is mutation in *katG*, while ETH is activated by the monooxygenase EthA [Laborde et al., *Org. Biomol. Chem.* **14**:8848-8858 (2016)]. These observations may imply that the RB's antibacterial mechanisms share one or more INH metabolic enzymes to form bactericidal species.

To elucidate a potential mechanism of action, a whole-genome sequencing analysis of an RB-resistant *M. smegmatis* ATCC607 strain was performed using the next-generation of DNA sequencing technologies [Lei et al., *Microbiol. Resour. Announc.* **8**:e00551 (2019)]. One identified insertion mutation occurred in anti-sigma E factor gene (*rseA:* evidenced TG:104 vs. T:0) and aquaporin family protein gene (evidenced GCACCCT:71 vs. G:0), respectively.

Consequently, these insertion mutations caused the reading frame changes in the corresponding proteins and generated the truncated

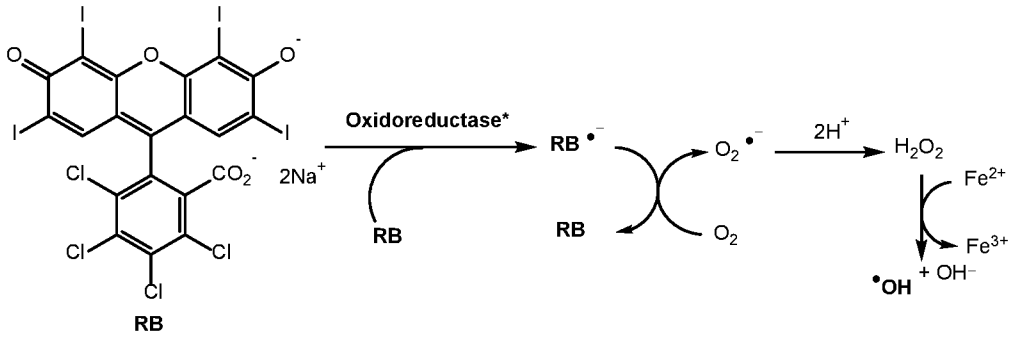
proteins compared to its parental strain. It has been reported that RseA functions as a specific anti-sigma E factor in *Mycobacterium tuberculosis* and that the sigma E factor (SigE) enables the mycobacterial organisms to tolerate a variety of stress responses [Boldrin et al., *Sci. Rep.* **9**:17643 (2019); and Wu et al., *J. Bacteriol.* ***179***:2922-2929 (1997)]. Thus, the expression of a non-functional RseA in the RB-resistant mutant may affect the activity of SigE, increasing the bacterial tolerance to RB. On the other hand, the aquaporin family proteins exist in various organisms and play a critical role in bidirectional flux of water and uncharged solutes cross cell membranes.

It was reported that a null mutation of the *Streptococcal* aquaporin homolog increased the intracellular H_2O_2 retention, indicating that aquaporin mediates transporting H_2O_2 in *Streptococcal* spp. [Tong et al., *J. Biol. Chem.* **294**:4583-4595 (2019)]. Therefore, we hypothesize that RB may inhibit the aquaporin function, leading to accumulation of H_2O_2 within the bacterial cells. Interestingly, a single nucleotide deletion causing the frameshift mutation was observed in molybdopterin-dependent oxidoreductase (evidenced T:74 vs. TC:0) of the RB-resistant strain.

The oxidoreductase systems can form superoxide by reduction of molecular oxygen or NO by reduction of inorganic nitrate. RB may serve as a single-electron acceptor in the redox of the oxidoreductases that will produce the radical anion ($RB^{\bullet-}$) or RB triplet state, undergoing electron-transfer reaction with oxygen. As such, the involvement of molybdopterin-dependent

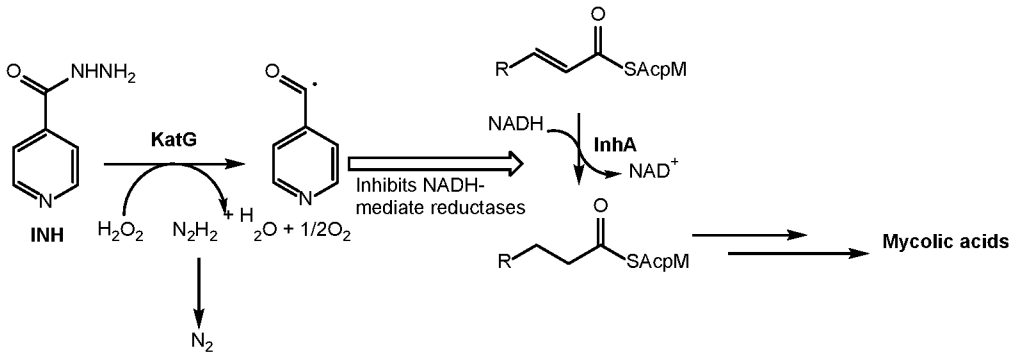
oxidoreductase is proposed in the generation of reactive oxygen or nitrogen spp. through the excitation of RB under dark conditions (Scheme 2, below). The oxidoreductase in Scheme 2 is molybdopterin-dependent oxidoreductase xanthine dehydrogenase family protein. The "·OH + OH^-" in Scheme 2 is a reactive oxygen species (ROS) or a reactive nitrogen species.

Scheme 2



The *KatG* gene was intact in the RB-resistant strain. Thus, it remains difficult to speculate a mechanism that confers the cross-resistance with INH. However, mutations in several transcriptional regulators of the RB-resistant strain were observed that may affect the expression level of KatG, suppressing the INH-activation. It generates hydroxy radicals (reactive oxygen species) through the Fenton reaction of H_2O_2. Requirement of relatively high concentration of RB to display bactericidal activity against Gram-positive bacteria including Mycobacterial spp. may imply that affinity of RB with catalases is moderate. Similarly, a cytotoxicity mechanism of RB in mammalian cells may be explained. As shown in Scheme 3, below.

Scheme 3



RB has been used for over 50 years to diagnose eye and liver disorders. It is often useful as a stain in diagnosing certain medical issues, such as conjunctival and lid disorders (*vide supra*). In these applications, 0.1-2.0% RB (i.e., 1,000-20,000 µg/mL) has been used.

RB in concentrations below 2.0% are considered to be safe under natural and artificial lights [Whitcher et al., *Am. J. Ophthalmol.* **149**:405-415 (2010)]. A healthy cell line, Vero (the kidney of an African green monkey) cells was chosen to determine *in vitro* cytotoxicity of RB under the fluorescent light.

Large data sets of cytotoxicity of antibacterial and anticancer agents against Vero cells have been generated, which allow comparison of the toxicity level of new molecules [Siricilla et al., *J. Med. Chem.* **60**:2869-2878 (2017); and Siricilla et al., *J. Antibiot.* **68**:271-278 (2015)]. In a 24h study under the dark, RB showed the IC_{50} value of 300 µM (292 µg/mL) against Vero cells.

Therapeutic concentrations of RB are likely to be about 0.1 µM to about 10 µM. Thus, these *in vitro* cytotoxicity tests imply that skin infections can be treated with RB without causing

cytotoxicity of host cells under illumination conditions.

Conclusions

Provectus has established a manufacturing and purification process for pharmaceutical-grade RB that fulfills both cGMP and ICH requirements. We have evaluated the antibacterial activity and cytotoxicity of a pharmaceutical-grade RB formulated product (PV-10®) as an exemplary RB compound in illuminating and dark conditions. The comprehensive MIC data for RB via saline-diluted PV-10® summarized here indicate that RB is very effective in killing most Gram-positive bacteria (MIC 0.39-3.1 μg/mL), except *Streptococcus pneumoniae* sp.

S. pneumonia is one of very few Gram-positive bacteria that is susceptible to colistin (polymixin E), an anti-Gram-negative drug. We have studied the relationship between RB's bactericidal effect and colistin-resistance in both Gram-positive and -negative bacteria. The resistance mechanism of *S. pneumoniae* against RB will be reported elsewhere.

We confirm that RB is an excellent agent to eradicate biofilms of Gram-positive bacteria, including drug-resistant strains. Under fluorescent and dark conditions, RB significantly reduced a number of viable cells of the drug-resistant strains of *S. aureus* and *E. faecium* in a concentration-dependent manner. These studies indicate that RB has the potential antibacterial agent to kill drug-resistant bacteria in any growth phases.

Materials and Methods

Antibiotics

All antibiotics were purchased from commercial sources [amikacin disulfate salt (Sigma Aldrich, A1774-1G), capreomycin sulfate (Sigma Aldrich, C4142-1G), ciprofloxacin hydrochloride monohydrate (TCI, C2227), ethionamide (TCI, E0695), isoniazid (Sigma Aldrich, I3377-5G), linezolid (Chem-Impex, 29723), meropenem trihydrate (Ark Pharm, AK161987), rifampicin (Sigma Aldrich, R3501-1G)] and used without further purification unless otherwise noted. APPB (aminouridylphenoxypiperi-dinylbenzyl butanamide) was synthesized according to the reported procedure. [Mitachi et al., *ACS Omega* **2018**, *3*:1726-1739; and Mitachi et al., *MethodsX* **2019**, *6*:2305-2321.]

Formulation of pharmacological grade rose bengal in saline (PV-10®).

Rose bengal disodium salt was synthesized according to Provectus' proprietary procedure. The detailed procedure was as described in U.S. Patents No. 8,530,675, No 9,273,022 and No. 9,422,260.

Acquisition of bacteria

The drug susceptible bacteria and yeasts used in this program were purchased from ATCC (The American Type Culture Collection, Manassas, VA). The drug-resistant strains were acquired from BEI Resources (NIAID).

Log phase bacterial culture

All liquid bacterial culturing was performed with a conical flask with an air filter.

A single colony of a bacterial strain was grown according to the conditions recommended by ATCC. Seed cultures and larger cultures of bacteria were obtained using media recommended by ATCC. *M. smegmatis* (ATCC 607) was cultured on a 0.5% Tween® 80 Middlebrook 7H10 nutrient agar (0.4% glycerol) [46]. The culture flasks were incubated for 3-4 days for *M. smegmatis* (ATCC 607), and for 10-12 days for *M. tuberculosis* $H_{37}Rv$ in a shaking incubator at 37 °C with a shaking speed of 200 rpm and cultured to mid-log phase (optical density – 0.5). The optical density was monitored at 600 nm using a 96 well microplate reader.

MIC assays

All testing follows the guidelines set by the Clinical & Laboratory Standards Institute (CLSI) ["Determining laboratory reference intervals: CLSI guideline makes the task manageable," *Lab. Med.* **40**:75-76 (2009).]. Minimum inhibitory concentrations (MICs) were determined by broth dilution microplate alamar blue assay or by OD measurement. All compounds were stored in DMSO or saline (1 mg/100 μL concentration). This concentration was used as the stock solution for all MIC studies. Each compound from stock solution was placed in the first well of a sterile 96 well plate and a serial dilution was conducted with the culturing broth (total volume of 10 μL). The bacterial suspension at log phase (190 μL) was added to each well (total volume of 200 μL), and was incubated for 24 h at 37 °C 20 μL of resazurin (0.02%) was added to each well and incubated for 4

h, (National Committee for Clinical Laboratory Standards (NCCLS) method (pink = growth, blue = no visible growth)). The OD measurements were performed for all experiments prior to performing colorimetric assays. The absorbance of each well was also measured at 570 and 600 nm via UV-Vis.

Minimal bactericidal concentration (MBC) assays

A single colony of a specific bacterium (grown on an agar plate) was inoculated into the culture broth. A bacterial culture was grown overnight (about 18 hours), then diluted in growth-supporting broth to a concentration between 1 x 10^5 and 1 x 10^9 CFU/mL. Based on the MIC values, agar plates containing a drug (MIC, 2x – 20x MIC) were prepared. A series of drug-containing agar plates were inoculated with equal volumes of the specific bacterium. The agar plates were incubated at the appropriate temperature and duration. CFU/mL were counted. The MBC values were determined by reduction of >99.9% of bacteria.

Mammalian cell lines and culturing

Vero cells (ATCC™ CCL-81) were purchased from the ATCC. HEK cells (SCCE020) were purchased from MilliporeSigma. The cell lines were cultured and maintained in the media as recommended by the suppliers.

Vero cells were cultured in Eagle's Minimum Essential Medium (MEM) (CORNING®, 10-009-CV) supplemented with 10% FBS, 1% Penicillin-Streptomycin Solution (cellgro®, 30-002-CI), 1% HyClone™ MEM Non-Essential Amino Acids Solution (100X) (GE Healthcare Life Sciences, SH30238.01) and

1% HyClone™ Sodium Pyruvate 100 mM Solution, (GE Healthcare Life Sciences, SH30239.01) and incubated in a 37 °C incubator with 100% humidity and 5% CO_2. Cultures were refreshed with fresh medium every 2 days until the culture reaches 100% confluence, which takes approximately 5 days depending on the proliferation rate.

Human Epidermal Keratinocytes, Neonatal (MILLIPORE®, Catalog# SCCE020) were cultured in EpiGRO™ Human Epidermal Keratinocyte Complete Culture Media Kit (MILLIPORE®, SCMK001) and incubated in a 37 °C incubator with 100% humidity and 5% CO_2. Refresh with fresh medium after 2 days and three times weekly until the culture reaches 100% confluence.

Cytotoxicity assay with Vero cells

All testing follows the guidelines set by the Clinical & Laboratory Standards Institute (CLSI) with minor modifications. Cytotoxicity assays for PV-10 were performed in a 24-well plate. Into each well (1 mL medium/well), 1 μL of drug concentration was added. After 1, 2, 3 and 4h of incubation under the light at room temperature (r.t.), the medium was removed, and the cells were washed with PBS (x3). After adding the medium (1 mL/well), 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added and incubated for another 3h at 37 °C (5% CO_2). The medium was removed, and DMSO (1 mL/well) was added. Viability was assessed on the basis of cellular conversion of MTT into a purple formazan product. The absorbance of the colored formazan product was measured at 570 nm by a BioTek Synergy™ HT

Spectrophotometer [Mitachi et al., *J. Med. Chem.* **63**:10855-10878 (2020)].

Vero cells [5 x 10^4 cells/well (in 196 μL of the culture medium)] were plated in a 96-well plate and the cell cultures were incubated for 4 days to form the monolayer (100% confluence). Into each well RB (0-300 mM) was added.

Images were obtained every hour using an IncuCyte® Live-Cell Imaging System (Essen BioScience, Ann Arbor, MI). Cell proliferation was quantified using the metric phase object confluence (POC), a measurement of the area of the field of view that is covered by cells, which is calculated

Whole-genome sequencing of *M. smegmatis* strains

RB-resistant *M. smegmatis* (ATCC607™) strains were prepared according to the procedures reported previously [Kaser et al., *Cold Spring Harb Protoc.* (2010)]. To identify single nucleotide polymorphisms (SNPs) that may contribute to the bacterial resistance to RB, the genomic DNAs were purified from the stationary cultures of RB-resistant mutant and its parental control *M. smegmatis* 607™ according to the procedure reported previously [Lelovic et al., *J. Antibiot.* **73**:780-789 (2020)].

The purified genomic DNA was submitted to the University of Minnesota Genomic Center (UMGC) for quality control analysis, the library preparation and DNA sequencing using an advanced Illumina MiSeq™ DNA-seq technology. Sequence reads from the mutant and the control were evaluated for their quality using FastQC. Low quality tails and adapters were removed with Trimmomatic [Lei et al.,

Methods Mol. Biol. **2069**:125-138 (2020)]. The whole-genome sequence of *M. smegmatis* strain FDAARGOS_679 was used as a reference, and SNPs or other variants such as deletion and insertions were called by using a bioinformatic tool Snippy [https://github.com/tseemann/snippy].

Each of the patents, patent applications and articles cited herein is incorporated by reference. The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.

CLAIMS

1. A topical ophthalmic system for treating a Gram-positive bacterial infection of the eye of a mammal in need that comprises an ophthalmic composition containing a halogenated fluorescein or a pharmaceutically acceptable salt or ester thereof dissolved or dispersed in an aqueous ophthalmic carrier and present in a bacterial keratitis-treating effective concentration of about 0.2 μg/mL to about 50 μg/mL in the composition, said composition having a pH value of about 6.5 to about 7.6, a viscosity of about 10 to about 300 cps and an osmolality of about 270 mOsm/kg to about 340 mOsm/kg, and said composition being present in a vessel opaque to actinic light.

2. The ophthalmic system according to claim 1, wherein said composition further contains glycerin or propylene glycol or both glycerin and propylene glycol up to a total of 2.5 weight percent of the composition.

3. The ophthalmic system according to claim 1, wherein at least some said osmolality is provided by the presence of one or more water-soluble sodium, potassium, calcium, and magnesium chlorides, phosphates, and nitrates.

4. The ophthalmic system according to claim 1, wherein said viscosity is provided at least in part by one or more polymer free of an ionic charge at the pH value of the composition.

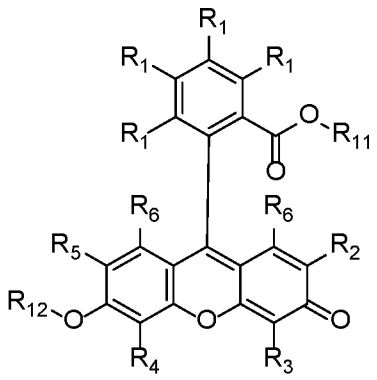
5. The ophthalmic system according to claim 4, wherein said polymer free of an ionic charge at the pH value of the composition is selected from the group consisting of one or more of PEG 400, PEG 1000, dextran, dextrin, poly(vinylpyrrolidone), hypromellose, methyl cellulose, hydroxyethyl cellulose, and poly(vinyl alcohol).

6. The ophthalmic system according to claim 1, wherein said viscosity is provided at least in part by one or more polymer bearing an anionic charge at the pH value of the composition.

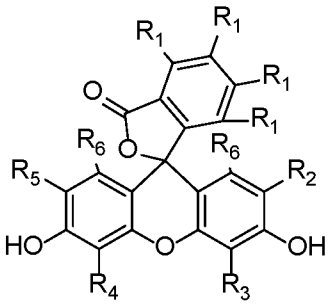
7. The ophthalmic system according to claim 6, wherein said polymer bearing an anionic charge at the pH value of the composition is selected from the group consisting of one or more of a) hyaluronic acid, b) water-soluble partially neutralized cross-linked acrylic acid, methacrylic acid and co-polymers cross-linked with allyl sucrose or allyl pentaerythritol, wherein each cross-linker contains an average of at least three allyl groups per molecule, c) water-swellable but water-insoluble partially neutralized reaction product of the copolymerization of at least 80 weight percent monoethylenically unsaturated carboxy-functional monomer and about 0.05 to about 1.5 weight percent of a cross-linking agent that is 3,4-dihydroxy-1,5-hexadiene, or 2,5-dimethyl-1,5-hexadiene, and d) partially neutralized carboxymethyl cellulose.

8. The ophthalmic system according to claim 1, wherein said halogenated fluorescein or

pharmaceutically acceptable salt or ester thereof has the chemical formula of one or both of Formulas 1 and/or 2



FORMULA 1



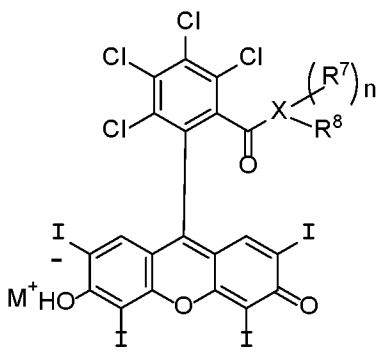
FORMULA 2

wherein R_1 is independently F, Cl, Br, I, H or C_1-C_4 alkyl;

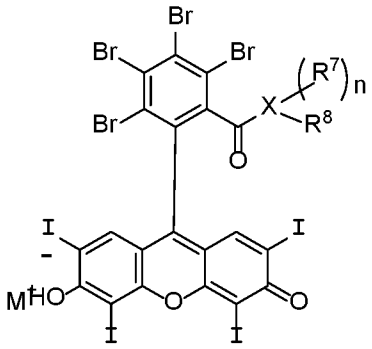
R_2, R_3, R_4, and R_5 are independently Cl, H or I with at least one substituent selected from R_2, R_3, R_4, R_5 being I; and

R_6 is independently H or C_1-C_4 alkyl; R^{11} is H or C_1-C_4 alkyl; R^{12} is H or C_1-C_7 acyl; and all (a) tautomeric forms; (b) atropisomers, (d) enantiomers of lactone forms depicted in **Formula 2**, and (e) pharmaceutically acceptable salts thereof.

9. The ophthalmic system according to claim 8, wherein R_1 of said halogenated fluorescein of **Formulas 1** and **2**, above, is either a chloro or a bromo substituent, whereas each of R_2, R_3, R_4, and R_5 of those formulas is an iodo substituent as are present in Formulas **I** and **II**, below, X is oxygen or nitrogen, "n" is zero or 1 such that when X is



Formula I



Formula II

oxygen, n is zero and R^7 is absent forming an aromatic ester, whereas when X is nitrogen, n is 1 and R^7 is present forming an aromatic amide;

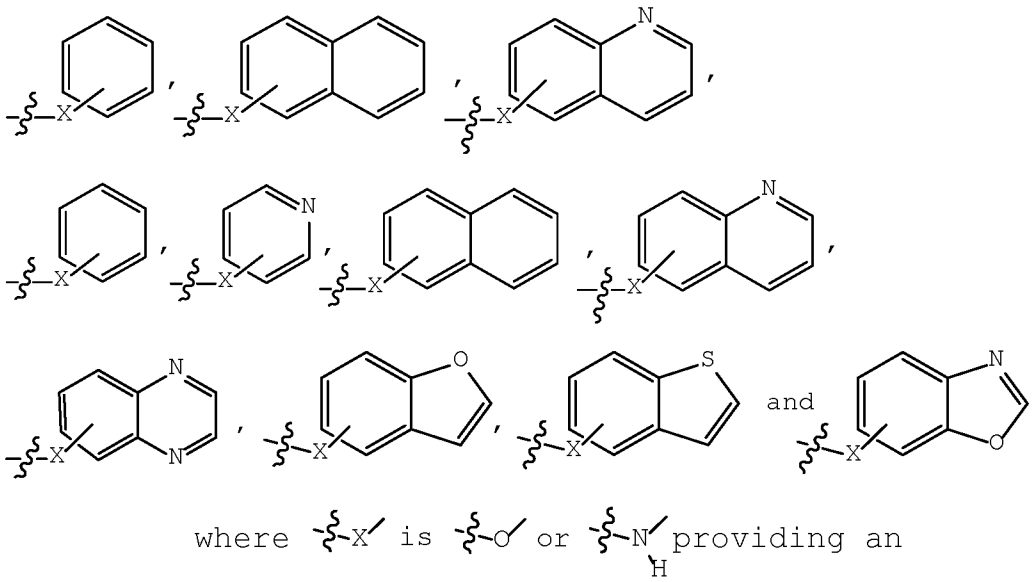
when X is oxygen, R^7 is selected from the group consisting of hydrogen (H), M^+ that is a pharmaceutically acceptable cation, C_1-C_4 alkyl, and an aromatic ring-containing substituent;

wherein said aromatic ring-containing substituent is a single ring containing 5- or 6-members, or a 5,6- or 6,6-fused aromatic ring system, in which the single aromatic ring or fused aromatic ring system contains 0, 1 or 2 hetero ring atoms that are independently nitrogen, oxygen or sulfur;

when X is nitrogen, R^7 and R^8 are the same or different and are selected from the group consisting of hydrogen, C_1-C_4 alkyl, an aromatic ring-containing substituent or together with amido nitrogen atom R^7 and R^8 together with amido nitrogen atom form a 5- or 6-membered ring.

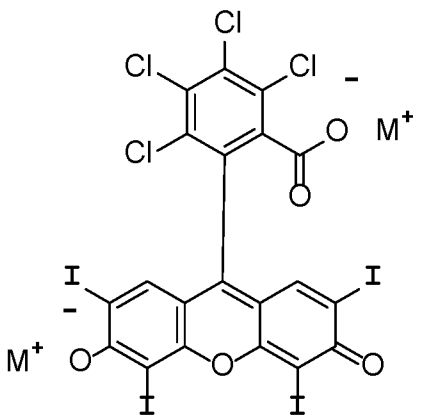
10. The ophthalmic system according to claim 9, wherein said aromatic ring-containing substituent of said aromatic ester or aromatic amide

unsubstituted, and is selected from the group consisting of the following

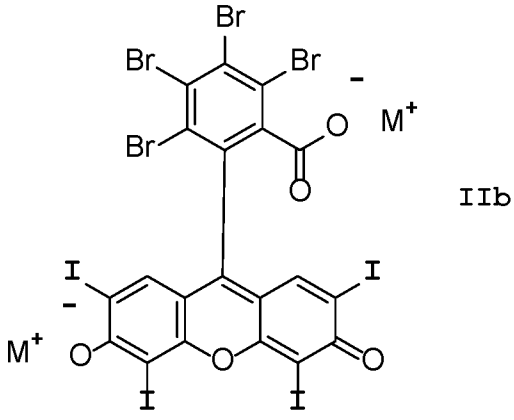


where is or providing an ester or the R^8 group is hydrogen forming a monosubstituted amide, respectively.

11. The ophthalmic system according to claim 1, wherein said halogenated fluorescein or pharmaceutically acceptable salt or ester thereof has the chemical formula of one or both of **Formulas Ia** and/or **IIb**



Ia



IIb

wherein M^+ is a pharmaceutically acceptable cation.

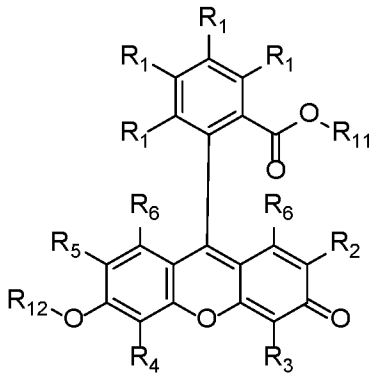
12. The ophthalmic system according to claim 1, wherein said halogenated fluorescein or pharmaceutically acceptable salt or ester thereof is rose bengal disodium.

13. A method of treating a mammalian subject having a Gram-positive bacterially-infected eye that exhibits keratitis comprising the steps of treating said microbially-infected eye by administering to that microbially-infected eye a keratitis-treating effective amount of an ophthalmic composition containing a halogenated fluorescein or pharmaceutically acceptable salt or ester thereof dissolved or dispersed in an aqueous ophthalmic carrier and present in a bacterial keratitis-treating effective concentration of about 0.2 μg/mL to about 50 μg/mL in the composition, said ophthalmic composition having a pH value of about 6.5 to about 7.6, a viscosity of about 10 to about 300 cps and an osmolality of about 270 mOsm/kg to about 340 mOsm/kg, and maintaining said treated eye in the substantial absence of actinic light for a time period of about 3 to about 12 hours.

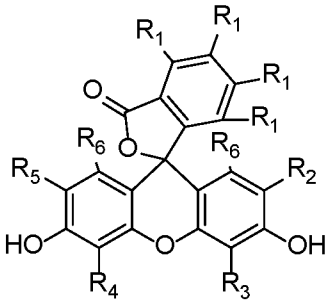
14. The method according to claim 13, wherein said treatment is carried out in the substantial absence of actinic light.

15. The method according to claim 13, wherein said ophthalmic composition is maintained in a vessel opaque to actinic light prior to said administration.

16. The method according to claim 13, wherein said halogenated fluorescein or pharmaceutically acceptable salt or ester thereof has the chemical formula of one or both of Formulas 1 and/or 2



FORMULA 1



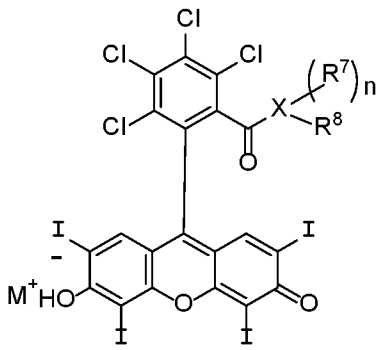
FORMULA 2

wherein R_1 is independently F, Cl, Br, I, H or C_1-C_4 alkyl;

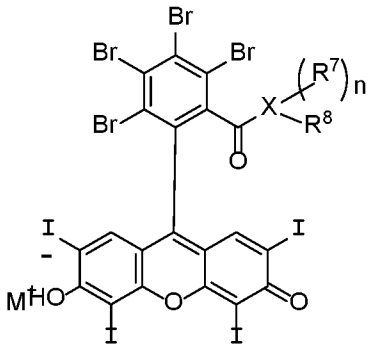
R_2, R_3, R_4, and R_5 are independently Cl, H or I with at least one substituent selected from R_2, R_3, R_4, R_5 being I; and

R_6 is independently H or C_1-C_4 alkyl; R^{11} is H or C_1-C_4 alkyl; R^{12} is H or C_1-C_7 acyl; and all (a) tautomeric forms; (b) atropisomers, (d) enantiomers of lactone forms depicted in **Formula 2**, and (e) pharmaceutically acceptable salts thereof.

17. The method according to claim 16, wherein R_1 of said halogenated fluorescein of **Formulas 1** and **2**, above, is either a chloro or a bromo substituent, whereas each of R_2, R_3, R_4, and R_5 of those formulas is an iodo substituent as are present in Formulas **I** and **II**, below, X is oxygen or nitrogen, "n" is zero or 1 such that when X is



Formula I



Formula II

oxygen, n is zero and R^7 is absent forming an aromatic ester, whereas when X is nitrogen, n is 1 and R^7 is present forming an aromatic amide;

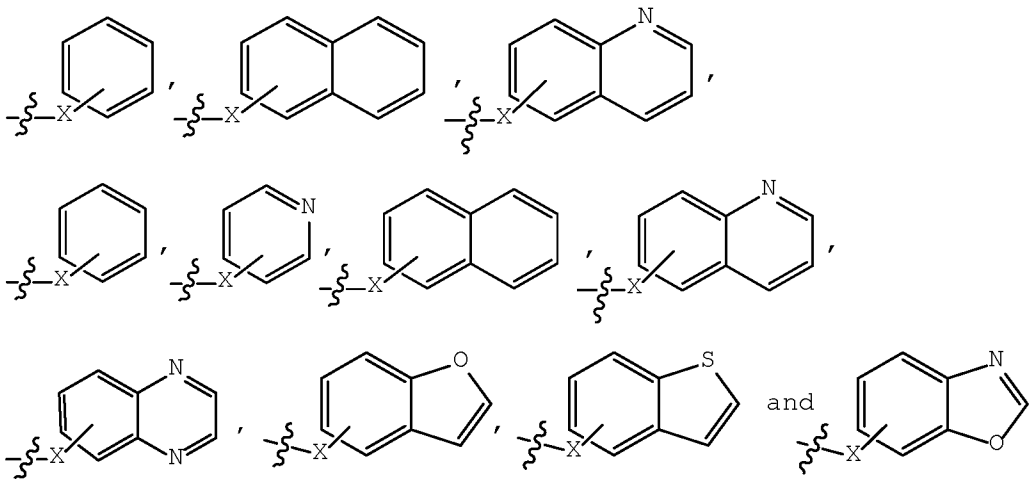
when X is oxygen, R^7 is selected from the group consisting of hydrogen (H), M^+ that is a pharmaceutically acceptable cation, C_1-C_4 alkyl, and an aromatic ring-containing substituent;

wherein said aromatic ring-containing substituent is a single ring containing 5- or 6-members, or a 5,6- or 6,6-fused aromatic ring system, in which the single aromatic ring or fused aromatic ring system contains 0, 1 or 2 hetero ring atoms that are independently nitrogen, oxygen or sulfur;

when X is nitrogen, R^7 and R^8 are the same or different and are selected from the group consisting of hydrogen, C_1-C_4 alkyl, an aromatic ring-containing substituent or together with amido nitrogen atom R^7 and R^8 together with amido nitrogen atom form a 5- or 6-membered ring.

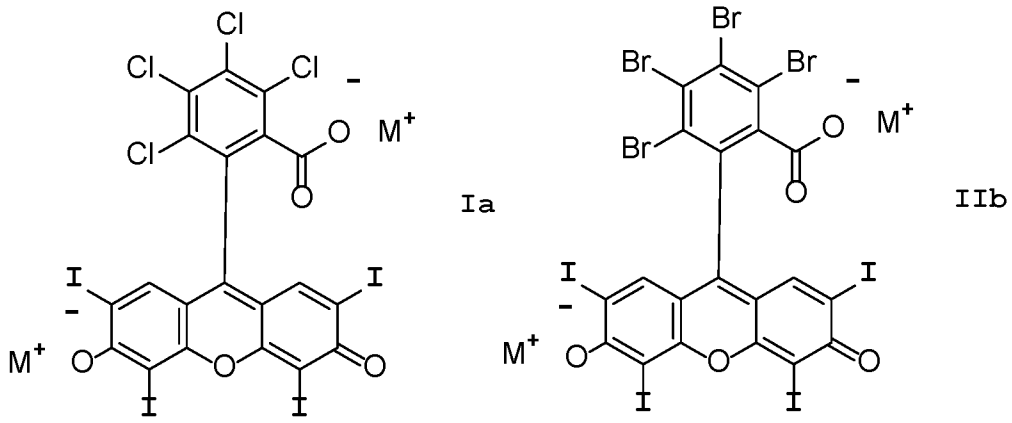
18. The method according to claim 17, wherein said aromatic ring-containing substituent of

said aromatic ester or aromatic amide unsubstituted, and is selected from the group consisting of the following



where  is  or  providing an ester or the R^8 group is hydrogen forming a monosubstituted amide, respectively.

19. The method according to claim 13, wherein said halogenated fluorescein or pharmaceutically acceptable salt or ester has the chemical formula of one or both of **Formulas Ia** and/or **IIb**



Ia IIb

wherein M^+ is a pharmaceutically acceptable cation.

20. The method according to claim 13, wherein said halogenated fluorescein or pharmaceutically acceptable salt or ester thereof is rose bengal disodium.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 22/54076

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. A61K 31/37, A61K 31/335 (2023.01)
ADD. C09B 11/08, A61P 27/02, A61P 31/04 (2023.01)

CPC - INV. A61K 31/37, A61K 31/335
ADD. C09B 11/08, A61P 27/02, A61P 31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2008/0118578 A1 (DEES, et.al.) 22 May 2008 (22.05.2008) para [0016];[0019];[0034];[0035];[0040];[0045];[0046];[0051];[0057];[0070]; [0074];[0076];[0080]; pg. 9, Table 1; pg. 10, Table 2; Figure 1b	1-9, 11-12 ---------- 10, 13-20
Y	NARANJO, et.al. Rose Bengal Photodynamic Antimicrobial Therapy For Patients with Progressive Infectious Keratitis: A Pilot Clinical Study in Am. J. Ophthalmol., 2019, Vol. 208, pp. 387-396. abstract; pg. 388, Col 2, para 3 to pg. 389, Col 1, para 1	13-20
Y	US 7,229,737 B2 (FUKUSHIGE, et.al.) 12 June 2007 (12.06.2007) Col 1, ln 7-17; Col 13	10, 18
A	US 2014/0343296 A1 (PROVECTUS PHARMACEUTICALS, Inc.) 20 November 2014 (20.11.2014) ENTIRE DOCUMENT	1-20
A	US 2019/0358142 A1 (KLOX TECHNOLOGIES, INC.) 28 November 2019 (28.11.2019) ENTIRE DOCUMENT	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 MARCH 2023 (05.03.2023)	APR 25 2023
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Kari Rodriquez Telephone No. PCT Helpdesk: 571-272-4300

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A61P 31/04(2006.01)

权利要求书5页 说明书22页

(54)发明名称

含卤代氧杂蒽的局部抗革兰氏阳性细菌的眼科组合物和方法

(57)摘要

本发明设想一种用于治疗革兰氏阳性细菌感染的哺乳动物的眼睛的局部眼科系统。所述系统包含眼科组合物,所述眼科组合物含有溶解或分散在水性眼科运载体中的卤代荧光素或其药学上可接受的盐或酯,并且所述卤代荧光素或其药学上可接受的盐或酯以约0.2μg/mL至约50μg/mL的治疗抗细菌性角膜炎有效浓度存在于所述组合物中。所述眼科组合物具有约6.5至约7.6的pH值,约10cps至约300cps的粘度,约270mOsm/kg至约340mOsm/kg的渗透压。所述眼科组合物存在于光化光不透过的容器中。本发明还设想一种治疗患有眼睛革兰氏阳性细菌感染的哺乳动物的方法,所述方法通过向受感染的眼睛施用眼科组合物并使所治疗的眼睛在基本上不存在光化光的情况下保持约3小时至约12小时而进行。

CN 118488836 A

1.一种用于治疗有需要的哺乳动物的眼睛的革兰氏阳性细菌感染的局部眼科系统,所述局部眼科系统包含眼科组合物,所述眼科组合物含有溶解或分散在水性眼科运载体中的卤代荧光素或其药学上可接受的盐或酯,并且所述卤代荧光素或其药学上可接受的盐或酯以约0.2μg/mL至约50μg/mL的治疗细菌性角膜炎有效浓度存在于所述组合物中,所述组合物具有约6.5至约7.6的pH值,约10cps至约300cps的粘度,约270mOsm/kg至约340mOsm/kg的渗透压,并且所述组合物存在于光化光不透过的容器中。

2.根据权利要求1所述的眼科系统,其中所述组合物还含有多至共占组合物2.5重量%的甘油或丙二醇或甘油和丙二醇两者。

3.根据权利要求1所述的眼科系统,其中至少一些所述渗透压由一种或多种水溶性的钠、钾、钙和镁的氯化物、磷酸盐和硝酸盐的存在而提供。

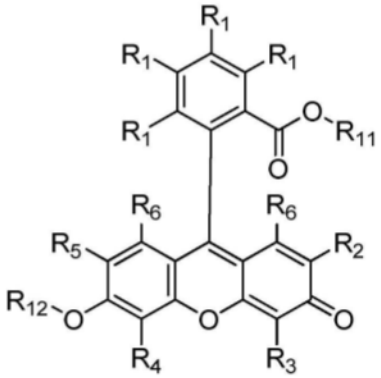
4.根据权利要求1所述的眼科系统,其中所述粘度至少部分由在组合物的pH值下不带离子电荷的一种或多种聚合物提供。

5.根据权利要求4所述的眼科系统,其中在组合物的pH值下不带离子电荷的所述聚合物选自由以下一种或多种组成的组:PEG400,PEG1000,右旋糖酐,糊精,聚(乙烯基吡咯烷酮),羟丙甲纤维素,甲基纤维素,羟乙基纤维素和聚(乙烯醇)。

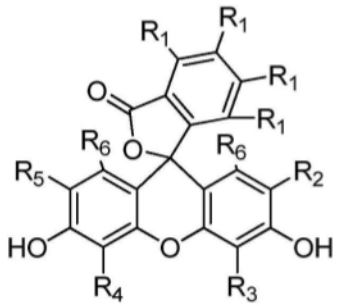
6.根据权利要求1所述的眼科系统,其中所述粘度至少部分由在组合物的pH值下带有阴离子电荷的一种或多种聚合物提供。

7.根据权利要求6所述的眼科系统,其中在组合物的pH值下带有阴离子电荷的所述聚合物选自由以下一种或多种组成的组:a)透明质酸,b)水溶性的部分中和的交联丙烯酸、甲基丙烯酸和共聚物,其与烯丙基蔗糖或烯丙基季戊四醇交联,其中每个交联剂平均每个分子含有至少三个烯丙基,c)至少80重量%的单烯基不饱和羧基官能单体和约0.05重量%至约1.5重量%的交联剂共聚的水溶胀性但不溶于水的部分中和的反应产物,所述交联剂是3,4-二羟基-1,5-己二烯或2,5-二甲基-1,5-己二烯,和d)部分中和的羧甲基纤维素。

8.根据权利要求1所述的眼科系统,其中所述卤代荧光素或其药学上可接受的盐或酯具有式1和/或式2的一种或两种的化学式:



式 1



式 2

其中R_1独立地是F、Cl、Br、I、H或C_1-C_4烷基;

R_2、R_3、R_4和R_5独立地是Cl、H或I,其中至少一个选自R_2、R_3、R_4、R_5的取代基是I;并且

R_6独立地是H或C_1-C_4烷基;R_{11}是H或C_1-C_4烷基;R_{12}是H或C_1-C_7酰基;和

所有(a)互变异构形式;(b)阻转异构体,(d)式2所示的内酯形式的对映异构体,和(e)

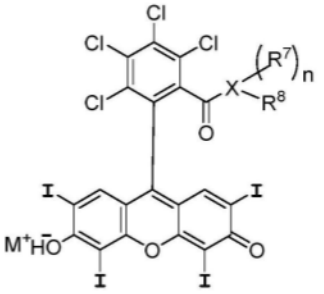
其药学上可接受的盐。

9.根据权利要求8所述的眼科系统,其中上述式1和式2的所述卤代荧光素中的R_1是氯取代基或溴取代基,而这些式中的R_2、R_3、R_4和R_5中的每一个都是碘取代基,所述碘取代基如以下式I和式II所示,X是氧或氮,"n"是0或1,使得当X是氧时,n是0并且R^7不存在,从而形成芳香酯,而当X是氮时,n是1并且R^7存在,从而形成芳酰胺;

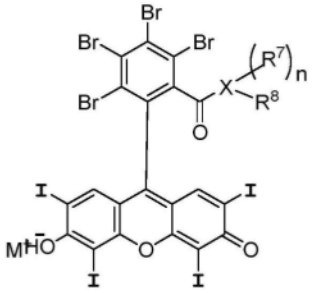
当X是氧时,R^7选自由以下组成的组:氢(H),药学上可接受的阳离子M^+,C_1-C_4烷基和含芳环的取代基;

其中所述含芳环的取代基是含有5个或6个成员的单环、或5,6-或6,6-稠合芳环系统,其中单芳环或稠合芳环系统含有0、1或2个杂环原子,所述杂环原子独立地是氮、氧或硫;

当X为氮时,R^7和R^8相同或不同,并且选自由以下组成的组:氢,C_1-C_4烷基,含芳环的取代基,或R^7和R^8与酰胺氮原子一起形成5元或6元环;

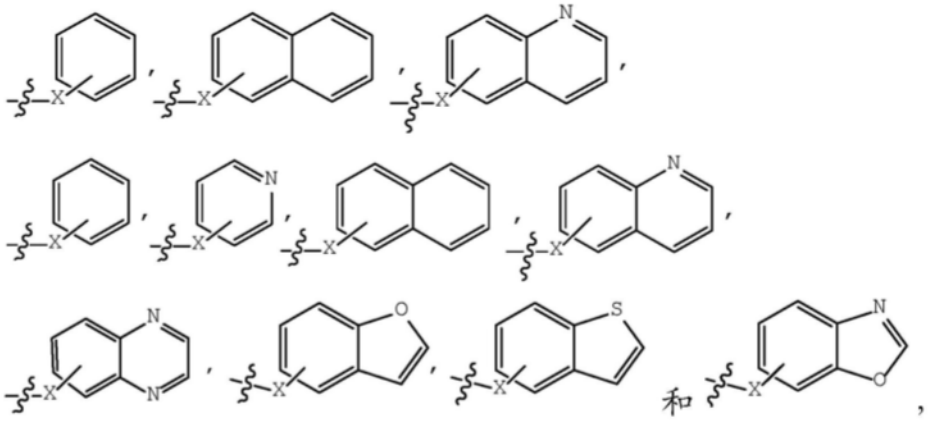


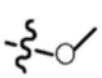
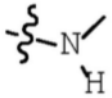
式 I



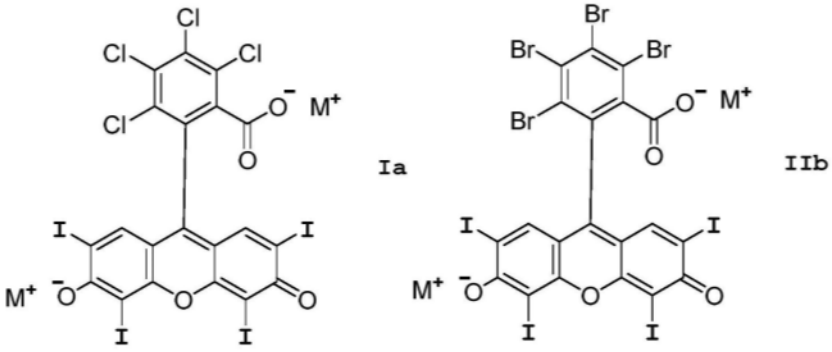
式 II。

10.根据权利要求9所述的眼科系统,其中所述芳香酯或芳酰胺的所述含芳环的取代基没有被取代,并且选自由以下组成的组:



其中  是  或  分别提供酯或当R^8基团是氢时形成单取代酰胺。

11.根据权利要求1所述的眼科系统,其中所述卤代荧光素或其药学上可接受的盐或酯具有式Ia和/或式IIb的一种或两种的化学式:



Ia　　IIb

其中M^+是药学上可接受的阳离子。

12.根据权利要求1所述的眼科系统,其中所述卤代荧光素或其药学上可接受的盐或酯是玫瑰红二钠。

13.一种治疗具有表现为角膜炎的革兰氏阳性细菌感染的眼睛的哺乳动物受试者的方法,所述方法包括以下步骤:

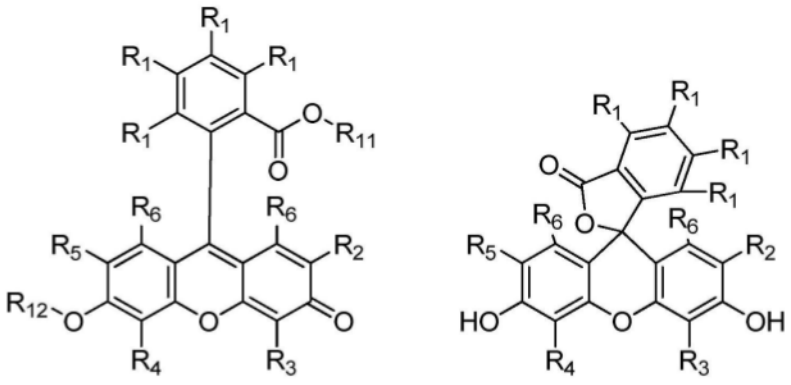
通过向微生物感染的眼睛施用治疗角膜炎有效量的眼科组合物来治疗所述微生物感染的眼睛,所述眼科组合物含有溶解或分散在水性眼科运载体中的卤代荧光素或其药学上可接受的盐或酯,并且所述卤代荧光素或其药学上可接受的盐或酯以约0.2μg/mL至约50μg/mL的治疗细菌性角膜炎有效浓度存在于所述组合物中,所述眼科组合物具有约6.5至约7.6的pH值,约10cps至约300cps的粘度,约270mOsm/kg至约340mOsm/kg的渗透压,并且

使所述治疗的眼睛在基本上不存在光化光的情况下保持约3小时至约12小时的时间段。

14.根据权利要求13所述的方法,其中所述治疗是在基本上不存在光化光的情况下进行的。

15.根据权利要求13所述的方法,其中所述眼科组合物在所述施用之前保持在光化光不透过的容器中。

16.根据权利要求13所述的方法,其中所述卤代荧光素或其药学上可接受的盐或酯具有式1和/或式2的一种或两种的化学式:



式 1　　式 2

其中R_1独立地是F、Cl、Br、I、H或C_1-C_4烷基;

R_2、R_3、R_4和R_5独立地是Cl、H或I,其中至少一个选自R_2、R_3、R_4、R_5的取代基为I;并且

R_6独立地是H或C_1-C_4烷基;R_{11}是H或C_1-C_4烷基;R_{12}是H或C_1-C_7酰基;和

所有(a)互变异构形式;(b)阻转异构体,(d)式2所示的内酯形式的对映异构体,和(e)

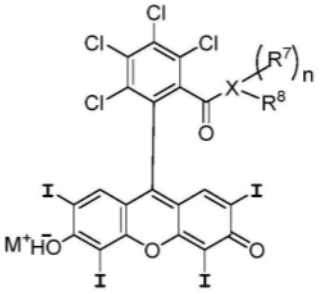
其药学上可接受的盐。

17.根据权利要求16所述的方法，其中上述式1和式2的所述卤代荧光素中的R_1是氯取代基或溴取代基，而这些式中的R_2、R_3、R_4和R_5中的每一个都是碘取代基，所述碘取代基如以下式I和式II所示，X是氧或氮，“n”是0或1，使得当X是氧时，n是0并且R^7不存在，从而形成芳香酯，而当X是氮时，n是1并且R^7存在，从而形成芳酰胺；

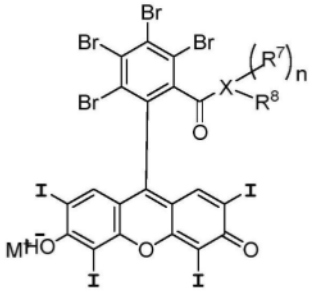
当X是氧时，R^7选自由以下组成的组：氢(H)，药学上可接受的阳离子M^+，C_1-C_4烷基和含芳环的取代基；

其中所述含芳环的取代基是含有5个或6个成员的单环、或5,6-或6,6-稠合芳环系统，其中单芳环或稠合芳环系统含有0、1或2个杂环原子，所述杂环原子独立地是氮、氧或硫；

当X为氮时，R^7和R^8相同或不同，并且选自由以下组成的组：氢，C_1-C_4烷基，含芳环的取代基，或R^7和R^8与酰胺氮原子一起形成5元或6元环；

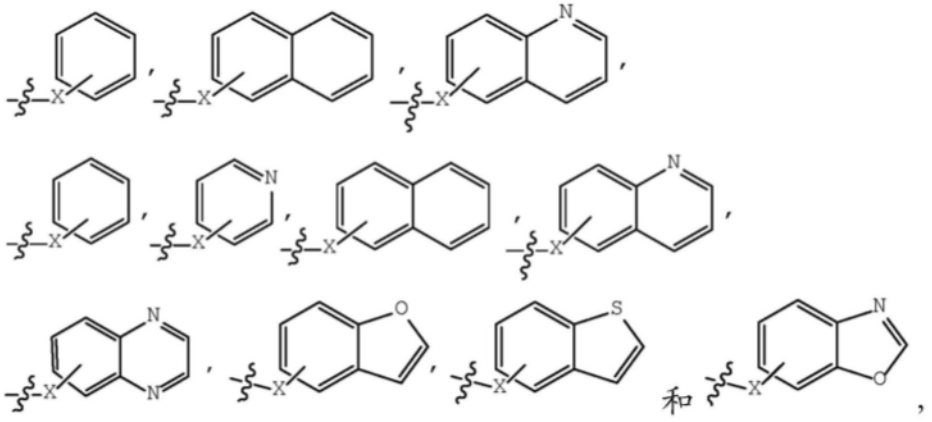


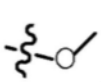
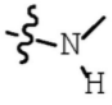
式 I



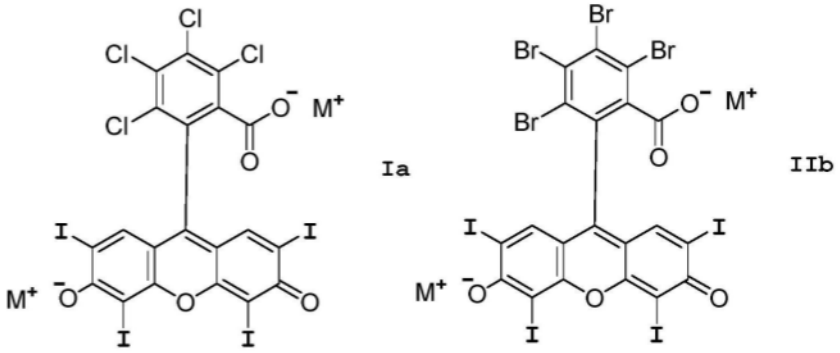
式 II。

18.根据权利要求17所述的方法，其中所述芳香酯或芳酰胺的所述含芳环的取代基没有被取代，并且选自由以下组成的组：



其中  是  或  ，分别提供酯或当R^8基团是氢时形成单取代酰胺。

19.根据权利要求13所述的方法，其中所述卤代荧光素或其药学上可接受的盐或酯具有式Ia和/或式IIb的一种或两种的化学式：



Ia IIb

其中M^+是药学上可接受的阳离子。

20.根据权利要求13所述的方法,其中所述卤代荧光素或其药学上可接受的盐或酯是玫瑰红二钠。

含卤代氧杂蒽的局部抗革兰氏阳性细菌的眼科组合物和方法

[0001] 对相关申请的交叉引用

[0002] 本申请要求于2021年12月28日提交的美国申请系列号63/294252的优先权,该申请的公开内容通过引用并入本文。

背景技术

[0003] 角膜疾病是全世界单眼失明的主要原因,尤其影响边缘化的人群。角膜混浊主要由传染性角膜炎造成,是全球失明的第四大原因,在世界上资源最少的国家中,角膜混浊占可避免视力障碍的10%。仅在印度,每年就有约200万人患角膜溃疡。在美国,传染性角膜炎通常与佩戴隐形眼镜相关,但在资源不足的国家,其更常由农业工作期间持续的眼创伤造成(Austin等人,Ophthalmology 124(11):1678-1689(2017))。

[0004] 一种角膜溃疡,即角膜炎,是角膜上的开放性伤,并且是常见的人类眼睛病况。它可能是由创伤造成的,特别是由植物物质,以及化学伤害、隐形眼镜和感染造成的。其它眼睛病况(诸如睑内翻、双行睫、角膜营养不良和干燥性角膜结膜炎(干眼症))可造成角膜溃疡。

[0005] 许多微生物造成感染性角膜溃疡。其中包括细菌、真菌、病毒、原生动物和衣原体。

[0006] 细菌性角膜炎可由多种细菌造成,包括金黄色葡萄球菌(Staphylococcus aureus)、草绿色链球菌(Streptococcus viridans)、大肠杆菌(Escherichia coli)、肠球菌(Enterococci)、假单胞菌(Pseudomonas)、诺卡菌(Nocardia)、淋病奈瑟菌(N.gonorrhoea)和许多其它细菌。多重耐药性(MDR)革兰氏阳性细菌的出现的增加是主要的公共卫生威胁(Bassetti等人,Annal.Clinic.Microbiol.Antimicrob.12:1-15(2013);Butler等人,J.Antibiot.66:571-591(2013);和Woodford等人,J.Infect.59:S4-16(2009))。特别地是,葡萄球菌、肠球菌和链球菌属的MDR菌株对发病率和死亡率有显著影响(Dupont等人,J.Antimicrob.Chemother.66:2379-2385(2011))。

[0007] 真菌性角膜炎造成深部的和严重的角膜溃疡。其通常由曲霉属(Aspergillus sp)、镰孢菌属(Fusarium sp)、念珠菌属(Candida sp.)以及根霉(Rhizopus)、毛霉菌(Mucor)和其它真菌造成。真菌性角膜炎的典型特征是缓慢发作和逐渐进展,其中迹象远多于症状。溃疡周围的小卫星病变是真菌性角膜炎的常见特征,并且观察到涉及眼前房中的炎性细胞的病况(眼前房积脓)。

[0008] 病毒性角膜炎造成角膜溃疡。其最常见地是由单纯疱疹、带状疱疹和腺病毒造成的。其也可能由冠状病毒和许多其它病毒造成。

[0009] 疱疹病毒造成树枝状溃疡,所述树枝状溃疡可在个体的一生中复发和重现。单纯疱疹病毒(HSV)角膜炎在美国影响估计500000人,并且在全球影响估计150万人。疱疹病毒是大多数发达国家中单侧感染性角膜失明的最常见的原因(Austin等人,Ophthalmology124(11):1678-1689(2017))。

[0010] 病毒性角膜炎与细菌性和真菌性角膜炎的不同之处在于病毒性角膜炎可以变成慢性和复发性的。HSV角膜炎除了是一种痛苦的、威胁视力的感染外,即使当患者没有经历

活动性感染时,其也会显著影响生活质量。较不常见形式的病毒性角膜炎包括水痘带状疱疹病毒(VZV)角膜炎和巨细胞病毒(CMV)角膜炎(Austin等人,Ophthalmology124(11):1678-1689(2017))。

[0011] 原生动物(诸如棘阿米巴原虫(Acanthamoeba))感染角膜炎的特征是剧烈疼痛,且与在游泳池中游泳的隐形眼镜使用者有关。

[0012] 浅表溃疡涉及部分上皮的脱落。深部溃疡延伸到基质中或穿过基质,可导致严重的瘢痕和角膜穿孔。当溃疡延伸穿过基质时,就会发生角膜后弹力层膨出(descemetoceles)。这种类型的溃疡特别危险,如果不及时治疗,会迅速导致角膜穿孔。

[0013] 溃疡的位置在一定程度上取决于病因。中央溃疡通常是由创伤、干眼症或从面神经麻痹或眼球突出的暴露造成的。睑内翻、严重干眼症和倒睫(睫毛的向后生长)可能造成周围角膜的溃疡。免疫介导的眼病可在角膜和巩膜的边缘导致溃疡。这些免疫病包括类风湿性关节炎、玫瑰痤疮和系统性硬化症,它们会引起一种特殊类型的角膜溃疡,称为蚕蚀性角膜溃疡。蚕蚀性角膜溃疡在角膜边缘内有一个环形凹坑状的角膜凹陷,通常有一个悬垂的边缘。

[0014] 正确的诊断对最佳治疗至关重要。溃疡(无论是感染性的还是非感染性)的造成原因尚待确定。

[0015] 细菌性角膜溃疡通常需要大量加强的抗生素疗法来治疗感染。真菌性角膜溃疡需要大量施用局部抗真菌剂。疱疹病毒造成的病毒性角膜溃疡可能对抗病毒剂有反应,例如每天至少局部滴注五次阿昔洛韦软膏。此外,通常会施用止痛药等支持疗法,包括阿托品或后马托品等局部睫状肌麻痹药,以扩张瞳孔,从而停止睫状肌痉挛。

[0016] 浅表溃疡可能在不到一周的时间内治愈。深部溃疡和角膜后弹力层膨出可能需要结膜移植物或结膜瓣、软性隐形眼镜或角膜移植。通常建议适当的营养,包括蛋白质摄入和维生素C。在角膜软化症的情况下,角膜溃疡是由于维生素A的缺乏引起的,通过口服或肌内途径给予维生素A补充。通常在角膜溃疡中禁用的药物是局部皮质类固醇(Alhassan等人,Cochrane Database Syst Rev.1(1):CD006131(2014))和麻醉剂,这些药物不应用于任何类型的角膜溃疡,因为它们会防止愈合,可能引起真菌和其它细菌的重复感染,并且通常会使病况更加恶化。

[0017] “红眼”、“结膜炎”和“角膜溃疡/角膜炎”是常提到的问题。结膜炎是造成结膜血管扩张并导致炎症的常见病况。病毒性和细菌性结膜炎均伴有红眼,并且具有高度传染性。评估应包括检查视力和用手电筒或裂隙灯检查。应将荧光素滴剂滴入结膜囊中,并用裂隙灯的钴蓝光或眼底镜观察眼睛,以排除任何角膜溃疡或感染的迹象。

[0018] 病毒性结膜炎是造成感染性结膜炎最常见的原因。这种感染在成人中比在儿童中更常见。大多数病例是由腺病毒造成的。有时,单纯疱疹或带状疱疹病毒是造成这种情况的原因。通常可以告知患者病毒性结膜炎是自限性的,因为没有具体的治疗方法。

[0019] 细菌性结膜炎虽然是结膜炎的较不常见的原因,但细菌性结膜炎在儿童中更常见。最常见的细菌是流感嗜血杆菌(Haemophilus influenza)、肺炎链球菌(Streptococcus pneumoniae)和金黄色葡萄球菌。使用抗生素滴眼液与改善的临床和微生物缓解率有关。通常建议使用广谱局部抗生素。

[0020] 角膜感染(微生物性角膜炎)是一种眼科急症,需要立即关注,因为其可以迅速发

展。角膜感染是处在工作年龄的成年人视力损伤的最常见的原因之一。在美国,每年报告约30000例微生物性角膜炎(Sharma等人,Ocul Surf 15:670-679(2017))。

[0021] 细菌感染是感染性角膜炎最常见的原因。常见的致病细菌包括金黄色葡萄球菌、凝固酶阴性葡萄球菌、肺炎链球菌和铜绿假单胞菌(Pseudomonas aeruginosa)(Teweldmedhin等人,BMC Ophthalmol17:212(2017))。铜绿假单胞菌(P.aeruginosa)是隐形眼镜佩戴者中与细菌性角膜炎有关的最常见的微生物。

[0022] 尽管如下所述,细菌性溃疡通常对可用的局部抗生素滴剂治疗有反应,但北美的抗生素耐药性(诸如耐甲氧西林金黄色葡萄球菌(MRSA))感染率的增加已经引起关注。美国疾病控制中心(CDC)估计,每年有200万人感染耐药性微生物。据报道,在美国,大约80%的MRSA的眼部分离株对最常用的抗生素类氟喹诺酮类处方药具有耐药性。(Austin等人,Ophthalmology 124(11):1678-1689(2017))

[0023] 局部抗生素是目前的主要治疗方法,并且选项包括使用氟喹诺酮类(每小时1-2滴环丙沙星0.3%或氧氟沙星0.3%,持续48小时,然后每4小时一次,直至痊愈)的单一疗法或加强的氨基糖苷类/头孢菌素组合(每小时1-2滴加强的头孢噻啉5%加庆大霉素0.9%,持续48小时,然后根据治疗反应减少频率)。这些方案具有相似的效果,但氟喹诺酮类降低化学性结膜炎的风险和眼部不适。与氧氟沙星相比,环丙沙星增加白色角膜沉淀物的风险。有时,可能需要角膜移植来根除生物体或修复损伤。虽然明显有效,但对于许多患者来说,每小时的滴眼液治疗是一种难以遵循的方案。

[0024] 氯霉素是用于红眼最常见的一线处方抗生素。有几种商业产品可用,作为滴眼液和眼膏使用。示例性地,含有0.25%和0.5%氯霉素的眼科溶液和含有1%氯霉素的眼膏可从Sandoz Canada Inc.以商品名PrPENTAMYCETIN®获得。

[0025] 眼科溶液的剂量和施用说明书仅规定"每3小时或更频繁(如有必要)向受感染的眼睛滴2滴眼科溶液"。眼膏的剂量和施用说明书规定:"每3小时或更频繁(如有必要)向受感染的眼睛施用少量软膏。"同样,需要高频率重复施用。(处方信息,PrPENTAMYCETIN®,PrPENTAMYCETIN/HC®,Sandoz Canada Inc,修订日期:2018年6月14日)。

[0026] 天然存在的和合成的染料已被用作抗菌剂或抗原生动物剂(Zheng等人,BMC Microbiol 20(2020))。例如,亚甲基蓝和氯苯吩嗪仍被认为是重要的孤儿药(Ginimuge等人,J.Anaesthesiol.Clin.Pharmacol.26:517-520(2010);和Ammerman等人,J.Antimicrob.Chemother.72:455-461(2017))。

[0027] 玫瑰红(RB)是一种明亮的玫瑰红色的氧杂蒽衍生物化合物,其于19世纪首次合成用作羊毛染料,随后在日本用作食品染料(食品红105号)(Mizutani等人,J.Environ.Public Health.2009,953952)。更具体地,RB是氧杂蒽化合物荧光素的衍生物。与荧光素相比,RB具有两种类型的额外的卤素:四个氯化物取代基和四个碘化物取代基。

[0028] 1914年首次描述了使用RB进行人类眼表损伤的视觉诊断(通过眼部滴注)(Feenstra等人,Ophthalmology 99:606-617(1992))。RB后来被引入作为一种静脉内施用的相对快速的诊断辅助手段,用于评估单次100mg剂量后的人类肝脏的功能能力(Wachter等人,Lasers Surg Med.32:101-110(2003))。1971年,^{131}I RB(Robengatope®,玫瑰红钠^{131}I注射液USP)被美国食品和药物管理局(FDA)批准用于确定肝功能的诊断辅助手段

(Baroyan等人,Eksperimental'naya Meditsina(Riga)20:74-78(1985);和Mincev等人,Folia Medica(Plovdiv)16:35-41(1974))。

[0029] 2009年,由于出现了更新的肝脏成像方法,诸如计算机断层扫描,Robengatope的制造商Bracco Diagnostics Inc.正式从美国市场撤回了RB诊断产品。1974年,Barnes-Hind Pharmaceuticals Inc.(Barnes-Hind)推出了在水溶液中1%RB的医疗器械产品,用于披露角膜损伤、诊断角膜炎、角膜结膜炎和干燥综合征以及检测眼睛中的异物(Gilger等人,Vet.Ophthalmol.16:192-197(2013))。1981年,Barnes-Hind推出了用于相同适应症的相同浓度的眼用试纸条。虽然这两种诊断产品都获得了美国食品和药物管理局(FDA)的上市许可,但因为它们的上市时间早于FDA的正式审查和批准,因此溶液和试纸条器械及其各自的声明均未获得批准。

[0030] 商业级RB(市售染料含量可在80%至95%RB之间变化,包括总污染物和物质相关杂质)是使用Gnehm在19世纪80年代开发的历史工艺制造的。据认为,用于诊断应用的RB是一种含有一些杂质的商业级RB(Paczkowski等人,Free Radic.Biol.Med.1:341-351(1985))。美国药典(USP)先前将RB列为分析标准品。RB于2019年从USP中移除。因此,商业级RB在现代诊断和治疗环境中缺乏关联性。因此,批准将RB应用于治疗人类疾病存在重大的监管挑战。

[0031] Singer等人的美国专利号8,530,675、9,273,022和9,422,260描述并要求合成高度纯化的玫瑰红,以及类似地纯化的类似化合物,所述化合物含有不同的卤素取代基和不同数量的卤素取代基,以及它们的内酯形式。这些化合物在本文中统称为"卤代氧杂蒽"。

[0032] 玫瑰红(RB)染料(4,5,6,7-四氯-2',4',5',7'-四碘荧光素)已被临床研究用于治疗黑色素瘤和其它实体癌(Maker等人,J.Clin.Cell.Immunol.6:343-349(2015;Liu等人,Oncotarget.7:37893-37905(2016);Patel等人,J.Clin.Oncol.38:3143(2020);Kim等人,J.Control.Release.156:315-322(2011);和Qin等人,Cell Death Dis.8:e2584(2017))。RB的光动力抗菌特性已有零星报道(Pérez-Laguna等人,Photodiagnosis Photodyn.Ther.21:211-216(2018);Uekubo等人,Laser Ther.25:299-308(2016);Anju等人,Photodiagnosis Photodyn.Ther.24:300-310(2018);Gavara等人,Front.Med.8:494(2021);Joanna等人,Front.Microbiol.9:1949(2018);Hirose等人,Arch.Oral Biol.122:105024(2021);Dai等人,Photodiag.Photodyn.Ther.6:170-188(2009);Ghorbani等人,Laser Ther.27:293-302(2018);Kim等人,J.Food Sci.73:C540-545(2008);Manoi等人,J.Photochem.Photobiol.B.162:258-265(2016);Nakonieczna等人,Front.Microbiol.9:1949(2018);Sabbhi等人,Appl.Water Sci.8:56(2018);Santos等人,Antibiotics 8:211(2019);和Worzella等人,In Cancer Cell Culture;Humana Press:Totowa,NJ,USA,285-291.(2011))。

[0033] 例如,Dees等人的美国专利号8,974,363教导了将10μM-100μM(即10μg/mL至100μg/mL)的RB局部制剂与500nm-600nm波段的绿光照射结合使用,以对抗革兰氏阳性和革兰氏阴性抗生素耐药性细菌,且对光源、其强度或照射持续时间没有具体要求。

[0034] Naranjo等人,Am J Ophthalmol 208:387-396(2019)报道了对18例对标准疗法无反应的进行性感染性角膜炎患者的眼睛使用玫瑰红光动力抗微生物疗法(RB-PDAT)。将含有0.1%或0.2%玫瑰红(RB)的组合物应用于去上皮化的角膜30分钟,然后用来自LED源的

5.4J/cm^{2}的绿光照射15分钟。棘阿米巴原虫(一种阿米巴原虫)是最常见的微生物(10/17;59%),其次是镰孢菌属(一种真菌;4/17;24%)、铜绿假单胞菌(一种革兰氏阴性细菌;2/17;12%)和弯孢菌属(Curvularia spp.)(一种真菌;1/17;6%),以及一名具有没有确诊的微生物学诊断的患者。据报道,在72%的病例中成功实现了RB-PDAT(避免治疗性角膜移植术),RB-PDAT后平均临床消退(通过再上皮化和浸润物消退减轻疼痛和炎症)时间为46.9±26.4天。

[0035] Amescua等人,Cornea 36(9):1141-1144(2017)教导了在375nm或518nm照射15分钟后,约0.1%的玫瑰红(RB)被光激活,可以在体外抑制多重耐药性角膜镰刀菌(Fusarium keratoplasticum)的真菌生长。在多次连续的抗真菌药物治疗后,使用上述浓度的RB滴在人类患者的镰刀菌感染的清创的角膜上皮30分钟,然后使用518nm的光进行照射以提供0.9J/cm^{2}的总能量来进行研究。照射后36小时注意到疼痛消退,并且角膜浸润物在接下来的两周内缩小。治疗后四天,浸润物在所有方向上都小了大约1mm。

[0036] 在照射治疗后第13天,浸润物几乎消退,并进行了第二次玫瑰红RB照射治疗(1.8J/cm^2)。在局部皮质类固醇治疗后,到第一次RB治疗后的第246天,患者表现出治疗的眼的健康且稳定的眼表以及清晰的角膜。Martinez等人,Cornea 37(10):e46-e48(2018)报道了对治疗的患者的眼睛的随访研究。

[0037] Halili等人,Am J Ophthalmol 166:194-202(2016)报道了对在琼脂上的从患者分离的耐甲氧西林金黄色葡萄球菌(MRSA)进行的体外研究:使用在黑暗中以0.1%和0.03%溶解在高纯度水中的RB,在环境室内光下照射30分钟,和在LED绿光下照射34分钟,以提供5.4J/cm^2。然后将样品在培养箱中在避光的盒子中生长72小时。以下均显示出对两种MRSA菌株的完全生长抑制:(1)在环境和绿色LED照射下的两种玫瑰红浓度,和(2)在黑暗中的0.1%的玫瑰红浓度。在黑暗条件下,0.03%的玫瑰红显示出对菌株2的完全抑制,但对菌株1的抑制不完全。

[0038] Arboleda等人,Am J Ophthalmol 158(1):64-70(2014)特别报道了在照射(518nm;5.4J/cm^2)和黑暗条件下使用RB然后孵育三天对真菌性角膜炎患者分离株的体外生长抑制。研究了腐皮镰孢菌(Fusarium solani)、烟曲霉(Aspergillus fumigatus)和白色念珠菌(Candida albicans)的分离株。下表汇总了该研究的数据。

[0039]

	第 3 天的生长抑制百分比			
生物体	RB + 518nm 照射	仅 RB	仅 518nm 照射	仅真菌
腐皮镰孢菌	78.2±2.1	7.3±1.1	6.8±9.3	9.8±4.7
烟曲霉	79.8±9	6.6±0.	0.6±0.2	0
白色念珠菌	95.6±3	42.2±3.7	35.6±6.7	24.2±2.6

[0040] 如下文所公开的,本发明根据需要提供温和且更容易使用的治疗。此外,该治疗设想在基本没有光的情况下(例如当治疗的受试者闭着眼睛睡觉时)使用,当光与物质(光化光)相互作用时,所述光会产生可识别或可测量的改变。

发明内容

[0041] 在一个方面,本发明设想了一种光化光不透过的容器的眼科系统,所述容器含有

卤代氧杂蒽(荧光素)诸如玫瑰红或其药学上可接受的盐或酯的局部眼科组合物,所述卤代氧杂蒽(荧光素)或其药学上可接受的盐或酯溶解或分散在水性眼科运载体中,并且以约0.2μg/mL至约50μg/mL(或约0.00002wt.%至约0.005wt.%)的治疗细菌性角膜炎有效浓度存在于所述组合物中。在优选的实践中,所述组合物具有约6.5至约7.6的pH值且优选地约7.0至约7.4的pH值(正常泪液的pH值)。设想的组合物优选地进一步包括增稠剂和电解质。更优选地,还存在透明质酸。

[0042] 在本发明的另一个方面中,用上述局部眼科组合物治疗有需要的哺乳动物受试者,通过在基本上不存在光化光的情况下将治疗角膜炎有效量的所述组合物放置在受试者的微生物感染的眼睛的角膜表面上(诸如每只眼睛一滴或两滴),然后使这样治疗的受试者在不存在光化光的情况下保持约3小时至约12小时的时间段。治疗通常在哺乳动物受试者入睡之前实施。这种治疗方案通常重复多次,直到微生物感染被克服。

[0043] 如本文所用,短语"光化光"表示可造成局部眼科组合物的成分中的一种或多种的光化学反应的光。根据该定义,所述光化光的波长被组合物的受体分子吸收,并且存在足够的所吸收的波长的光子通量以引起在吸收受体卤代荧光素分子中或由吸收受体卤代荧光素分子诱导的可检测化学反应。举例说明性化学反应包括分解和光敏化。

[0044] 术语"基本上不存在光化光"在本文中用于表示在施用于受感染的眼睛后,组合物经受环境光约两分钟或更短的时间段。因此,在施用后,闭合眼睑,在经治疗的眼睛上放置遮光眼罩,关闭环境光等。类似地,吸收的波长的光子通量不足以引起在吸收受体卤代荧光素分子中或由吸收受体卤代荧光素分子诱导的可检测化学反应。

[0045] 感染的微生物源可以是细菌、病毒、真菌或阿米巴。优选地,感染源是细菌性的,并且感染性细菌优选地是革兰氏阳性细菌。

具体实施方式

[0046] 在一个方面,本发明设想了一种光化光不透过的容器的眼科系统,所述容器含有卤代氧杂蒽(荧光素)诸如玫瑰红或其药学上可接受的盐或酯的局部眼科组合物,所述卤代氧杂蒽(荧光素)或其药学上可接受的盐或酯溶解或分散在水性眼科运载体中,并且以约0.2μg/mL至约50μg/mL(或约0.00002wt.%至约0.005wt.%)的治疗细菌性角膜炎有效浓度存在于所述局部眼科组合物中。优选地,卤代荧光素(氧杂蒽)的浓度为约0.5μg/mL至约20μg/mL。在优选的实践中,所述组合物具有约6.5至约7.6的pH值,更优选地约7.0至约7.4的pH值(正常泪液的pH值)。设想的组合物优选地进一步包括增稠剂和电解质。

[0047] 值得注意的是,与之前讨论的Naranjo等人、Amescua等人、Martinez等人、Halili等人和Arboleda等人的论文中使用的玫瑰红的浓度相比,设想的组合物含有该浓度约十分之一至约千分之一的卤代氧杂蒽。

[0048] 更优选地,还存在透明质酸,所述透明质酸是由D-葡萄糖醛酸和N-乙酰基-D-葡萄糖胺组成的二糖聚合物,所述二糖聚合物通过交替的β-(1→4)和β-(1→3)糖苷键连接。透明质酸为组合物提供一些增稠效果、缓冲和张力,并有助于维持施用的组合物的水与眼睛表面接触。

[0049] 在本发明的另一方面,用上述局部眼科组合物治疗有需要的哺乳动物受试者:通过在不存在额外提供的光化光的情况下将治疗角膜炎有效量的所述组合物放置在受试者

的微生物感染的眼睛的表面上(诸如每只眼睛一滴或两滴),然后使这样治疗的受试者在不存在光化光的情况下保持约3小时至约12小时的时间段。所述治疗通常在哺乳动物受试者入睡之前实施。当治疗的受试者在施用后处在存在光化光的情况或预期处在存在光化光的情况时,也可以在施用后使用光化光不透过的眼罩覆盖经治疗的眼。该治疗方案通常重复多次,直到微生物感染被克服。

[0050] 局部眼科药物组合物

[0051] 水性眼科运载体

[0052] 设想的组合物主要是(按重量计)水性眼科运载体,其它成分溶解或分散在水性眼科运载体中。所述组合物的主成分是无菌水,诸如USP注射用无菌水。

[0053] 设想的组合物具有至少一种助强剂(builder)或增稠剂,所述助强剂或增稠剂以提供以下粘度的含量存在:约10cps至约300cps,优选地约30cps至约120cps,更优选地约50cps至约80cps。尽管诸如甘油和丙二醇等溶剂也可以为组合物提供一些增稠作用,但典型的增稠剂是聚合物。

[0054] 甘油和丙二醇单独或一起可占组合物的0重量%至约2.5重量%。更优选地,一种或两者一起以约0.4重量%至约2重量%存在。甘油和丙二醇均可与水混溶。

[0055] 提供了含有一种或多种溶解的溶质的设想的局部眼科组合物,所述溶解的溶质足以为药物组合物提供约270mOsm/kg至约340mOsm/kg的渗透压(Osm),并且优选地渗透压为约300mOsm/kg至325mOsm/kg,其大致为人泪液展现的渗透压的范围。

[0056] 通过水溶性的钠、钾、钙和镁的氯化物、磷酸盐和硝酸盐的存在,可以获得至少部分渗透压。钠,诸如氯化钠的形式,由于其固有的生理相容性,是作为电解质的优选的实施方案。优选这种电解质以约0.1%至约2%的浓度、并且更优选地以约0.5%至约1.5%的浓度、更优选地以约0.8%至约1.2%的浓度、并且最优选地以约0.9%的浓度存在于组合物中。水性0.9N氯化钠的渗透压为308mOsmol/升。

[0057] 当需要治疗的受试者出现角膜肿胀时,可以使用含有约2%至约5%氯化钠的高渗组合物来减轻角膜肿胀同时治疗感染。含有2%和5%氯化钠的眼睛治疗组合物的渗透压分别计算为684mOsm和1711mOsm(Yorek等人,Invest Ophthalmol Vis Sci.57:2412-2419(2016))。这些举例说明性产品由Bausch&Lomb(Bridgewater,New Jersey)以Muro128®商标销售。

[0058] 低分子量聚(乙二醇)(PEG)诸如PEG 400或PEG 1000可以为组合物提供润滑和对干眼症状的一些缓解。由于这些聚合物缺乏离子电荷,这些聚合物还增加相对少量的渗透压。取决于设想的组合物中存在的其它溶质,低分子量PEG可能不存在或以约1mg/mL至约10mg/mL存在。

[0059] 右旋糖酐是一种具有糖苷键的复杂的含支链的非离子带电的微生物来源的聚α-d-葡萄糖苷,所述糖苷键主要为C-1→C-6,具有α-1,3键形成的支链。右旋糖酐链的长度不同(从约3千道尔顿至约2000千道尔顿)。鉴于其分子量为约40000kDa和70000kDa,因此两种常用的右旋糖酐被分别称为右旋糖酐40和右旋糖酐70。

[0060] 糊精是由α-1,4或α-1,6键连接的水溶性的直链聚葡萄糖。糊精是由淀粉和糖原的水解产生的混合物,且不含离子电荷。其特征性分支将右旋糖酐与糊精区分开来,糊精是由α-1,4或α-1,6键连接的直链葡萄糖聚合物。

[0061] 类似于PEG,存在于设想的组合物中的右旋糖酐和糊精不带电荷,不会极大地改变组合物的渗透压。右旋糖酐可以为眼睛提供一些润滑,并且可以不存在,或者以约0.5mg/mL至约2mg/mL存在。

[0062] 通常作为聚维酮在商业上销售的聚(乙烯基吡咯烷酮)是另一种非离子电荷聚合物,类似于右旋糖酐,其已被用作血液增量剂用于静脉内应用,用在眼科药物中,并且存在时不会明显改变设想的组合物的渗透压。当存在时,聚维酮以约1mg/mL至约2mg/mL存在,并且更优选地以约7mg/mL至约15mg/mL存在。

[0063] 另一种有用的非离子聚合物是羟丙基甲基纤维素(HPMC),也作为羟丙甲纤维素(Hypromellose)在商业上销售。美国专利号5,679,713教导在药物组合物中可以使用与治疗干眼症的活性剂胆碱能化合物一起使用的羟丙甲纤维素、甲基纤维素、聚乙烯醇或透明质酸。上述专利的无结果实例使用了0.5重量%至1重量%的甲基纤维素和1.4重量%的聚乙烯醇。

[0064] 另一种水溶性的非离子聚合物增稠剂是羟乙基纤维素(HEC)。这种材料有几种分子量可用,每种分子量提供不同量的增稠作用/存在的wt%。以Natrosol™250的名称出售的HEC的一个来源是Covington,KY的Ashland Specialty Chemicals。

[0065] 以商品名**Refresh® Tears®**和**Refresh®** Relieva™出售的市售干眼症药物均含有0.5%的阴离子羧甲基纤维素钠作为活性成分,而**Refresh®**Relieva™产品也含有0.9%的甘油作为活性成分。活性成分与溶解在纯化水中的许多非活性成分一起存在。这些产品由Allergan,Inc.(AbVie公司,North Chicago,IL)销售。

[0066] 透明质酸(HA)是一种阴离子、非硫酸化糖胺聚糖,广泛分布于结缔组织、上皮组织和神经组织中。所述透明质酸是由D-葡萄糖醛酸和N-乙酰基-D-葡萄糖胺组成的重复单元的聚合物,通过交替的β-(1→4)和β-(1→3)糖苷键连接。它在糖胺聚糖中是独特的,因为它是非硫酸化的,并且分子量可以达到数百万道尔顿。因为它是阴离子的,并且每个分子通常含有几个羧基,所以HA可以对设想的组合物的渗透压具有相对大的影响。

[0067] 另一种阴离子增稠聚合物是部分中和的水分散性羧甲基纤维素。这种材料可以以不同分子量从许多供应商那里获得,以提供水中的每给定重量百分比的不同粘度。

[0068] 短语"部分中和"与含羧基的聚合物结合使用,所述含羧基的聚合物的羧基官能团与碱反应形成带阴离子电荷的羧酸基,并有助于为聚合物提供水溶性或分散性。

[0069] 还可以使用其它阴离子增稠剂,诸如部分中和的交联丙烯酸、甲基丙烯酸和共聚物,诸如美国专利号3,074,852、3,330,729和4,226,848的一项或多项的水溶性的交联丙烯酸酯聚合物,例如Lubrizol Corp.(Wickliffe,Ohio)以**CARBOLOL®**934P NF或974P NF商标出售的材料。这些材料是与聚烯基聚醚(诸如烯丙基蔗糖或烯丙基季戊四醇)交联的丙烯酸均聚物,每个交联剂平均每个分子含有至少三个烯丙基。据报道,这两种聚合物都是水溶性的,并且当以0.5重量%存在时,在pH值为7.5时提供粘度。934P NF在苯中聚合,而974P NF在作为溶剂的乙酸乙酯中聚合。

[0070] 另一类有用的增稠剂是一种水溶胀性但不溶于水的交联丙烯酸聚合物,所述交联丙烯酸聚合物在美国专利号3,202,577和4,615,697中有描述,并且在文献中常被称为聚卡波非和作为生物粘附剂。这些聚合物可以定义为至少80重量%的单烯不饱和羧基官能单体和约0.05重量%至约1.5重量%的交联剂(即3,4-二羟基-1,5-己二烯或2,5-二甲基-1,5-

己二烯)共聚的反应产物。该聚合物的羧基在组合物的pH值下也是至少部分中和的。

[0071] 这些生物粘附聚合物不含前面讨论的聚烯基聚醚交联物。下面讨论可以存在以构成100重量%的单体的剩余单体。

[0072] 除了上述两种成分外,生物粘附聚合物也可以包括聚合的单烯不饱和重复单元,诸如上述一种或多种的酸的C_1-C_6烷基酯(诸如丙烯酸己酯、甲基丙烯酸丁酯和巴豆酸甲酯);上述酸的羟基亚烷基官能酯平均每个分子含有1至约4个含2-3个碳原子的氧化烯基,诸如甲基丙烯酸羟乙酯、丙烯酸羟丙酯和四乙二醇单丙烯酸酯;甲基丙烯酰胺、丙烯酰胺及其C_1-C_6单烷基和二烷基衍生物,诸如N-甲基丙烯酰胺、N-丁基甲基丙烯酰胺和N,N-二甲基丙烯酰胺;苯乙烯;以及本领域已知的可与上述含羧基官能团的单体和交联剂共聚的类似物。生物粘附聚合物最优选地仅由单烯不饱和羧基官能单体和交联剂制备。

[0073] 本文中使用的生物粘附剂可以通过使用引发剂诸如过氧化苯甲酰、偶氮二异丁腈等的常规自由基聚合技术制备,并且也可以在水性介质中聚合,并且不通过蒸汽作用聚集。最近的上文段落中提到的两项专利提供了有用的生物粘附剂的示例性制备,该专利的公开内容通过引用并入本文。

[0074] 如前所述,所述生物粘附剂可以在水性介质中聚合。在优选的实践中,水性介质是碱土金属盐(诸如硫酸镁)的饱和溶液。

[0075] 碱土金属盐具有至少两种功能。第一,它增加了聚合介质的密度,使得聚合的生物粘附剂漂浮在水性介质的表面上,并且可以容易地从中除去。第二,特别是硫酸镁的使用减少了生物粘附剂在水性介质中的膨胀,从而促使了聚合和回收。经过几次水冲洗聚合物后,生物粘附剂通常含有约0.5%至约1%的碱土金属离子。

[0076] 美国专利号5,221,722教导了被称作增强型聚卡波非的制备。该聚合物在适合的引发剂和二乙烯乙二醇交联剂(诸如3,4-二羟基-1,5-己二烯)的存在下,在选自丙酮和烷基乙酸酯(烷基中含有1至6个碳原子)的溶剂中制备。据说以这种方式制备的聚卡波非具有生物粘附的特性,并且当在水中测量其1重量%的粘液时,其粒径小而不需研磨,并且粘度超过20000cPs。

[0077] 为了防止聚合物在聚合中胶凝并推进离散颗粒的形成,至少一部分羧基被教导用氢氧化物、氧化物或碳酸盐等形式的IA族金属化合物中和。这些的例子包括钠、钾等,以及与氨和某些胺类(包括吗啉、单乙醇胺、二乙醇胺和三乙醇胺;单丙醇胺;以及部分聚合物的盐不易溶于反应介质的其它胺)的反应。优选地,单体上大于0.1重量%的羧基被中和或形成上文所列的材料的盐。更优选地,在聚合前中和大于1重量%和多至约10重量%(尤其是小于约5%)的羧基或将其转化为等效盐。

[0078] 据报道,上述合成提供了平均粒径小于10μm的聚合物,使得颗粒将通过635号网(ASTM-E11)。上述一家商业供应商是Lubrizol Corporation,该公司以Noveon®AA-1(聚卡波非,USP)的名称销售该聚合物以用于医药用途。

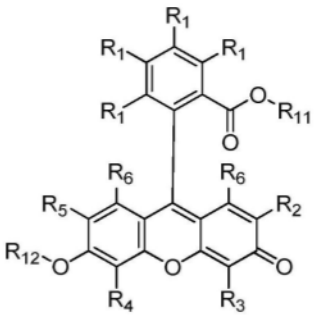
[0079] 任何上述聚合物的具体的量与聚合物作为增稠剂和/或生物粘附剂的作用无关。因此,所用聚合物的量通常彼此不同,因为它们具有不同的增稠和生物粘附能力。通常来说,使用增稠有效量和/或生物粘附有效量。

[0080] 卤代氧杂蒽(荧光素)

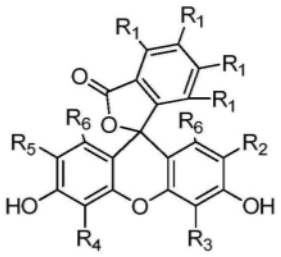
[0081] 设想的局部眼科组合物的卤代氧杂蒽(荧光素)化合物可以是下式1的化合物,其

中R_1独立地是F、Cl、Br、I、H或C_1-C_4烷基；R_2、R_3、R_4和R_5独立地是Cl、H或I，其中至少一个选自R_2、R_3、R_4、R_5的取代基是I；并且R_6独立地是H或C_1-C_4烷基；R_{11}是H或C_1-C_4烷基；R_{12}是H或C_1-C_7酰基；和所有(a)互变异构形式；(b)阻转异构体，(c)如式2(下文)所示的封闭内酯形式，(d)如式2所示的内酯形式的对映异构体，和(e)其药学上可接受的盐。

[0082]



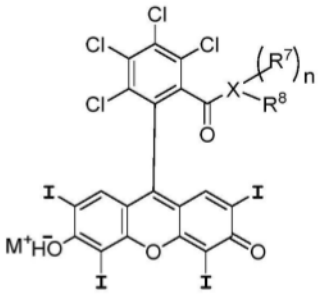
式 1



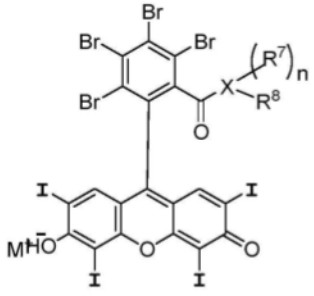
式 2

[0083] 由于荧光素是氧杂蒽的衍生物，因此人们更相信设想的卤代氧杂蒽被更准确地称为卤代荧光素。在优选的实践中，上述式1和2的卤代荧光素的每个R_1是氯取代基或溴取代基，而这些式的R_2、R_3、R_4和R_5中的每个是碘取代基，如以下式I和II所示。在下式中，X是氧或氮，“n”是0或1，使得当X是氧时，n是0并且不存在R^7，而当X是氮时，n是1并且存在R^7。当X是氧时，R^7选自由以下组成的组：氢(H)、M^+(其是药学上可接受的阳离子)，C_1-C_4烷基和本文下文定义的含芳环的取代基。当X是氮时，R^7和R^8相同或不同，并且选自由以下组成的组：氢、C_1-C_4烷基、含芳环的取代基，或R^7和R^8与酰胺氮原子一起形成5元环或6元环，以及含芳环的取代基。含芳环的取代基是含有5或6个成员的单环、或5,6或6,6稠合的芳环系统，所述单芳环或稠合芳环系统含有0、1或2个杂环原子，所述杂环原子独立地是氮、氧或硫。

[0084]



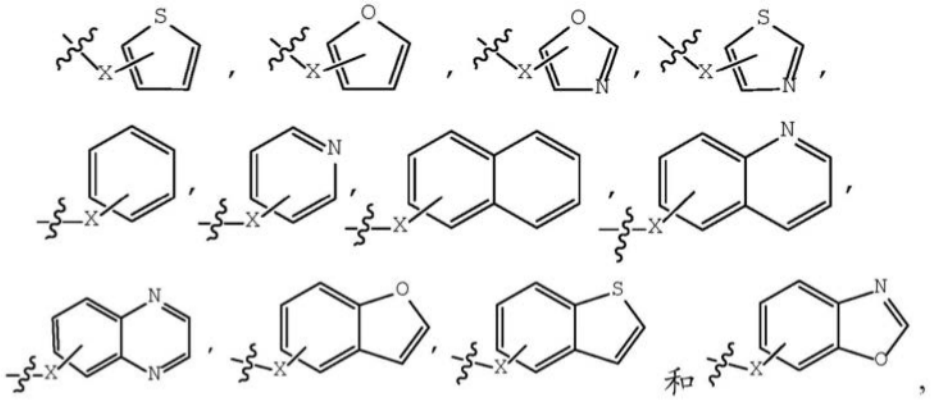
式 I



式 II

[0085] 为了便于描述，芳香酯或芳酰胺统称为芳香族衍生物。因此，这些衍生物由具有单个5元或6元芳环或5,6-或6,6-稠合的芳环系统的醇或胺(优选单取代的)形成。

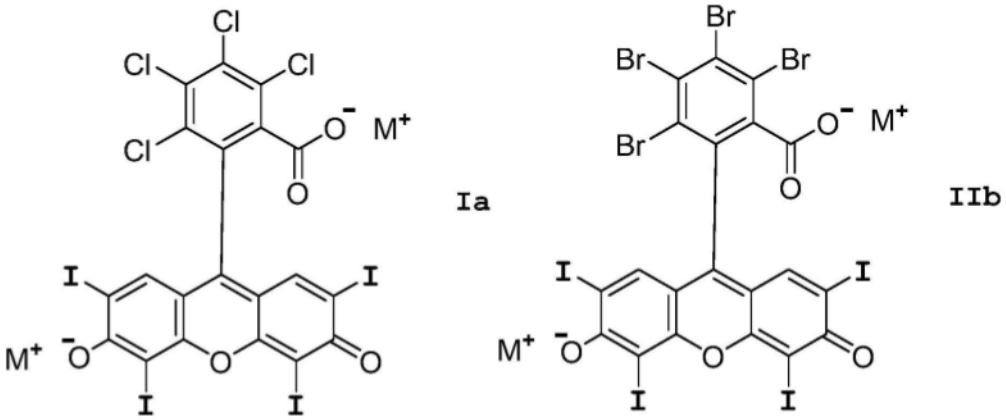
[0086] 示例性芳环取代基的结构式如下所示：

[0087] 

[0088] 其中  分别提供酯或单取代酰胺。

[0089] 玫瑰红(RB)是本文使用的优选的化合物,并且其二钠盐(即,玫瑰红二钠(RBD))是最优选的RB化合物。这些化合物在本文中举例说明性地用于RB化合物的组。

[0090] 玫瑰红的化学名称是4,5,6,7-四氯-2',4',5',7'-四碘荧光素。一种优选形式的玫瑰红二钠(RBD)具有以下结构式Ia:

[0091] 

Ia IIb

[0092] 美国专利号8,530,675、9,273,022和9,422,260讨论了高度纯化的卤代荧光素分子(诸如玫瑰红(上面的式Ia)和四溴-四碘类似物(上面的式IIb))的制备。美国专利号5,998,597、6,331,286、6,493,570、7,390,668和8,974,363公开了RBD在光化学诱导的治疗中的应用。优选地,上面的结构式中的阳离子M^+是钠(Na^+)或钾(K^+)。

[0093] 各种语法形式的术语"生理上可接受的盐"和"药学上可接受的盐"是指制药工业中常用的任何无毒的阳离子,诸如碱金属、碱土金属和铵盐,包括钠、钾、锂、钙、镁、钡、铵和鱼精蛋白锌盐,它们可以通过本领域已知的方法制备。这些盐可以通过使用碱金属、碱土金属或氢氧化铵中和设想的卤代荧光素的酸形式或通过与氢氧化物形式的离子交换树脂混合而有用地制备。如此形成的阳离子卤代荧光素盐优选地可溶于最终配制的局部眼科组合物。

[0094] 设想的阳离子提供了可溶于组合物的氧杂蒽(荧光素或fluorescienate)盐。优选地,盐是单取代或二取代盐形式的钠、钾、钙和铵盐。读者可参考Berge,J.Pharm.Sci.1977 68(1):1-19列出的常用的生理上(或药学上)可接受的酸和碱,所述酸和碱与药物化合物形成生理上可接受的盐。

[0095] 卤代荧光素以治疗角膜炎有效浓度存在于组合物中。在通常的实践中,治疗角膜炎有效浓度在局部眼科组合物中为约50μg/mL至约3000μg/mL,优选地约100μg/mL至约2000μg/mL,更优选地约500μg/mL至约1500μg/mL,以DRB测量。

[0096] 因此,如果使用除DRB以外的卤代荧光素,则使用量基于DRB的分子量(为1017.6g/摩尔)计算。例如,如果四个氯取代基被四个溴取代基取代,则分子量将为1195.2克/摩尔。分子量差异为约17.45%。因此,与给定的DRB量相比,待使用的四溴四碘荧光素的当量将增加约17.45%。

[0097] 设想的卤代荧光素化合物诸如玫瑰红是二取代盐,具有2.52和1.81的pKa值。几种设想的卤代荧光素的pKa值确定可以在Batsitela等人,Spectrochim Acta Part A 79(5):889-897(2011年9月)中找到。

[0098] 卤代氧杂蒽(荧光素)药物组合物的pH值可以通过本领域技术人员已知的任何合适手段进行调控或调节。可以通过添加酸或碱等来缓冲组合物或调节pH值。由于卤代氧杂蒽(荧光素)或其生理上可接受的盐是弱酸(取决于卤代黄嘌呤(荧光素)浓度和/或电解质浓度),组合物的pH值可能不需要使用缓冲液和/或pH值调节剂。然而,特别优选的是,该组合物不被缓冲,以允许组合物一旦施用就顺应生物环境,例如泪液。

[0099] Abelson等人,Arch Ophthalmol 99(2):301(1981)报道了测量44名正常受试者的下盲囊的泪液的pH值,并且发现pH值为约6.5至约7.7,平均值为约7.0。因此,设想的组合物优选地具有约6.5至约7.6且优选约7.0至约7.4的pH值。

[0100] 还优选的是药物组合物不包括任何防腐剂,许多防腐剂可以有害地干扰药物组合物或其制剂,或者可以与卤代氧杂蒽组合物活性成分复合或以其它方式相互作用或干扰卤代氧杂蒽组合物活性成分的递送。就使用防腐剂的程度而言,咪唑烷基脲是优选的防腐剂,因为它在药物组合物中或施用时不与卤代氧杂蒽相互作用。

[0101] 一种设想的治疗方法用于治疗有需要的哺乳动物受试者。“有需要的哺乳动物”是出现眼睛感染(诸如细菌、病毒、真菌或阿米巴感染)的哺乳动物。这种感染通常是细菌感染,并且感染细菌通常是革兰氏阳性细菌。

[0102] 举例说明性的经治疗的革兰氏阳性细菌包括药物敏感和耐药性的金黄色葡萄球菌、表皮葡萄球菌(S.epidermis)、粪肠球菌(E.faecalis)和屎肠球菌(E.faecium)中的一种或多种,以及枯草芽孢杆菌(Bacillus subtilis)、蜡样芽孢杆菌(Bacillus cereus)和唾液链球菌(Streptococcus salivarius)中的一种或多种。当治疗(接触细胞)受感染的眼睛时,革兰氏阳性细菌存在于眼睛的哺乳动物细胞内或细胞上。

[0103] 一些革兰氏阳性细菌菌株在本文中被称为“抗性”或“耐药性”或抗性的其它语法变体。这里的“抗性”是指细菌对一种或多种抗菌药物产品的治疗的抗性/耐药性,所述抗菌药物产品通常被认为在已知浓度和细菌细胞密度下对该类型细菌具有杀菌作用。因此,既有“药物敏感”菌株,也有“耐药性”菌株,诸如金黄色葡萄球菌(S.aureus)、表皮葡萄球菌(S.epidermis)、粪肠球菌(E.faecalis)和屎肠球菌(E.faecium)。几种常见细菌菌株已经产生耐药性的举例说明性药物包括万古霉素、甲氧西林和庆大霉素。

[0104] 还设想了类似的方法来治疗革兰氏阴性细菌,所述革兰氏阴性细菌优选地是伯克霍尔德氏菌(Burkholderia)、沙门氏菌(Salmonella)和变形杆菌(Proteus)中的一种或多种。在这里,上述RB化合物以约10μg/mL至约100μg/mL的浓度,并且优选以约20μg/mL至约50

μg/mL的浓度溶解或分散存在于水性药物组合物中。

[0105] 如本文所用,词语“施用”用于意指治疗方案的开始,其中将组合物应用于感染的眼的外表面。因此,施用通常需要将一定剂量的本文所述的眼科组合物放置在眼睛表面上,或通过拉下下眼睑放入形成的口袋或囊中,然后闭合眼睑以使眼科组合物分布在治疗的眼球的外表面上。

[0106] 治疗方法

[0107] 设想的治疗方法包括使有需要的受试者的角膜表面与含有抗革兰氏阳性细菌量的卤代荧光素化合物的组合物接触。使用玫瑰红作为此类化合物的示例,将抗革兰氏阳性杀菌有效量的RB施用于有需要的哺乳动物受试者,并且可以使用增稠的水性液体、凝胶或如前所述的其它形式进行配制。

[0108] 此后,使治疗的眼睛在基本上不存在光化光的情况下保持约3小时至约12小时的时间段。因此,例如,在施用后,闭合眼睑、在经治疗的眼睛上放置遮光眼罩、关闭环境光等。也许最容易的是,在受试者晚上入睡前对眼睛进行治疗。

[0109] 在一个实施方案中,革兰氏阳性细菌细胞存在于受试哺乳动物上或体内(例如感染)。举例说明性地,受试哺乳动物可以具有皮肤病学革兰氏阳性细菌感染,诸如酿脓链球菌(Streptococcus pyogenes)感染,或者特别是在局部治疗开放性伤口的情况下,作为目前感染的治疗和对随后的革兰氏阳性细菌感染的预防。

[0110] 治疗的受试哺乳动物可以是灵长类动物(诸如人)、猿(诸如黑猩猩或大猩猩)、猴(诸如食蟹猴或猕猴)、实验动物(诸如大鼠、小鼠或兔)、伴侣动物(诸如狗、猫、马)、或食用动物(诸如牛或公牛、羊、羔羊、猪、山羊、美洲驼)等。

[0111] 通常重复每次设想的组合物的施用,直到治疗的细菌疾病(感染)减少到所需程度,诸如不可检测。因此,根据医师的指导,对有需要的受试哺乳动物的施用可以在一天内、每天、每周、每月或几个月至几年的时间内进行多次。

[0112] 按照参与治疗的医务人员的指导,可以在施用设想的组合物之前对感染(例如角膜溃疡)周围区域的角膜上皮进行清创,以获得去上皮的区域从而增加玫瑰红的吸收。

[0113] 结果

[0114] 医药级玫瑰红(RB)

[0115] Provectus Biopharmaceuticals,Inc.(Knoxville,TN)(Provectus)使用商业级RB,并寻求一种制造工艺来制备纯形式的RB作为药物。然而,由于在染料制造过程中发生的商业级反应以及其它失去一个或多个碘化物取代基的副产物,在RB的纯化中发现了一些挑战。

[0116] 得出的结论是,商业级RB不能有效产出足够数量的医药级材料,不足以支持FDA和其它全球药物监管机构的临床开发和注册。Provectus(Singer等人,美国专利号8,530,675、美国专利号9,273,022和美国专利号9,422,260)建立了一种合成和制造RB的新型多步骤方法。这项工作的一个关键因素是消除引起形成关键的历史性杂质的条件。

[0117] 这些合成和纯化方法已应用于良好生产规范(GMP),生产出医药级RB。这种RB,**PV-10®**,是可从Provectus获得的无菌的在0.9%水性盐水(生理盐水)中的10%玫瑰红二钠(RBD)溶液,所述PV-**10®**是在人用药品技术要求国际协调理事会(The International Council for Harmonization of Technical Requirements for Pharmaceuticals for

Human Use,ICH)的指导下制造的,并且设计为作为可注射药物施用。医药级RBD也存在于不同配制的局部药物中(美国专利号9,422,260;Innamarato等人,BMC Cancer 21:756(2021);Thompson等人,Melanoma Res.31:232-241(2021);和Swift等人,Onco Targets Ther.12:1293-1307(2019)),被称为**PH-10®**。两种制剂均使用玫瑰红二钠(RBD)作为活性成分,其结构式如前所述。因为除了二钠盐外还可以使用许多玫瑰红的可溶性盐,所以本文使用"RB"作为任何药学上可接受的玫瑰红盐的名称,而名称"RBD"专门指玫瑰红二钠。

[0118] 微生物

[0119] 感染的微生物源可以是细菌、病毒、真菌或阿米巴。在通常的实践中,感染源是细菌,并且感染细菌优选地是革兰氏阳性细菌。

[0120] 几个研究小组已经通过光动力方法研究了RB的抗菌活性(参见Kurosu等人,Molecules 27:322(2022)及其引用)。RB的光动力疗法的应用不仅限于皮肤感染,包括蜂窝织炎、丹毒、脓疱病、毛囊炎以及疖和痈。然而,RB的活性谱、杀伤率和生物膜根除活性已有零星报道。

[0121] 如上述Kurosu等人重新研究了医药级RB在不同光源(荧光、LED和阳光)和黑暗条件下对包括革兰氏阳性、革兰氏阴性需氧细菌(包括分枝杆菌属(Mycobacterium spp.))的一系列微生物的杀菌活性。有趣的是,尽管用光化光照射表现出更大的即时功效,但RB在黑暗中也表现出抗菌活性。

[0122] 本发明使用本发明的药物组合物在黑暗中的抗菌活性。因此,需要治疗的哺乳动物受试者,诸如患有细菌性眼睛感染的人,在休息并闭上她/他/他们的眼睛之前的0至约5分钟内施用抗菌有效量的设想的药物组合物。

[0123] 使用上述Kurosu等人的论文中所述的在黑暗条件下覆盖铝箔的96孔板,通过培养液稀释法和琼脂稀释法在暗室中24小时(h)时间段获得的最小抑制浓度(MIC,以μg/mL为单位)汇总在以下表1中。表1中使用的RB是由Provectus Biopharmaceuticals,Inc.(Knoxville,TN,美国)提供的**PV-10®**的稀释的形式(Singer等人,美国专利号8,530,675、9,273,022和9,422,260)。

[0124] 表1

[0125] 通过培养液稀释法确定的一系列真菌、革兰氏阳性细菌和革兰氏阴性细菌的MIC

[0126]

条目编号	微生物	MIC (μg/mL)
1	枯草芽孢杆菌 ATCC6051	50
2	蜡样芽孢杆菌 NRRL B-569	50
3	蜡样芽孢杆菌 13061™	50
4	金黄色葡萄球菌 6538™	50.0
5	金黄色葡萄球菌金黄色亚种（Staphylococcus aureus subsp. Aureus）BAA-1683(MRSA 菌株)	50
6	金黄色葡萄球菌金黄色亚种 BAA-41™(MRSA 菌株)	25.0
7	金黄色葡萄球菌金黄色亚种 BAA-42™(MRSA 菌株)	50.0
8	金黄色葡萄球菌金黄色亚种 BAA-44™(MRSA 菌株)	50.0
9	金黄色葡萄球菌金黄色亚种 BAA-2094™(MRSA 菌株)	25.0
10	金黄色葡萄球菌 BAA-2313™(MRSA 菌株)	25.0
11	金黄色葡萄球菌金黄色亚种 33592™(耐甲氧西林和庆大霉素的菌株)	50.0
12	金黄色葡萄球菌 AIS2006032(耐万古霉素的菌株)	50.0
13	金黄色葡萄球菌 BR5(耐甲氧西林和万古霉素的菌株)	25.0
14	金黄色葡萄球菌 AIS 1000505 菌株 (VRS10，耐万古霉素的菌株)	25.0
15	金黄色葡萄球菌 USA100 菌株 71080(VRS8，耐万古霉素的菌株)	25.0

[0127]

16	表皮葡萄球菌（Staphylococcus epidermidis）35984™	25.0
17	粪肠球菌 19433™	25.0
18	屎肠球菌 349™	50.0
19	屎肠球菌 BAA-2320	25.0
20	屎肠球菌 NR-32065(耐万古霉素的菌株)	50.0
21	屎肠球菌患者#3-1, NR-31912(耐万古霉素的菌株)	50
22	屎肠球菌 UAA714(耐万古霉素的菌株)	50
23	唾液链球菌 Salivarius 亚种 7073™	25.0
24	肺炎链球菌 6301™	>100
25	耻垢分枝杆菌（Mycobacterium smagmatis）607™	25.0
26	鸟分枝杆菌鸟亚种（Mycobacterium avium subsp. Avium）2285	50.0
27	堪萨斯分枝杆菌感染（Mycobacterium kansasii）824™	50.0
28	牛分枝杆菌（Mycobacterium bovis）35734™(BCG)	50.0
29	脓肿分枝杆菌（Mycobacteroides abscessus）19977™	50.0
30	酿酒酵母（Saccharomyces cerevisiae）BY4743	125

[0128] 对表1中具有不同SCCmec类型的一组7种耐甲氧西林金黄色葡萄球菌(MRSA)(条目5-11)检验了RB的杀菌活性(Zuo等人,Sci.Rep.11:Article 5447(2021))。在荧光或LED灯下,表1中测试的所有MRSA菌株均被浓度为0.78μg/mL-3.1μg/mL的RB杀死。进一步针对4种耐万古霉素金黄色葡萄球菌菌株(条目12-16)检验RB;在任一光照条件下,所有耐万古霉素的菌株在低于1.0μg/mL的浓度下被杀死。

[0129] 表皮葡萄球菌是一种厌氧细菌,但在有氧条件下生长良好。它在有氧条件下被RB

有效杀死(条目16)。RB杀死了药物敏感和耐药性粪肠球菌(包括耐万古霉素的菌株)(条目17-21)。唾液链球菌对RB敏感(条目23),而肺炎链球菌对RB显示出耐受性(条目24)。目前尚不清楚这种耐受性的缘由。

[0130] 因此,在黑暗条件下,RB在25.0μg/mL-100μg/mL浓度下对条目1-23(表1)中列出的所有革兰氏阳性细菌显示出抗菌活性。在黑暗条件下,已知RB在高浓度下展示抗菌活性。表1中测定的所有革兰氏阴性细菌在黑暗条件下对RB不敏感。这些数据支持RB具有一种或多种未知机制(而不是通过三重态氧的激发机制,产生细胞毒性活性氧)来抑制细菌生长的想法。

[0131] 检验了RB对5种分枝杆菌属的抗菌活性(条目25-29)。有趣的是,在黑暗条件下,这些分枝杆菌被杀死的MIC与在光照条件下观察到MIC的相似。RB在黑暗条件下以(比抑制细菌)更高的浓度抑制酿酒酵母的生长(条目30)。

[0132] 通过琼脂稀释法确定的RB的MIC值不同于通过培养液稀释法确定的MIC值(表1)。通过琼脂稀释法确定的MIC值的选择的实例总结在表2中,该表2通过对测定的微生物重新编号而显示。

[0133] 表2

[0134] 通过琼脂稀释法确定的一系列革兰氏阳性细菌的MIC和分枝杆菌的MIC

[0135]

条目编号	微生物	MIC(μg/mL)
1	枯草芽孢杆菌ATCC6051	125
2	蜡样芽孢杆菌13061™	125
3	金黄色葡萄球菌6538™	25.0
4	金黄色葡萄球菌金黄色亚种BAA-44™(MRSA菌株)	50.0
5	耻垢分枝杆菌607™	50.0

[0136] 革兰氏阳性细菌诸如枯草芽孢杆菌、蜡样芽孢杆菌和金黄色葡萄球菌的生长在约25μg/mL至约125μg/mL浓度下被抑制(表2中的条目1-4),所述浓度大于通过培养液稀释法确定的MIC值。耻垢分枝杆菌(ATCC607™)在比培养液稀释法较低浓度的含药物的琼脂平板(或孔)上被杀死(条目29,表1;条目5,表2)(Lelovic等人,J.Antibiot 73:780-789(2020))。

[0137] 在黑暗条件下,通过琼脂稀释法确定的RB的MIC与培养液稀释法中测量的值展现出良好的一致性。RB对选择的细菌的最小杀菌浓度(MBC)也总结在上面的表2中。

[0138] 用于治疗和杀伤的示例性革兰氏阳性细菌包括药物敏感和耐药性金黄色葡萄球菌、表皮葡萄球菌、粪肠球菌和屎肠球菌中的一种或多种,以及枯草芽孢杆菌、蜡样芽孢杆菌和唾液链球菌中的一种或多种。

[0139] 医药级RB(HP-RBf)的抗生物膜活性

[0140] 在前节中观察到的RB的抗菌特性鼓励评估RB在革兰氏阳性细菌中的抗生物膜功效。上文总结的数据表明RB对革兰氏阳性细菌具有显著的药物亲和力或渗透性(渗透到其上或其内)。

[0141] 已经在生物膜条件下用光敏剂研究了抗微生物和抗真菌光动力疗法;然而,仅用RB检验了有限数量的细菌生物膜(Pérez-Laguna等人,Photodiagnosis Photodyn.Ther.21:211-216(2018))。

[0142] 在这里,在荧光和黑暗条件下研究了RB对药物敏感金黄色葡萄球菌6508™、耐药性金黄色葡萄球菌71080(VRS8)和屎肠球菌NR-32065的生物膜的功效。雷奈佐利不是根除革兰氏阳性细菌的生物膜的有效药物,但在预防生物膜形成方面具有有益作用(Reiter等人,J.Med.Microbiol.62:394-399(2013)),并且所述雷奈佐利被用作对照。

[0143] 在Kurosu等人,Molecules 27:322(2022)中报道的生物膜测定中,雷奈佐利作为阳性对照,其浓度非常高,为600μg/mL(浮游细胞>100xMIC)。这些作者报道了,他们证实,所有测试的菌株都在聚苯乙烯孔板上形成了强大的生物膜。在荧光灯下,RB可以根除金黄色葡萄球菌6508™的生物膜,且在30.0μg/mL(38xMIC)浓度下减少7个对数,这表明与雷奈佐利(600μg/mL)观察到的功效水平相同(Kurosu等人,出处同上,图3)。

[0144] RB在光照和黑暗条件下均显示出剂量依赖性方式的生物膜根除活性。尽管需要更高的浓度,但RB在黑暗条件下仍显示出生物膜根除活性。在50.0μg/mL(黑暗下2xMIC)浓度下,RB显著减少了活细菌的数量。在500μg/mL(20xMIC)浓度下,仅观察到约110CFU/mL至约150CFU/mL。在1000μg/mL浓度下没有出现活细菌(N.D.)。使用低得多的RB浓度,在耐药性金黄色葡萄球菌71080(VRS8)和屎肠球菌NR-32065的生物膜中观察到类似的趋势。

[0145] RB的抗菌机制

[0146] 几篇文章报道了RB的抗菌光动力疗法。RB通过渗透细菌细胞壁并且与细胞膜结合,然后产生活性氧,可能是光照条件下的杀菌机制(Nsubuga等人,Nanoscale Adv.3:1375(2021);和Lamber等人,Photochem.Photobiol.66:15-25(1997))。虽然需要相对高的浓度,但RB在黑暗条件下仍杀死大多数革兰氏阳性细菌,包括分枝杆菌属。它还有效地根除革兰氏阳性细菌的生物膜(上文)。RB在黑暗条件下的抗菌活性仍远未完全了解(Nakonechny等人,Int.J.Mol.Sci.29:3196(2019);和Kim等人,J.Enzyme.Inhibit.Med.Chem.35:1414-1421(2020))。

[0147] RB在光照条件下以12.5mg/mL-25.0mg/mL杀死分枝杆菌属,在黑暗条件下以25.0mg/mL-50.0mg/mL杀死分枝杆菌属(表1)。在两种条件下,RB杀死五种分枝杆菌的速度比杀死革兰氏阳性细菌慢。RB似乎具有对分枝杆菌细胞壁的低渗透性。由于甚至在黑暗条件下RB对革兰氏阳性细菌也具有快速杀菌作用,因此产生革兰氏阳性细菌的耐RB突变体是一项极其困难的任务。

[0148] 我们成功产生了耻垢分枝杆菌(M.smegmatis)的耐RB突变体,其MIC值为200mg/mL(Lelovic等人,J.Antibiot.73:780-789(2020))。耐RB菌株对大多数TB药物(阿米卡星、卷曲霉素、利福平、氨基尿苷基苯氧哌啶基苯甲基丁酰胺(aminouridylphenoxypiperidinylbenzyl butanamide,APPB)和乙硫异烟胺)敏感(以下的表3)。

[0149] 表3

[0150] RB和抗分枝杆菌剂对耻垢分枝杆菌及其PV-10耐药性菌株的MIC[a]

[0151]

药物	对耻垢分枝杆菌 607™（野生型）的 MIC（μg/mL）	对耻垢分枝杆菌 607™（RB 突变体）的 MIC（μg/mL）
玫瑰红	12.5	**200**
异烟肼 (INH)[b]	1.56	**25-50**
乙硫异烟胺 (ETH)	12.5	12.5
阿米卡星	0.78	0.78
卷曲霉素	3.13	3.13
利福平	1.56	1.56
APPB[c]	0.20	0.20

[0152] [a]在黑暗条件下确定MIC值。所有研究一式三份。

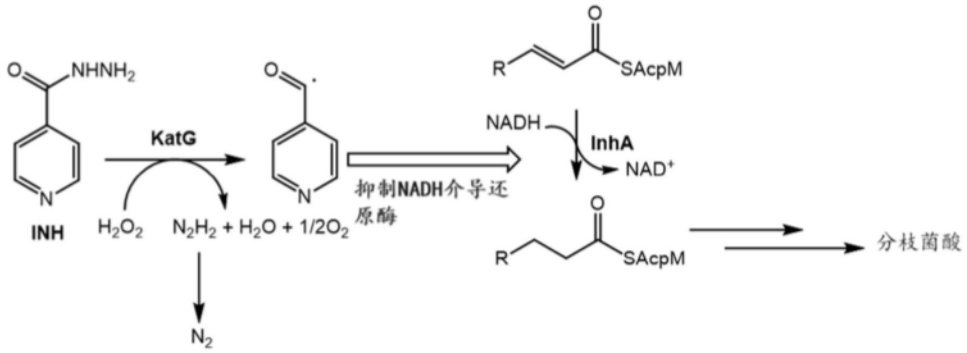
[0153] [b]分枝菌酸合成的抑制剂。

[0154] [c]APPB＝氨基尿苷基苯氧哌啶基苯甲基丁酰胺，一种MraY/WecA抑制剂。

[0155] 然而，其显示出对异烟肼(INH)的交叉耐受性。INH是一种需要通过属于过氧化氢酶-过氧化物酶家族的KatG酶进行氧化活化的前药。KatG氧化INH以形成亲电子物质，即异烟酰基自由基分子，其与NADH依赖性烯酰基-ACP(酰基运载体蛋白)还原酶反应，所述还原酶是参与分枝杆菌的分枝菌酸的生物合成(方案1，下文)的酶(Vilchèze等人，Microbiol.Spectr.2:MGM2-0014-2013(2014)；和Morlock等人，Antimicrob.Agents Chemother 47:3799-3805(2003))。

[0156] 方案1

[0157]



[0158] 耐RB耻垢分枝杆菌菌株获得中度INH耐受性，但未显示对乙硫那酰胺(ETH)的耐受性。INH耐受性的主要机制是katG中的突变，而ETH被单加氧酶EthA激活(Laborde等人，Org.Biomol.Chem.14:8848-8858(2016))。这些观察结果可能意味着RB的抗菌机制共享一种或多种INH代谢酶以形成杀菌物。

[0159] 为了阐明潜在的作用机制，使用下一代DNA测序技术对耐RB耻垢分枝杆菌ATCC607菌株进行了全基因组测序分析(Lei等人，Microbiol.Resour.Announc.8:e00551(2019))。一个鉴定的插入突变分别发生在抗sigma E因子基因(rseA；表现为TG:104相对于T:0)和水通道蛋白家族蛋白基因(表现为GCACCCT:71相对于G:0)中。

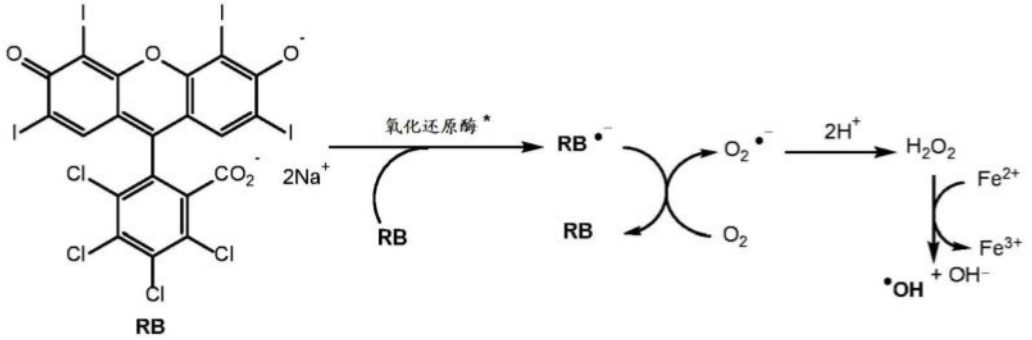
[0160] 因此，与其亲本菌株相比，这些插入突变导致相应蛋白质中的阅读框变化，并产生了截短的蛋白质。据报道，RseA在结核分枝杆菌(Mycobacterium tuberculosis)中起着特定的抗sigma E因子的作用，并且所述sigma E因子(SigE)使分枝杆菌生物体能够耐受多种应激反应(Boldrin等人，Sci.Rep.9:17643(2019)；和Wu等人，J.Bacteriol.179:2922-2929

(1997))。因此,耐RB突变体中的非功能性RseA的表达可能会影响SigE的活性,增加细菌对RB的耐受性。另一方面,水通道蛋白家族蛋白存在于各种生物体中,并在水和不带电的溶质的跨细胞膜的双向流动中起关键作用。

[0161] 据报道,链球菌水通道蛋白同源物的无效突变增加了细胞内H_2O_2滞留,表明水通道蛋白介导了链球菌中H_2O_2的运输(Tong等人,J.Biol.Chem.294:4583-4595(2019))。因此,我们假设RB可能抑制水通道蛋白功能,引起H_2O_2在细菌细胞内的积累。有趣的是,在耐RB菌株的亚钼嘌呤(molybdopterin)依赖性氧化还原酶(表现为T:74相对于TC:0)中观察到造成移码突变的单核苷酸缺失。

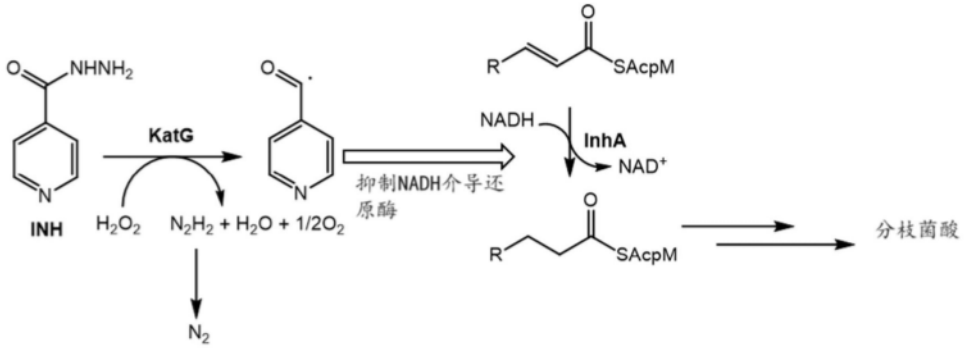
[0162] 氧化还原酶系统可以通过还原分子氧来形成超氧化物或通过还原无机硝酸盐来形成NO。RB可以作为氧化还原酶的氧化还原中的单电子受体,所述氧化还原酶的氧化还原产生自由基阴离子($RB^{\bullet -}$)或RB三重态,与氧发生电子转移反应。因此,通过在黑暗条件下激发RB,提出了亚钼嘌呤依赖性氧化还原酶参与活性氧或氮物质的产生(以下的方案2)。方案2中的氧化还原酶是亚钼嘌呤依赖性氧化还原酶黄嘌呤脱氢酶家族蛋白。方案2中的".OH+OH^-"是活性氧物质(ROS)或活性氮物质。

[0163] 方案2

[0164] 

[0165] 在耐RB菌株中,katG基因是完好的。因此,仍然难以推测赋予对INH交叉耐受性的机制。然而,观察到耐RB菌株的几种转录调节因子中的突变可能影响KatG的表达水平,抑制INH活化。其通过H_2O_2的芬顿反应产生羟基自由基(活性氧物质)。需要相对高浓度的RB来展现对革兰氏阳性细菌(包括分枝杆菌属)的杀菌活性可能意味着RB与过氧化氢酶的亲和力是中等的。类似地,可以解释RB在哺乳动物细胞中的细胞毒性机制。如以下的方案3所示。

[0166] 方案3

[0167] 

[0168] RB用于诊断眼睛和肝脏疾病已有50多年。其通常可用作用于诊断某些医学问题(诸如结膜和眼睑病症)的染色剂(见上文)。在这些应用中,使用了0.1%-2.0%(即1000μg/mL-20000μg/mL)的RB。

[0169] 在自然光和人工光下,浓度低于2.0%的RB被认为是安全的(Whitcher等人,Am.J.Ophthalmol.149:405-415(2010))。选择健康的细胞系Vero(非洲绿猴的肾脏)细胞来确定RB在荧光下的体外细胞毒性。

[0170] 已经产生了对Vero细胞的抗菌剂和抗癌剂的细胞毒性的大量数据集,以允许比较新分子的毒性水平(Siricilla等人,J.Med.Chem.60:2869-2878(2017);和Siricilla等人,J.Antibiot.68:271-278(2015))。在黑暗条件下的24小时研究中,RB显示出对Vero细胞的IC_{50}值为300μM(292μg/mL)。

[0171] RB的治疗浓度可能为约0.1μM至约10μM。因此,这些体外细胞毒性测试意味着可以用RB治疗皮肤感染,而不会在光照条件下造成宿主细胞的细胞毒性。

[0172] 结论

[0173] Provectus已经建立了医药级RB的制造和纯化工艺,所述工艺同时满足cGMP和ICH的要求。我们已经评估了医药级RB配制的产品(**PV-10®**)作为示例性RB化合物在光照和黑暗条件下的抗菌活性和细胞毒性。这里总结的通过生理盐水稀释的**PV-10®**关于RB的综合MIC数据表明,除肺炎链球菌外,RB非常有效地杀死大多数革兰氏阳性细菌(MIC 0.39μg/mL-3.1μg/mL)。

[0174] 肺炎链球菌是极少数对抗革兰氏阴性药物粘菌素(多粘菌素E)敏感的革兰氏阳性细菌之一。我们已经研究了革兰氏阳性细菌和革兰氏阴性细菌中的RB杀菌作用与粘菌素耐药性之间的关系。肺炎链球菌对RB的耐药机制将在其它地方报道。

[0175] 我们证实RB是根除革兰氏阳性细菌(包括耐药性菌株)的生物膜的优秀药物。在荧光和黑暗条件下,RB以浓度依赖性方式显著减少了金黄色葡萄球菌和屎肠球菌的耐药性菌株的许多活细胞。这些研究表明,RB是在任何生长阶段杀死耐药性细菌的潜在抗菌剂。

[0176] 材料和方法

[0177] 抗生素

[0178] 所有抗生素(阿米卡星二硫酸盐(Sigma-Aldrich,A1774-1G)、硫酸卷曲霉素(Sigma-Aldrich,C4142-1G)、盐酸环丙沙星一水合物(TCI,C2227)、乙硫异烟胺(TCI,E0695)、异烟肼(Sigma-Aldrich,I3377-5G)、雷奈佐利(Chem-Impex,29723),美洛培南三水合物(Ark Pharm,AK161987),利福平(Sigma-Aldrich,R3501-1G))均购自商业来源,除非另有说明,否则无需进一步纯化即可使用。根据报道的程序合成APPB(氨基尿苷基苯氧哌啶基苯甲基丁酰胺)(Mitachi等人,ACS Omega 2018,3:1726-1739;和Mitachi等人,MethodsX 2019,6:2305-2321)。

[0179] 生理盐水中医药级玫瑰红的制剂(**PV-10®**)

[0180] 玫瑰红二钠盐是根据Provectus的专有程序合成的。详细程序如美国专利号8,530,675、9,273,022和9,422,260中所述。

[0181] 获取细菌

[0182] 该计划中使用的药物敏感细菌和酵母购自ATCC(美国典型培养物保藏中心,Manassas,VA)。耐药性菌株获自BEI资源(NIAID)。

[0183] 对数期细菌培养物

[0184] 所有液体细菌培养均使用带有空气过滤器的锥形瓶进行。细菌菌株的单个菌落根据ATCC推荐的条件生长。使用ATCC推荐的培养基获得细菌的种子培养物和较大的培养物。

耻垢分枝杆菌(ATCC 607)在0.5％Tween®80Middlebrook 7H10营养琼脂(0.4％甘油)(46)上培养。在振荡培养箱中在37℃以200rpm的振荡速度,将培养瓶中的耻垢分枝杆菌(ATCC 607)孵育3-4天,结核分枝杆菌H_{37}Rv孵育10-12天,并培养至对数中期(光密度-0.5)。使用96孔酶标仪在600nm处监测光密度。

[0185] MIC测定

[0186] 所有测试均遵循美国临床和实验室标准协会(CLSI)制定的指南("Determining laboratory reference intervals:CLSI guideline makes the task manageable," Lab.Med.40:75-76(2009).)。通过培养液稀释微型板块拉马尔蓝测定(broth dilution microplate alamar blue assay)或通过OD测量确定最小抑制浓度(MIC)。将所有化合物储存在DMSO或盐水中(1mg/100μL浓度)。该浓度用作所有MIC研究的储备溶液。将来自储备溶液的每种化合物置于无菌96孔板的第一孔中,并用培养液(总体积为10μL)进行连续稀释。将对数期的细菌悬浮液(190μL)添加到每个孔中(总体积为200μL),并在37℃下孵育24小时,向每个孔中加入20μL刃天青(0.02％)并孵育4小时,(美国国家临床实验室标准委员会(NCCLS)方法(粉红色＝生长,蓝色＝无可见生长))。在进行比色测定之前,对所有实验进行OD测量。还通过UV-Vis在570nm和600nm处测量每个孔的吸光度。

[0187] 最小杀菌浓度(MBC)测定

[0188] 将特定细菌的单个菌落(在琼脂平板上生长)接种到培养液中。将细菌培养物生长过夜(约18小时),然后在生长支持液中稀释至$1x10^5$ CFU/mL和$1x10^9$ CFU/mL的浓度。基于MIC值,制备含有药物(MIC,2xMIC-20xMIC)的琼脂平板。将等体积的特定细菌接种在一系列含药物的琼脂平板上。将琼脂平板在适当的温度和持续时间下孵育。进行CFU/mL计数。通过减少>99.9％的细菌来确定MBC值。

[0189] 哺乳动物细胞系和培养

[0190] Vero细胞(ATCC™CCL-81)购自ATCC。HEK细胞(SCCE020)购自MilliporeSigma。按照供应商的建议,将细胞系培养并维持在培养基中。

[0191] 将Vero细胞在补充有10％FBS、1％青霉素-链霉素溶液(cellgro®,30-002-CI)、1％HyClone™MEM非必需氨基酸溶液(100X)(GE Healthcare Life Sciences,SH30238.01)和1％ HyClone™丙酮酸钠100mM溶液(GE Healthcare Life Sciences,SH30239.01)的Eagle极限必需培养基(MEM)(CORNING®,10-009-CV)中培养,并在37℃、100％湿度和5％ CO_2的培养箱中孵育。每2天对培养物更换新鲜培养基,直到培养物达到100％汇合度,这需要大约5天的时间(取决于增殖速率)。

[0192] 将新生儿人表皮角质形成细胞(MILLIPORE®,目录号#SCCE020)在EpiGRO™人表皮角质形成细胞完全培养基试剂盒(MILLIPORE®,SCMK001)中培养,并在37℃、100％湿度和5％ CO_2的培养箱中孵育。2天后更换新鲜培养基,每周三次,直到培养物达到100％汇合度。

[0193] 用Vero细胞进行细胞毒性测定

[0194] 所有测试均遵循美国临床和实验室标准协会(CLSI)制定的指南,稍作修改。PV-10的细胞毒性测定在24孔板中进行。向每个孔(1mL培养基/孔)中加入1μL的药物浓缩物。在室温(r.t.)下在光照下孵育1h、2h、3h和4h后,除去培养基,并用PBS洗涤(x3)细胞。在加入培养基(1mL/孔)后,加入10μl的3-(4,5-二甲基噻唑-2-基)-2,5-二苯基四唑溴化物(MTT)溶

液(在PBS中5mg/mL),并在37℃(5%CO_2)下再孵育3h。除去培养基,并加入DMSO(1mL/孔)。基于MTT向紫色甲臜产物的细胞转化来评估活力。通过BioTek Synergy™HT分光光度计在570nm处测量有色甲臜产物的吸光度(Mitachi等人,J.Med.Chem.63:10855-10878(2020))。

[0195] 将Vero细胞(5×10^4个细胞/孔(在196μL的培养基中))铺在96孔板中,并将细胞培养物孵育4天以形成单层(100%汇合度)。向每个孔中加入RB(0mM-300mM)。

[0196] 使用IncuCyte®活细胞成像系统(Essen BioScience,Ann Arbor,MI)每小时获得图像。使用计算的度量相位对象汇合(metric phase object confluence)(POC)对细胞增殖进行定量,所述度量相位对象汇合是对细胞覆盖的视野面积的测量。

[0197] 耻垢分枝杆菌菌株的全基因组测序

[0198] 根据先前报道的程序制备耐RB耻垢分枝杆菌(ATCC607™)菌株(Kaser等人,Cold Spring Harb Protoc.(2010))。为了鉴定可能促成细菌对RB耐受性的单核苷酸多态性(SNP),根据先前报道的程序,从耐RB突变体及其亲本对照耻垢分枝杆菌607™的静置培养物中纯化基因组DNA(Lelovic等人,J.Antibiot.73:780-789(2020))。

[0199] 将纯化的基因组DNA提交给明尼苏达大学基因组中心(UMGC),以使用先进的Illumina MiSeq™DNA-seq技术进行质量控制分析、文库制备和DNA测序。使用FastQC评估来自突变体和对照的序列读数的质量。用Trimmomatic去除低质量的尾和衔接子(Lei等人,Methods Mol.Biol.2069:125-138(2020))。以耻垢分枝杆菌菌株FDAARGOS_679的全基因组序列作为参照,并且使用生物信息学工具Snippy(https://github.com/tseemann/snippy)判断SNP或其它变体,诸如缺失和插入。

[0200] 本文引用的专利、专利申请和文章各自均通过引用并入本文。本文中的冠词“一个”和“一种”是指该冠词的语法宾语中的一个(一种)或多于一个(多于一种)(即至少一个(至少一种))。

[0201] 上述描述和实例旨在举例说明,不应被理解为限制。在本发明的精神和范围内的其它变化也是可行的,并且将容易地呈现给本领域技术人员。