(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 February 2016 (25.02.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/029199 A1

(51) **International Patent Classification**:
A61K 31/56 (2006.01)

(21) **International Application Number**:
PCT/US2015/046453

(22) **International Filing Date**:
24 August 2015 (24.08.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
62/040,721 22 August 2014 (22.08.2014) US
62/194,120 17 July 2015 (17.07.2015) US

(72) **Inventor; and**
(71) **Applicant** : **ZHANG, Kang** [US/US]; 9890 Blackgold Road, La Jolla, CA 92037 (US).

(72) **Inventor**: **HOU, Rui**; Building No. 17, Pujiang Garden, Hunnan District, Shenyang, Liaoning Province, 110000 (CN).

(74) **Agent**: **WANG, Xinsheng**; US-China Intellectual Property Counsel, 2827 Edgewood Drive, Sugar Land, TX 77479 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

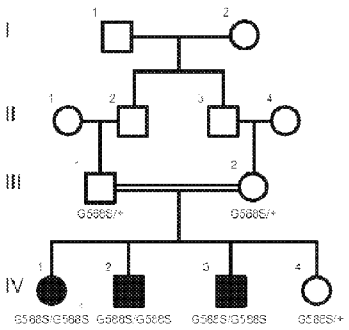
Published:

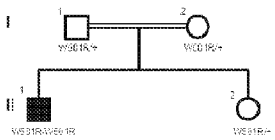
— *with international search report (Art. 21(3))*

(54) **Title**: COMPOSITIONS AND METHODS TO TREAT VISION DISORDERS

(57) **Abstract**: The present invention provides sterols and uses thereof to treat vision disorders. In one embodiment, composition comprising pharmaceutically effective amount of lanosterol is used to treat and/or prevent vision disorders in a subject. In another embodiment, composition comprising pharmaceutically effective amount of lanosterol is used to treat cataract or blindness/impaired vision in a subject. In yet another embodiment, composition comprising lanosterol is used to dissolve amyloid-like fibrils of crystallin proteins.

Fig.1A





WO 2016/029199 A1

COMPOSITIONS AND METHODS TO TREAT VISION DISORDERS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Application Number 62/040,721, filed August 22, 2014 and U.S. Provisional Application Number 62/194,120, filed July 17, 2015, the content of which are hereby incorporated herein by reference in their entirety.

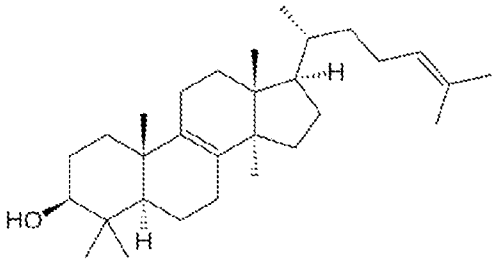
FIELD OF THE INVENTION

[0002] The disclosure generally relates to sterols and uses thereof to treat vision disorders that affect the normal function of the lens in the eye in a subject having or at risk of developing such vision disorders.

SUMMARY OF THE INVENTION

[0003] The invention provides a method of treating or preventing vision disorders, the method comprising administering to an individual in need thereof an effective amount of a lanosterol; and a prodrug or pharmaceutically acceptable salt thereof.

[0004] The invention also provides an ophthalmic pharmaceutical composition comprising a pharmaceutically acceptable ophthalmic carrier and lanosterol with a structure of formula I:



and a prodrug or pharmaceutically acceptable salt thereof.

[0005] In various aspects of the method, the vision disorder is a disorder of the eye that affects function, clarity and/or structure of the lens of the eye. Such eye diseases include, but are not limited to, cataracts of the eye, presbyopia of the eye, and nuclear sclerosis of the eye lens. In addition, vision disorders refer to retinal degeneration, such as as Refsum disease, Smith-Lemli-Opitz syndrome (SLOS) and Schnyder crystalline corneal dystrophy (SCCD), abetalipoproteinemia and familial hypobetalipoproteinemia.

[0006] In one embodiment, the present invention provides a method of ameliorating at least one symptom associated with a vision disorder by administering to a subject a therapeutically

or prophylactically effective amount of a sterol of formula 1. In various aspects of the method, the composition is administered topically, subconjunctivally, retrobulbarly, periocularly, subretinally, suprachoroidally, or intraocularly. Subjects that receive the invention sterol can include, but are not limited to mammals, avians, amphibians, reptiles and other vertebrates. In one embodiment, the subjects are horses, pigs, dogs, cats, rodents and/or other companion pets. In another embodiment, the subjects are humans.

[0007] In one embodiment, the present invention relate to an ophthalmic pharmaceutical composition comprising the invention sterol in an ophthalmic pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition comprises lanosterol, or derivatives thereof in an ophthalmic pharmaceutically acceptable carrier. In certain embodiments of the invention, the pharmaceutically acceptable carrier is water, a buffer or a solution of sodium chloride. In some embodiments, the pharmaceutically acceptable carrier is sterile. In other embodiments, the pharmaceutically carrier is an ointment. In still other embodiments, the pharmaceutically acceptable carrier is a gel. Gels can be formulated using gel formulating materials that are well known in the art, including but not limited to, high viscosity carboxymethylcellulose, hydroxypropylmethylcellulose, polyethylene oxide and carbomer. In some aspects of the composition, the pharmaceutically acceptable ophthalmic carrier is a cyclodextrin. In one embodiment, the cyclodextrin is (2-hydroxypropyl)-β-cyclodextrin.

[0008] Certain embodiments of the invention also contemplate kits that comprise components useful for treating and/or preventing a symptom associated with a vision disorder. Such kits comprise a container comprising invention sterol in a pharmaceutically acceptable carrier and instructions for administering the invention sterol such that at least one symptom associated with the vision disorder is ameliorated or prevented. Such vision disorder includes, but is not limited to, cataracts, presbyopia, and nuclear sclerosis of the eye lens. In addition, vision disorders refer to retinal degeneration, such as Refsum disease, Smith-Lemli-Opitz syndrome (SLOS) and Schnyder crystalline corneal dystrophy (SCCD), abetalipoproteinemia and familial hypobetalipoproteinemia. The containers included in some of the kits contemplated herein are droppers for the administration of eye drops. In other embodiments, the container is a tube for dispensing ointment or gel. In still other embodiments, the container is any appropriate container for drug delivery including, but not limited to, a syringe, or other container appropriate for delivery of a drug ophthalmically or topical application.

[0009] In other aspects, the invention provides a method for inhibiting or preventing protein aggregation. In various aspects of the method, the protein is an amyloid-forming protein or a

protein underlying a loss-of-function disease. In some aspects, the amyloid-forming protein is selected from the group consisting of Hsp27, αA-crystallin, αB-crystallin, βB2-crystallin, βB1-crystallin, γD-crystallin, Hsp22, Hsp20, tau, Alpha-synuclein, IAPP, beta-amyloid, PrP, Huntingtin, Calcitonin, Atrial natriuretic factor, Apolipoprotein AI, Serum amyloid A, Medin, Prolactin, Transthyretin, Lysozyme, Beta 2 microglobulin, Gelsolin, Keratoepithelin, Cystatin, Immunoglobulin light chain AL, and S-IBM. In other aspects, the protein underlying a loss-of-function disease is selected from the group consisting of mutant β-glucosidase, cystic fibrosis transmembrane receptor, hexosaminidase A, hexosaminidase B, β-galactosidase, and alpha-glucosidase.

[0010] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the disclosure, are given by way of illustration only, because various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description. The entire document is intended to be related as a unified disclosure, and it should be understood that all combinations of features described herein are contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document. In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. For example, if aspects of the invention are described as "comprising" a feature, embodiments also are contemplated "consisting of" or "consisting essentially of" the feature.

[0011] In one embodiment, this invention discloses the use of a composition for the preparation of medicament to treat and/or prevent vision disorders in a subject, said composition comprises a pharmaceutically acceptable ophthalmic carrier and a pharmaceutically effective amount of lanosterol. Said subject is having or at risk of developing a vision disorder that affects the normal structure of the lens in the eye. Said subject may be selected from the group consisting of amphibians, reptiles, avians, and mammals; wherein said mammal may be selected from the group consisting of rodents, cats, dogs, pigs, horses and humans. In another embodiment, said vision disorder is selected from the group consisting of cataract, congenital cataracts, cortical opacities, posterior subcapsular cataract, presbyopia nuclear sclerosis, retinal degenerative disorder, Refsum disease, Smith-Lemli-Opitz syndrome, Schnyder crystalline corneal dystrophy, drusen, age-related macular degeneration, and diabetic retinopathy, and lanosterol inhibits crystallin protein aggregation.

[0012] In yet another embodiment, this invention discloses the use of a composition for the preparation of medicament to treat cataract or blindness/impaired vision in a subject, said composition comprises a pharmaceutically acceptable ophthalmic carrier and a pharmaceutically effective amount of lanosterol, wherein said lanosterol dissolves lens crystallin protein aggregate(s) in the eye of said subject; wherein the lens crystallin protein is any of α–crystallin, β–crystallin or γ–crystallin. The above mentioned composition may be formulated as an ophthalmic solution, an ophthalmic ointment, an ophthalmic wash, an intraocular infusion solution, a wash for anterior chamber, an internal medicine, an injection, or preservative for extracted cornea.

[0013] In yet another embodiment, this invention discloses a method for dissolving amyloid-like fibrils of crystallin proteins, comprising the step of contacting the amyloid-like fibrils with lanosterol in a sufficient amount and duration so as to dissolve the amyloid-like fibrils of crystalline proteins, wherein the method may be performed in situ, in vitro or in vivo. The method may be performed on a subject selected from the group consisting of amphibians, reptiles, avians, and mammals; wherein said mammal may be selected from the group consisting of rodents, cats, dogs, pigs, horses and humans.

[0014] In another embodiment, this invention discloses a kit for treating and/or preventing vision disorders that affect the normal structure of the eye in a subject, comprising a formulation of a pharmaceutically effective amount of lanosterol, a pharmaceutically acceptable carrier and instructions for administering said formulation such that said administration treats and/or prevents said vision disorder. In yet another embodiment, this invention discloses an ophthalmic pharmaceutical composition for treating and/or preventing vision disorders in a subject, said composition comprises a pharmaceutically acceptable ophthalmic carrier and a pharmaceutically effective amount of lanosterol; wherein said composition may be formulated as an ophthalmic solution, an ophthalmic ointment, an ophthalmic wash, an intraocular infusion solution, a wash for anterior chamber, an internal medicine, an injection, or preservative for extracted cornea.

[0015] In another embodiment, this invention discloses a method for identifying and/or treating a subject at risk of developing cataract or blindness/impaired vision associated with formation of lens crystallin protein aggregate(s) in an eye, comprising: a) assaying for amount of lanosterol synthase activity in the subject; b) determining whether the amount of lanosterol synthase activity is less than that of a control population without cataract or blindness/impaired vision, wherein an amount of lanosterol synthase activity less than that of a control population

is indicative of a higher risk of developing cataract or blindness /impaired vision associated with the formation of lens crystallin protein aggregate(s); and c) treating the subject with lanosterol in an effective amount and duration so as to prevent or reverse formation of lens crystallin protein aggregate(s) in an eye of the subject, thereby identifying and treating the subject at risk of developing cataract or blindness/impaired vision associated with formation of lens crystallin protein aggregatc(s) in the eye of the subject.

[0016] In another embodiment, this invention discloses a method of identifying and/or treating a subject at risk of developing cataract or blindness/impaired vision associated with formation of lens crystallin protein aggregate(s) in an eye of the subject, comprising: a) determining whether both alleles of the lanosterol synthase gene are affected with a mutation which decreases lanosterol synthase expression or activity, wherein presence of a mutation in both alleles of the lanosterol synthease increases the risk of developing cataract or blindness/impaired vision associated with formation of lens crystallin protein aggregate(s) in an eye of a subject; and b) treating the subject with lanosterol in an effective amount and duration so as to prevent or reverse formation of lens crystallin protein aggregate(s) in an eye of the subject, thereby identifying and treating the subject at risk of developing cataract or blindness/impaired vision associated with formation of lens crystallin protein aggregate(s) in the eye or the subject. In one embodiment, the mutation in lanosterol synthase gene is at codon 581 changing tryptophan (W) to arginine (R) or codon 588 changing glycine (G) to serine (S).

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] **Figure 1** shows identification of mutations in LSS causing congenital cataracts. Fig. 1A, Pedigrees of affected families and cataract phenotype. Squares and circles indicate males and females respectively. 1, wild-type allele; W581R and G588S are the two mutations. Fig 1B, Upper panel, DNA sequencing data of an unaffected individual and an affected child (II-1) with a homozygous W581R mutation; lower panel, DNA sequencing data of an unaffected individual and an affected child (IV-1) with a homozygous G588S mutation. The underlined sequence indicates the nucleic acid change. Fig 1C, Left, colour photograph of patient 1's right eye in the first pedigree (IV-1) with a total cataract; right, colour photograph of patient 2's right eye in the same pedigree (IV-3) with a cataract.

[0018] **Figure 2** shows LSS mutations abolished the cyclase enzymatic function. Fig 2A, Conservation of W581R and G588 in LSS across several species: Homo sapiens, Pan troglodytes, Bos taurus, Mus musculus, Rattus norvegicus, Gallus gallus and Danio rerio. Fig

2B, Computer modelling of LSS structure and impact of the LSS W581R and G588S mutations. A computer modelling analysis identifies a loop originating from C584 and ending at E578 with the key side chain of W581 at the tip of the loop stabilizing the sterol. The loop is fixed by an S–S bridge and the E578–R639 salt bridge. Amide nitrogen N of G588 interacts with the C584 from the previous helical turn and the Ca hydrogen of G588 is in close proximity to the critical E578, which then forms a strong salt bridge with R639 of the same supporting helix. The mutation G588S causes the side chain of the serine to clash into the E578 residue of the loop and is incompatible with the structure. Arrow indicates the location of the mutant side chain. Fig. 2C, Effect of engineered expression of the wild-type protein (WT LSS) and LSS mutants on sterol content. Wild-type LSS markedly increased lanosterol production, whereas neither W581R nor the G588S mutant exhibited any cyclase activity. n=3 in each group; ***P<0.001.

[0019] Figure 3 shows lanosterol reduced intracellular aggregation of various crystallin mutant proteins. Fig 3A, Confocal images of crystallin protein aggregates in human lens progenitor cells. The cataract-causing Y118D mutant of aA-crystallin formed p62-positive intracellular inclusion bodies or aggresomes. Green, eGFP–crystallin proteins; red, p62; blue, nuclei. Cells transfected with peGFP- N1 were used as a control. Fig 3B Confocal images of inhibitory effect of LSS on crystalline aggregates. Fig 3C, Inhibition of crystallin mutant aggregation by wild-type LSS (WT LSS) and lanosterol, but not mutant LSS or cholesterol. Fig. 3D, Increase in soluble aA-crystallin (Y118D) mutant protein by co-expression of wild-type LSS but not LSS mutants (Y118D co-expressed with pcDNA3.1–N- Flag was used as a control). Quantitative analysis was performed using densitometry of crystallin proteins by western blot analysis of the supernatant or insoluble fraction of cell lysates. n=3 in each group; representative western blot analysis is shown in - Fig. 9c; *P<0.05, **P<0.01. Fig 3E, Confocal images of the re-dissolution of pre-formed crystalline aggregates by lanosterol. Fig 3F, Lanosterol significantly reduced the intracellular aggregation by various cataract-causing mutant crystallin proteins in a concentration-dependent manner (n=3, $P<1\times10^{-4}$). Cholesterol did not reduce intracellular aggregation (n=3, $P>0.1$). Fig. 3G Lanosterol increased the soluble fractions of various crystallin mutants in human lens progenitor cells. n=3; P<0.001. Fig. 3H, Effects of DMSO, cholesterol or lanosterol on αA-crystallin Y118D aggregates in human lens progenitor cells by serial live cell imaging. Fig. 3I, Effect of lanosterol on dissolution of intracellular crystallin aggregates over time (n=22 from 3 biological replicates). The mean ±

SD values are shown as black symbols. The data are best fitted by the single exponential decay process (red line).

[0020] **Figure 4** shows lanosterol re-dissolved pre-formed amyloid-like fibrils of crystallin proteins. Fig 4A, Negatively stained TEM photographs of aggregates of aA-crystallin mutant proteins treated by a liposome vehicle, cholesterol or lanosterol in liposomes. Images in the right column of the lanosterol group show a 5X magnification of the image on their right. Fig.4B, Effect of lanosterol on the re-dissolution of crystallin aggregates by ThT fluorescence (n=3). Fig.4B (i), b/gamma-crystallin mutants; Fig.4B (ii), a-crystallin mutants. Each bar results from three independent samples.

[0021] **Figure 5** shows lanosterol reduced cataract severity and increased clarity. Fig 5A, Photographs of a cataractous rabbit lens treated with lanosterol showing increased lens clarity. Fig 5A(i), left, before treatment; Fig 5A(ii), right, after treatment. Fig 5B, Boxplot of the quantification of the treatment effect of lanosterol (n=13). Fig 5C, Photographs of a cataractous dog lens treated with lanosterol showing increased lens clarity. Fig 5C(i), left, before treatment; Fig 5C(ii), right, after treatment. d, Boxplot of the quantification of the treatment effect of lanosterol (n=7). Range, median (horizontal line) and mean (circle) are presented. Crosses indicate the maximum and minimum cataract grades measured. Whiskers indicate the standard deviation and the box encompasses a 40% confidence interval.

[0022] **Figure 6A** shows homozygosity mapper plots the genome-wide homozygosity as bar charts. To emphasize regions of interest, any score higher than 80% of the maximum score reached in this project is coloured in red. **Figure 6B** shows the homozygosity scores were plotted against the physical position on chromosome 21, which contains the *LSS* gene. Red bars indicate regions with highest scores. The right side of the chromosome contains a long continuous homozygous region, where the *LSS* gene is located.

[0023] **Figure 7** shows representative confocal images of cells co-transfected with Flag–LSS and eGFP. Human lens progenitor cells were co-transfected with either the wild-type or the mutated *LSS* gene and the *eGFP* gene for 4 h and cultured for 16 h in fresh culture medium. The cellular distribution of LSS was then visualized using an anti-Flag antibody (purple). The distribution of eGFP (green) was used as a control. The nuclei were stained and visualized by Hoechst 33342 (blue).

[0024] **Figure 8** shows representative confocal images of cells co-transfected with LSS and various cataract-causing crystallin mutants. Fig 8A, R116C mutant of αA-crystallin. Fig 8B, R120G mutant of αB-crystallin. Fig 8C, V187E mutant of βB2-crystallin. Fig 8D, G129C

mutant of γC-crystallin. Fig 8AE, W43R mutant of γD-crystallin. Human lens progenitor cells were co-transfected with either the wild-type or the mutated Flag–LSS gene and the mutant GFP–crystallin gene for 4 h and cultured for 16 h in fresh culture medium. All crystallin mutants formed p62-positive aggregates as indicated by the co-localization of the mutant crystallins and p62. Cells co-transfected with GFP–crystallin and pcDNA3.1-N-Flag were used as controls. The formation of intracellular aggregates of various crystallin proteins was visualized by fluorescence of GFP (green). Wild-type or mutated LSS was detected with an anti-Flag antibody (red), p62 was stained using an anti-p62 antibody, while the nuclei were stained and visualized by Hoechst 33342 staining (blue). Quantitative analysis of cells with aggregates is summarized in Fig 3c.

[0025] **Figure 9** shows inhibition of crystallin mutant aggregation by wild-type LSS and lanosterol in HLEB-3 cells (Fig 9A) or HeLa cells (Fig 9B). Cells co-transfected with LSS and crystallin mutant constructs were cultured for 24 h before assaying for aggregates. The rescue experiments were performed by addition of 40∈μM sterols (lanosterol or cholesterol) to the cell culture medium for 2 h, the sterol medium was then replaced with fresh culture medium and the cells were cultured for a further 12 h. The percentage of cells with crystallin aggregates were calculated from ten randomly selected viewing fields. The values of the wild-type LSS group, mutant group, or mutant plus lanosterol group were calculated. Aggregates were significantly lower in the wild-type LSS and lanosterol groups compared to the control group ($P < 1 \times 10^{-4}$), while aggregates in mutant LSS or cholesterol groups showed no difference to the control group ($P > 0.1$). Fig. 9C, Human lens progenitor cells were co-transfected with wild-type or mutant LSS plus αA-crystallin (Y118D). αA-crystallin (Y118D) co-expressed with pcDNA3.1-N-Flag was used as a control. After transfection for 4 h and incubation in fresh culture medium for another 24 h, the cells were lysed and centrifuged to separate supernatant and insoluble fractions. LSS and crystallin fusion proteins were detected by antibodies against Flag and GFP, respectively. Red arrows indicate higher crystalline content in the soluble fraction versus in the insoluble fraction in cells containing the WT-LSS. Data were quantified from three independent experiments and summarized in Fig 3D.

[0026] **Figure 10** shows lanosterol significantly reduced the intracellular aggregation caused by various cataract-causing mutant crystallin proteins in a concentration-dependent manner when assayed in HLEB-3 or HeLa cells. Fig 10A, Representative confocal images of HLEB-3 cells transfected with various cataract-causing crystallin mutants. Fig 10B, Representative confocal images of HeLa cells transfected with various cataract-causing crystallin mutants.

Cells were transfected with various crystallin constructs for 4 h and cultured for an additional 24 h in fresh culture medium. Then the cells were treated with 10, 20 and 40 μM lanosterol in 1% (HLEB-3 cells) or 2% DMSO (HeLa cells) for 2 h and cultured for another 12 h. Cells treated with 1% (HLEB-3 cells) or 2% DMSO (HeLa cells) were used as the controls. Formation of intracellular aggregates of various crystallin proteins was visualized by fluorescence of GFP (green) and the nuclei were stained with Hoechst 33342 (blue). Typical intracellular aggregates are indicated by arrows. Fig 10C, Concentration dependence of the aggregation-dissolving effects of lanosterol when assayed in HLEB-3 cells. Fig 10D, Concentration dependence of the aggregation-dissolving effects of lanosterol when assayed in HeLa cells.

[0027] **Figure 11** shows treatment by lanosterol, but not cholesterol, increased cataract-causing mutant crystallins in soluble fractions when compared to a control group or a mutant LSS group. Fig 11A, Human lens progenitor cells were transfected with mutant crystallin genes for 4 h, and then incubated in fresh culture medium for another 24 h. The cells were harvested and lysed. Supernatant and insoluble fractions were separated by centrifugation and analyzed by western blot analysis. LSS and crystallin fusion proteins were identified by antibodies against Flag and GFP tags, respectively. The lanosterol-treated group is highlighted by red boxes. Cells treated with 1% DMSO were used as a control. β-Actin was used as an internal protein loading control of total cell lysates (TCL). S, supernatant; P, insoluble fraction. Fig 11B, Effect of DMSO ($n = 4$) and cholesterol ($n = 7$) on the size changes of αA-crystallin (Y118D) aggregates in human lens progenitor cells evaluated by single-particle tracking in live-cell imaging. Fig 11C, Evaluation of the effect of lanosterol on the dissolution of crystallin aggregates by turbidity. Crystallin aggregates were formed by incubating 5 mg ml^{-1} protein solution at 60 °C for 2 h (α-crystallins) or 37 °C for 48 h (β- and γ-crystallins) in the presence of 1 M guanidine chloride. The preformed aggregates were re-suspended in PBS at a final protein concentration of 0.2 mg ml^{-1} and were treated with 500 μM sterols in 500 μM DPPC liposome and incubated at 37 °C for 24 h. Aggregates treated with 500 μM DPPC liposome only were used as the controls. Fig 11D, Concentration-dependent effect of lanosterol on the re-dissolution of amyloid-like fibrils by αA-crystallin mutants evaluated by ThT fluorescence. Aggregates treated with 500 μM DPPC liposome only were used as the controls.

[0028] **Figure 12** shows grading system of cataractous lenses. Fig 12A, Lenses were placed above a grid and photographed. The degree of transparency was scored as 0, a clear lens and absence of opacification (gridlines clearly visible, a′); 1, a blurry lens and a slight degree of

opacification (minimal clouding of gridlines, with gridlines still visible, b′); 2, a cloudy lens and presence of diffuse opacification involving almost the entire lens (moderate clouding of gridlines, with main gridlines visible, c′); or 3, an opaque lens and presence of extensive thick opacification involving the entire lens (total clouding of gridlines, with gridlines not seen at all, d′). Fig 12 B, Lanosterol reduced cataract severity and increased clarity in isolated cataractous rabbit lenses. Rabbit lenses (n = 13) were dissected and incubated with lanosterol for 6 days and subsequently assessed for lens clarity and transparency. Pairs of photographs of each cataractous rabbit lens showing before and after treatment with scores underneath are shown. Fig 12C, Lanosterol reduced cataract severity and increased lens clarity in dogs. Dog eyes with cataracts (n = 7) were treated with lanosterol for 6 weeks and assessed for lens clarity and transparency. A pair of photographs of each study eye before and after treatment is shown with scores underneath. Three control eyes treated with vehicles alone are also presented.

DETAILED DESCRIPTION

[0029] Reference will now be made in detail to specific embodiments of the invention including the best modes contemplated by the inventors for carrying out the invention. Examples of these specific embodiments are illustrated in the accompanying drawings. While the invention is described in conjunction with these specific embodiments, it will be understood that it is not intended to limit the invention to the described embodiments. On the contrary, it is intended to cover alternatives, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims. In the following description, specific details are set forth in order to provide a thorough understanding of the present invention. The present invention may be practiced without some or all of these specific details. In addition, well-known features may not have been described in detail to avoid unnecessarily obscuring the invention.

[0030] The present invention relates to a method of and compositions for treating or preventing vision disorders that affect the normal structure of the eye in a subject having or at risk of developing such vision disorders, comprising administering to such subject a composition comprising a pharmaceutically acceptable carrier and a pharmaceutically effective amount of a sterol having the formula I. For example, an exemplary compound of the invention comprises administering to a patient an opthalmological pharmaceutically effective amount of lanosterol (3β-Hydroxy-8,24-lanostadiene; 8,24-Lanostadien-3β-ol).

[0031] In other embodiments, the present disclosure describes sterols and methods of using sterols. For example, the sterols of formula I are formulated in ophthalmic pharmaceutical

compositions comprising a pharmaceutically acceptable ophthalmic carrier to inhibit crystallin protein aggregation. In certain other embodiments, the present disclosure describes methods of using sterols of formula 1 to inhibit crystallin protein aggregation. In yet other embodiments, compounds of the invention are able to reverse aggregation of crystallin protein and inhibit further aggregation of crystallin protein.

Methods of Treating or Preventing Vision Disorders

[0032] The present invention provides ophthalmic pharmaceutical compositions and methods of using the present invention in preventing and/or treating vision disorders that affect the normal structure of the lens in the eye in a subject having or at risk of developing such vision disorders. As described herein, a vision disorder that affects the normal structure of the lens in the eye (referred herein as the phrase "vision disorder") refers to conditions that affect the structure of the lens as to cause vision dysfunction, such as changes to the clarity or rigidity of the lens of the eye. Such conditions include cataracts, presbyopia and nuclear sclerosis. In addition, vision disorders refer to retinal degeneration, such as as Refsum disease, Smith-Lemli-Opitz syndrome (SLOS) and Schnyder crystalline corneal dystrophy (SCCD), abetalipoproteinemia and familial hypobetalipoproteinemia. In certain embodiments, the present invention provides compositions and methods of use thereof to alleviate or reverse crystalline protein aggregation. In alternative embodiments, there are provided compositions and methods for inhibiting, preventing and/or treating the disruption of intra- or inter-protein interactions that form the macro-structure essential for lens transparency and refractive index.

[0033] The term "cataract" as referred to in the present invention means a disease or condition that exhibits symptoms of causing cloudiness or opacity on the surface and/or the inside of the lens or inducing the swelling of the lens, and it includes both congenital cataract and acquired cataract (cf. PDR Staff, "PDR of Ophthalmic Medicines 2013", PDR Network, 2012). In some embodiments, the cataract is an age-related cataract, a diabetic cataract, a cataract associated with surgery, a cataract resulting from exposure to radiation, a cataract resulting from a genetic illness, a cataract resulting from an infection, or a cataract resulting from medication. In some embodiments, the individual has a hereditary form of cataract with early onset. Concrete examples of such are congenital cataract such as congenital pseudo-cataract, congenital membrane cataract, congenital coronary cataract, congenital lamellar cataract, congenital punctuate cataract, and congenital filamentary cataract; and acquired cataract such as geriatric cataract, secondary cataract, browning cataract, complicated cataract, diabetic cataract, traumatic cataract, and others inducible by electric shock, radiation, ultrasonic, drugs, systemic

diseases, and nutritional disorders. Acquired cataract further includes postoperative cataract with symptoms of causing cloudiness in the posterior encapsulating a lens inserted to treat cataract.

[0034] Nuclear sclerosis refers to a condition, generally in older animals, that results similarly in opacity of the lens. It is an age-related change in the density of the crystalline lens nucleus that is caused by compression of older lens fibers in the nucleus by new fiber formation.

[0035] Presbyopia refers to a vision condition in which the crystalline lens of the eye loses its flexibility, which makes it difficult to focus on close objects.

[0036] In some embodiments, the invention provides a method of treating or preventing a vision disorder, the method comprising administering to an individual in need thereof an effective amount of a composition comprising a compound having a structural formula I. In some embodiments, the compound is a sterol having a structural formula I.

[0037] An individual "in need of" treatment according to the invention is an individual that is suffering from a vision disorder that affects the normal function of the lens in the eye. For example, the individual may have or is at risk for developing an age-related cataract or a cataract. Individuals at risk of developing a cataract include, but are not limited to, individuals with a family history of developing cataracts, individuals with a mutation linked to a cataract, individuals exposed to radiation, diabetics, and the like. For example, in one aspect, the individual has been diagnosed with cataract in one eye, and the compound is administered to prevent or slow cataract formation in the contralateral eye. Similarly, an individual "in need of" treatment according to the invention is an individual that may have or is at risk for developing presbyopia. Similarly, an individual "in need of" treatment according to the invention is an individual that has or is at risk for developing nuclear sclerosis. Preferably the individual is human, however, animals that suffer from or who are at risk for an eye disease (animals in need of treatment) can also be identified by one skilled in the art. Mammals in need of treatment, such as cats, dogs, pigs, horses, cows and rodents can be identified. Additionally, animals such as avians, reptiles, amphibians, and fish that are in need of treatment can be identified.

[0038] "Treating" a vision disorder does not require a 100% abolition or reversal of a vision disorder. In some embodiments, "treating" vision disorders according to inventive method alleviates, inhibits, prevents and/or reverses dysfunction of the lens, e.g., opacity or inflexibility of the lens by, e.g., at least about 5%, at least about 10% or at least about 20% compared to levels observed in the absence of the inventive composition or method (e.g., in a biologically-matched control subject or specimen that is not exposed to the invention composition or

compound of the inventive method). In some embodiments, dysfunction (such as cataract formation, opacity or crystalline aggregation on or in the lens) is treated by at least about 30%, at least about 40%, at least about 50%, or at least about 60%, at least about 70%, at least about 80%, at least about 90%, or more (about 100%) compared to lens dysfunction in the absence of the compound of the inventive method. Lens dysfunction, such as opacity or cloudiness or cataracts, generally are detected using any of a number of optic tests including, but not limited to, visual acuity testing, ophthalmoscopy, slit-lamp examination, keratometry, tonometry, contrast testing, glare sensitivity, wavefront mapping.

[0039] Similarly, "prevention" does not require 100% inhibition or deterrence of a vision disorder. For example, any reduction in cloudiness or opacity, or deceleration of cataract progression constitutes a beneficial biological effect in a subject. Also exemplary, any decrease in crystalline aggregation in the lens of an eye constitutes a beneficial biological effect. In this regard, the invention reduces the vision disorder, e.g., at least about 5%, at least about 10% or at least about 20% compared to levels observed in the absence of the inventive method (e.g., in a biologically-matched control subject or specimen that is not exposed to the compound of the inventive method). In some embodiments, the vision disorder is reduced by at least about 30%, at least about 40%, at least about 50%, or at least about 60%, at least about 70%, at least about 80%, at least about 90%, or more (about 100%).

[0040] Inhibiting, preventing or reversal of dysfunction does not require a 100% inhibition, prevention, abolition or reversal. For example, any inhibition of aggregation constitutes a beneficial biological effect in a subject. In this regard, the invention inhibits a vision disorder that affects the normal function of the lens of the eye in a subject, e.g., at least about 5%, at least about 10% or at least about 20% compared to levels observed in the absence of the inventive method (e.g., in a biologically-matched control subject or specimen that is not exposed to the compound of the inventive method). In some embodiments, the vision disorder is inhibited, prevented and/or reversed by at least about 30%, at least about 40%, at least about 50%, or at least about 60%. In some embodiments, the inventive method inhibits amyloid formation by at least about 70%, at least about 80%, at least about 90%, or more (about 100%) compared to amyloid formation in the absence of the compound of the inventive method.

[0041] An "effective amount" of an ophthalmic pharmaceutical composition comprising a compound of formula 1 is an amount that inhibits, prevents or reverses dysfunction of the lens in an individual. An ophthalmic pharmaceutical composition of the present invention is being administered to a subject in need thereof at an effective amount to treat the vision disorder. As used herein, "therapeutically effective amount" means a dose that alleviates at least one of the

signs, symptoms, or causes of a vision disorder, or any other desired alteration of a biological system. In preventative applications, the term "prophylactically effective amount" means a dose administered to a patient susceptible to or otherwise at risk of a particular disease, which may be the same or different dose as a therapeutically effective amount. The effective amount of the composition for a particular individual can depend on the individual, the severity of the condition of the individual, the type of formulation being applied, the frequency of administration, and the duration of the treatment. In accordance with the present invention, administration of an ophthalmic pharmaceutical formulation of the present invention such as, e.g., lanosterol, even at relatively low concentrations in liquid drops, e.g., at least 10^{-9} M, at least 0.5 to 1×10^{-8} M, at least 0.5 to 1×10^{-7} M, at least 0.5 to 1×10^{-6} M, at least 0.5 to 1×10^{-5} M, at least 0.5 to 1×10^{-4} M, or at least 0.5 to 1×10^{-3} M, or any concentration falling in a range between these values (e.g., 10^{-9} M to 10^{-3} M), may reverse such vision disorders with only one, two, three or multiple, daily applications and does so rapidly.

Route of Administration

[0042] As will be understood by those skilled in the art, the most appropriate method of administering a compound to a subject is dependent on a number of factors. In various embodiments, the compound according to the invention is administered locally to the eye, e.g., topically, subconjunctivally, retrobulbarly, periocularly, subretinally, suprachoroidally, or intraocularly.

[0043] Pharmaceutical compositions that are particularly useful for administration directly to the eye include aqueous solutions and/or suspensions formulated as eye drops and thickened solutions and/or suspensions formulated as ophthalmic gels (including gel-forming solutions) or ointments, which is an ophthalmic solution, ophthalmic ointment, ophthalmic wash, intraocular infusion solution, wash for anterior chamber, internal medicine, injection, or preservative for extracted cornea. Other dosage forms for ophthalmic drug deliver include ocular inserts, intravitreal injections and implants. Injectable solutions can be directly injected into the cornea, crystalline lens and vitreous or their adjacent tissues using a fine needle. The composition also can be administered as an intraocular perfusate.

[0044] Additional contemplated routes of administration include, but are not limited to, one or more of: oral (e.g., as a tablet, capsule, or as an ingestible solution), mucosal (e.g., as a nasal spray or aerosol for inhalation), nasal, parenteral (e.g., by an injectable form), gastrointestinal, intraspinal, intraperitoneal, intramuscular, intravenous, intrauterine, intradermal, intracranial,

intratracheal, intravaginal, intracerebroventricular, intracerebral, subcutaneous, transdermal, rectal, buccal, epidural and sublingual.

[0045] In some embodiments, the mode for delivery of a composition of the invention to the eye is via a contact lens. The lens may be provided pre-treated with the desired compound. Alternatively, the lens is provided in a kit with components for preparing a coated lens, which are provided as lyophilized powders for reconstitution or as concentrated or ready-to-use solutions. The compositions can be provided as kits for single or multi-use.

[0046] In some embodiments, the mode for delivery of a composition of the invention to the eye is via an ophthalmic rod (Gwon et al., Ophthalmology. 1986 September; 93(9 Suppl):82-5). In some embodiments, the mode for delivery of a composition of the invention to the eye is via an intraocular lens-hydrogel assembly (Garty et al., Invest Ophthalmol Vis Sci, 2011 Aug. 3; 52(9):6109-16).

Dose

[0047] The composition comprising the compound is provided in a therapeutically effective amount that achieves a desired biological effect at a medically-acceptable level of toxicity. The dosage of the compositions may vary depending on the route of administration and the severity of the disease. The dosage may also be adjusted depending on the body weight, age, sex, and/or degree of symptoms of each patient to be treated. The precise dose and route of administration will ultimately be at the discretion of the attendant physician or veterinarian. It will be appreciated that it may be necessary to make routine variations to the dosage depending on the age and weight of the patient as well as the severity of the condition to be treated. The frequency of administration depends on the formulation and the aforementioned parameters. For example, it may be desirable to apply eye drops at least once per day, including 2, 3, 4, or 5 times per day.

[0048] Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of the particular pharmaceutical composition and the method of administration. Acceptable dosages can generally be estimated based on EC50 (effective concentration for 50% of the test group) found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the

patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the therapeutic compositions described herein are administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years. Exemplary doses of the compounds for administration to a human (of approximately 70 kg body weight) via systemic route are 0.1 mg to 5 g, e.g., 1 mg to 2.5 g of the compound per unit dose.

[0049] Preferred concentrations of the compound of formula I range from about 1 μg/ml to 500 μg/ml, for example, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 10 μg/ml, about 20 μg/ml, about 30 μg/ml, about 40 μg/ml, about 50 μg/ml, about 60 μg/ml, about 70 μg/ml, about 80 μg/ml, about 90 μg/ml, about 100 μg/ml, about 120 μg/ml, about 140 μg/ml, about 160 μg/ml, about 180 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, or about 500 μg/ml. The inhibitor may be provided in combination with other pharmaceutically active agents.

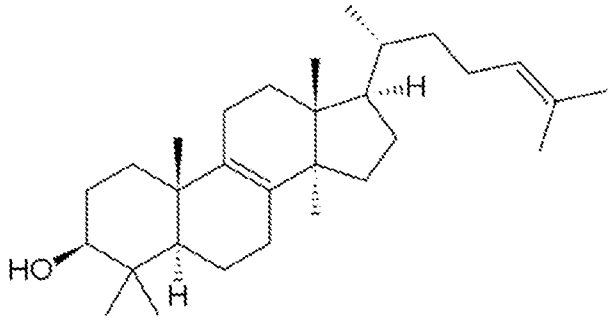
[0050] The pharmaceutical compositions described herein can be administered as a single dose or in multiple doses; administered either as individual therapeutic agents or in combination with other therapeutic agents; and combined with conventional therapies, which may be administered sequentially or simultaneously. In one embodiment of the invention, daily dosages in human and/or animal therapy of the present ophthalmic formulations are about 1 drop per eye, about 2 drops per eye, about 3 drops per eye, about 4 drops per eye, about 5 drops per eye, about 6 drops per eye, about 7 drops per eye, about 8 drops per eye, about 9 drops per eye, about 10 drops per eye, about 11 drops per eye, about 12 drops per eye or more than about 12 drops per eye. In another embodiment of the invention, daily administration schedule for the present ophthalmic formulations in human and/or animal therapy is about 1 time per day, about 2 times per day, about 3 times per day, about 4 times per day, about 5 times per day, about 6 times per day, about 7 times per day, about 8 times per day, about 9 times per day, about 10 times per day, about 11 times per day, about 12 times per day or more than about 12 times per day. Dosages can be standardized for instance by means of a standard pharmacopeial medicinal dropper of 3 mm in external diameter, which when held vertically delivers 20 drops of water of total weight of 0.9 to 1.1 grams at 25° C.

[0051] When administered according to the dosage schedule described above, the treatment regimen in humans and/or animals can continue indefinitely or until no further improvement is observed. Alternately, the treatment regimen can last for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27

days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days, 45 days, 46 days, 47 days, 48 days, 49 days, 50 days, 60 days, 70 days, 80 days, 90 days, 100 days, 150 days, 200 days, 250 days, 300 days, 400 days, 500 days, 750 days, 1000 days or more than 1000 days.

Compounds Effective in Treating or Preventing Cataract

[0052] In various embodiments, the compound of the inventive method or composition is lanosterol having a compound of formula I:



, or a prodrug or pharmaceutically acceptable salt thereof.

[0053] For example, the compound of the inventive method or composition is lanosterol; a prodrug or pharmaceutically acceptable salt thereof. In one embodiment, the compound is lanosterol. In another embodiment, any prodrug or pharmaceutically acceptable salt of the above compounds are contemplated to be within the scope of the invention.

Pharmaceutical Compositions

[0054] In some embodiments of the invention, pharmaceutical compositions of one or more therapeutic compounds can be prepared by formulating one or more of these therapeutic compounds in a pharmaceutically acceptable carrier. As used herein, "pharmaceutically or therapeutically acceptable carrier" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the host or patient. The type of carrier which is used in the pharmaceutical preparation will depend on the method by which the therapeutic compounds are to be administered. Many methods of preparing pharmaceutical compositions for various routes of administration are well known in the art.

[0055] As used herein, "pharmaceutically acceptable ophthalmic carrier" refers to a pharmaceutically acceptable excipient, carrier, binder, and/or diluent for delivery of the compound of the structural formula 1 directly or indirectly to, on or near the eye. Accordingly,

the invention further comprises a composition comprising the compound of the structural formula I and a pharmaceutically acceptable ophthalmic carrier.

[0056] Optionally, the composition includes a free acid, free base, salt (e.g., an acid or base addition salt), hydrate or prodrug of the compound of structural formula I. The phrase "pharmaceutically acceptable salt" or "pharmaceutically acceptable acid," as used herein, refers to pharmaceutically acceptable organic or inorganic salts or acids, respectively, of a compound of Formula I. The counter ion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt (or acid) may have more than one charged atom in its structure. Instances where multiple charged atoms are part of the pharmaceutically acceptable salt (or acid) can have multiple counter ions. Hence, a pharmaceutically acceptable salt (acid) can have one or more charged atoms and/or one or more counter ion.

[0057] Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (i.e., 1,1′-methylene-bis-(2-hydroxy-3-naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counter ion.

[0058] The prodrug is a material that includes the compound of structural formula I covalently bound to a carrier moiety. The carrier moiety can be released from the compound of structural formula 1, in vitro or in vivo to yield compound of structural formula I. Prodrug forms are well known in the art as exemplified in Sloan, K. B., Prodrugs, M. Dekker, New York, 1992; and Testa, B. and Mayer, J. M., Hydrolysis in drug and prodrug metabolism: chemistry, biochemistry, and enzymology, Wiley-VCH, Zurich, 2003.

[0059] In some embodiments of the invention, pharmaceutical compositions are prepared by dissolving the invention composition in an appropriate solvent. Appropriate solvents include, but are not limited to, water, saline solution (for example, NaCl), buffered solutions, ointments, gels or other solvents. In certain embodiments, the solvents are sterile.

[0060] Aqueous solutions and diluents for suspensions that are used in preparation of eye drops can include distilled water, physiological saline, and the like. These pharmaceutical compositions can be formulated by admixing, diluting or dissolving the compound, optionally, with appropriate pharmaceutical additives such as excipients, disintegrators, binders,

lubricants, diluents, buffers, antiseptics, moistening agents, emulsifiers, dispersing agents, stabilizing agents and dissolving aids in accordance with conventional methods and formulating in a conventional manner depending upon the dosage form. Non-aqueous solutions and diluents for suspensions can include edible (eg vegetable) oil, liquid paraffin, mineral oil, propylene glycol, p-octyldodecanol, polysorbate, macrogols, aluminum monostearate as well as similar solvents.

[0061] Various additives may be contained in eye drops, ophthalmic gels and/or ophthalmic ointments as needed. These can include additional ingredients, additives or carrier suitable for use in contact on or around the eye without undue toxicity, incompatibility, instability, irritation, allergic response, and the like. Additives such as solvents, bases, solution adjuvants, suspending agents, thickening agents, emulsifying agents, stabilizing agents, buffering agents, isotonicity adjusting agents, pH-adjusting agents, chelating agents, soothing agents, preservatives, corrigents, flavoring agents, coloring agents, excipients, binding agents, lubricants, surfactants, absorption-promoting agents, dispersing agents, preservatives, solubilizing agents, and the like, can be added to a formulation where appropriate.

[0062] For example, eye drops can be formulated by dissolving the compound in sterilized water in which a surface active agent is dissolved and optionally adding appropriate pharmaceutical additives such as a preservative, a stabilizing agent, a buffer, an antioxidant and a viscosity improver.

[0063] For example, buffering agents are added to keep the pH constant and can include pharmaceutically acceptable buffering agents such as borate buffer, citrate buffer, tartrate buffer, phosphate buffer, acetate buffer or a Tris-HCl buffer (comprising tris(hydroxymethyl) aminomethane and HCl). For example, a Tris-HCl buffer having pH of 7.4 comprises 3 g/l of tris-(hydroxymethyl)-aminomethane and 0.76 g/l of HCl. In yet another aspect, the buffer is 10× phosphate buffer saline ("PBS") or 5×PBS solution. Buffering agents are included in an amount that provides sufficient buffer capacity for the expected physiological conditions.

[0064] Other buffers include, but are not limited to, buffers based on HEPES (N-{2-hydroxyethyl}peperazine-N′-{2-ethanesulfonic acid}) having pK_a of 7.5 at 25° C. and pH in the range of about 6.8-8.2; BES (N,N-bis{2-hydroxyethyl}2-aminoethanesulfonic acid) having pK_a of 7.1 at 25° C. and pH in the range of about 6.4-7.8; MOPS (3-{N-morpholino}propanesulfonic acid) having pK_a of 7.2 at 25° C. and pH in the range of about 6.5-7.9; TES (N-tris{hydroxymethyl}-methyl-2-aminoethanesulfonic acid) having pK_a of 7.4 at 25° C. and pH in the range of about 6.8-8.2; MOBS (4-{N-morpholino}butanesulfonic acid) having pK_a of 7.6 at 25° C. and pH in the range of about 6.9-8.3; DIPSO (3-(N,N-bis{2-

hydroxyethyl}amino)-2-hydroxypropane)) having pK_a of 7.52 at 25° C. and pH in the range of about 7-8.2; TAPS ({(2-hydroxy-3{tris(hydroxymethyl)methylamino}-1-propanesulfonic acid)) having pK_a of 7.61 at 25° C. and pH in the range of about 7-8.2; TAPS ({(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino}-1-propanesulfonic acid)) having pK_a of 8.4 at 25° C. and pH in the range of about 7.7-9.1; TABS (N-tris(hydroxymethyl)methyl-4-aminobutanesulfonic acid) having pK_a of 8.9 at 25° C. and pH in the range of about 8.2-9.6; AMPSO (N-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid)) having pK_a of 9.0 at 25° C. and pH in the range of about 8.3-9.7; CHES (2-cyclohexylamino)ethanesulfonic acid) having pK_a of 9.5 at 25° C. and pH in the range of about 8.6-10.0; CAPSO (3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid) having pK_a of 9.6 at 25° C. and pH in the range of about 8.9-10.3; and CAPS (3-(cyclohexylamino)-1-propane sulfonic acid) having pK_a of 10.4 at 25° C. and pH in the range of about 9.7-11.1.

[0065] In addition to a buffer, isotonizers can be added to eye drops to make the preparation isotonic with the tear. Isotonizers include, but are not limited to, sugars such as dextrose, glucose, sucrose and fructose; sugar alcohols such as mannitol and sorbitol; polyhydric alcohols such as glycerol, polyethylene glycol and propylene glycol; and salts such as sodium chloride, sodium citrate, benzalkonium chloride, phedrine chloride, potassium chloride, procaine chloride, chloram phenicol, and sodium succinate. Isotonizers are added in an amount that makes the osmotic pressure of the eye drop equal to that of the tear.

[0066] Preservatives can be added to maintain the integrity of the eye drop and/or ophthalmic ointment. Examples of preservatives include, but are not limited to, sorbic acid, benzalkonium chloride, benzododecinium bromide, parabens, chlorobutanol, benzylic alcohol, phenylethyl alcohol, edentate disodium, sorbic acid, polyquaternium-1, or other agents known to those skilled in the art.

[0067] In some embodiments, thickeners are used to increase the viscosity of ophthalmic preparations such as eye drops, ophthalmic gels and/or ophthalmic ointments. Thickeners that can be used include, but are not limited to, glycerol, polyethylene glycol, carboxymethyl cellulose and carboxyvinyl polymers.

[0068] In addition to the above, in some embodiments, it is desirable to use additional agents which include, but are not limited to, stabilizers such as sodium sulfite, sodium carbonate, and propylene glycol; antioxidants such as ascorbic acid, sodium ascorbate, butylated hydroxy toluene (BHT), butylated hydroxyanisole (BHA), tocopherol, sodium thiosulfate; and/or chelating agents such as ethylene-diamine-tetra-acetic acid (EDTA), ethylene glycol-bis-(2-aminoethyl)-N,N,N,N-tetraacetic acid (EGTA) and sodium citrate.

[0069] Eye drops, ophthalmic gels and/or ophthalmic ointments can be prepared by aseptic manipulation or alternatively sterilization is performed at a suitable stage of preparation. For example, a sterile pharmaceutical composition can be prepared by mixing sterile ingredients aseptically. Alternatively, the sterile pharmaceutical composition can be prepared by first mixing the ingredients then sterilizing the final preparation. Sterilization methods can include, but are not limited to, heat sterilization, irradiation and filtration.

[0070] Ophthalmic ointments (eye ointments) can be aseptically prepared by mixing the active ingredient into a base that is used for preparation of eye ointments followed by formulation into pharmaceutical preparations with any method known in the art. Typical bases for eye ointments are exemplified by vaseline, jelene 50, plastibase and macrogol. In addition, surfactants may be added to increase hydrophilia.

[0071] A number of effective methods for controlled release of an active agent are available. See, for example, Wagh V. D., Inamdar B., Samanta M. K., Polymers used in ocular dosage form and drug delivery systems. Asian J Pharm 2, 2008, 12-17 and the literature references cited therein, the contents of which are incorporated herein by reference. The use of polymers (e.g., cellulose derivatives such as hydroxypropylmethylcellulose (HPMC) and hydroxypropylcellulose (HPC), poly (acrylic acid) (PAA), polyacrylates, cyclodextrins and natural gums, polyorthoesters (POEs) and mucoadhesive polymers); semisolids such as gels, films and other inserts; resins such as ion exchange resins; iontophoretic delivery; and colloidal particles such as microspheres and nanoparticles, are specifically contemplated.

[0072] The compounds of the invention may also be provided in combination with other therapeutic agents. In some embodiments, the compounds of the invention may be co-formulated with other active agents, including, but not limiting to, anti-infective agents, antibiotics, antiviral agents, anti-fungal, anti-protozoal agent, anti-inflammatory drugs, anti-allergic agents including anti-histamines, artificial tears vasoconstrictors, vasodilators, local anesthetics, analgesics, intraocular pressure-lowering agents, immunoregulators, anti-oxidants, vitamins and minerals, an enzyme inhibitor or alternatively, proteases and peptidases, a cytokine inhibitor, and the like.

[0073] In various embodiments, the compounds of the invention may also be provided in combination with an ocular therapeutic selected from the group consisting of Acular (ketorolac tromethamine ophthalmic solution) 0.5%, Acuvail (ketorolac tromethamine), AK-Con-A (naphazoline ophthalmic), Akten (lidocaine hydrochloride), Alamast, Alphagan (brimonidine), Alrex, Astepro (azelastine hydrochloride nasal spray), AzaSite (azithromycin), Bepreve (bepotastine besilate ophthalmic solution), Besivance (besifloxacin ophthalmic suspension),

Betaxon, BSS Sterile Irrigating Solution, Cosopt, Durezol (difluprednate), Eylea (aflibercept), Lotemax, Lucentis (ranibizumab), Lumigan (bimatoprost ophthalmic solution), Macugen (pegaptanib), Ocuflox (ofloxacin opthalmic solution) 0.3%, OcuHist, Ozurdex (dexamethasone), Quixin (levofloxacin), Rescula (unoprostone isopropyl ophthalmic solution) 0.15%, Restasis (cyclosporine ophthalmic emulsion), Salagen Tablets, Travatan (travoprost ophthalmic solution), Valcyte (valganciclovir HCl), Viroptic, Vistide (cidofovir), Visudyne (verteporfin for injection), Vitrasert Implant, Vitravene Injection, ZADITOR, Zioptan (tafluprost ophthalmic solution), Zirgan (ganciclovir ophthalmic gel), Zymaxid (gatifloxacin ophthalmic solution), Atropine, Flurbiprofen, Physostimine, Azopt, Gentamicin, Pilocarpine, Bacitracin, Goniosol, Polymyxin B, Betadine, Gramicidin, Prednisolone, Betaxolol, Humorsol, Proparacaine, Betoptic, Hylartin, Propine, Brinzolamide, Hypertonic NaCl, Puralube, BSS, Indocycanine Green, Rose Bengal, Carbachol, Itraconazole, Sodium Hyaluronate, Cefazolin, Latanoprost, Suprofen, Celluvisc, Mannitol, Terramycin, Chloramphenicol, Methazolamide, Timolol, Ciloxan, Miconazole, Tobramycin, Ciprofloxacin, Miostat, Triamcinolone, Cosopt, Muro 128, Trifluridine, Demecarium, Neomycin, Tropicamide, Dexamethasone, Neptazane, Trusopt, Dipivefrin, Ocuflox, Vidarabine, Dorzolamide, Ofloxacin, Vira-A, Epinephrine, Oxytetracycline, Viroptic, Fluorescein, Phenylephrine, and Xalatan.

Kits

[0074] Some embodiments of the invention relate to kits for preventing and/or ameliorating one or more symptoms associated with an eye disease. The kits can comprise one or more containers that contain one or more of the therapeutic compounds described herein. The compounds can be present in the container as a prepared pharmaceutical composition, or alternatively, the compounds can be unformulated. In such embodiments, the kit can include the unformulated compounds in a container that is separate from the pharmaceutically acceptable carrier. Prior to use, the compound in diluted or otherwise mixed with the pharmaceutically acceptable carrier.

[0075] Some embodiments of the kits provided herein also comprise instructions which describe the method for administering the pharmaceutical composition in such a way that one or more symptoms associated with an eye disease which includes, but is not limited to, retinal degeneration, presbyopia, cataracts and/or nuclear sclerosis of the eye lens. In some embodiments, the instructions also describe the procedure for mixing the therapeutic compounds contained in the kit with ophthalmic pharmaceutically acceptable carriers.

[0076] In some embodiments of the invention, the container that comprises the therapeutic compounds described herein is a container which is used for ophthalmic administration. In certain embodiments, the container is a dropper for administering eye drops. In other embodiments, the container is a tube for administering an ophthalmic gel or an ophthalmic ointment.

[0077] Some embodiments of this invention are further illustrated by the following examples that should not be construed as limiting. It will be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the embodiments of the invention described herein, and thus can be considered to constitute preferred modes for the practice of these embodiments. Those of skill in the art will, however, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed herein and still obtain a like or similar result without departing from the spirit and scope of the invention.

Devices

[0078] Some embodiments of the invention relate to devices for administering the invention sterol to a subject. In some embodiments, the devices include an interior portion, cavity or reservoir that contains the invention sterol formulated in a pharmaceutically acceptable carrier. In such embodiments, the pharmaceutically carriers include, but are not limited to, solutions, gels, and ointments. In certain embodiments, the interior portion, cavity or reservoir contains one or more of the invention sterol-containing pharmaceutical preparations described herein.

[0079] In some embodiments, the devices contemplated herein also comprise an applicator that is coupled to the interior portion, cavity or reservoir of the device. The applicator can be cylindrical, conical or any other shape that permits the invention sterol-containing pharmaceutical preparation to be delivered from the interior portion, cavity or reservoir to the eye. In a preferred embodiment, the applicator is a tapered cylinder wherein the wide end is coupled to the interior portion, cavity or reservoir and the tapered end forms the exit opening for passage of the invention sterol-containing pharmaceutical preparation to the eye.

[0080] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[0081] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

[0082] The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those mat might he used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLE 1

[0083] The human lens is comprised largely of crystallin proteins assembled into a highly ordered, interactive macro-structure essential for lens transparency and refractive index. Any disruption of intra- or inter-protein interactions will alter this delicate structure, exposing hydrophobic surfaces, with consequent protein aggregation and cataract formation. Cataracts are the most common cause of blindness worldwide, affecting tens of millions of people1, and currently the only treatment is surgical removal of cataractous lenses. The precise mechanisms by which lens proteins both prevent aggregation and maintain lens transparency are largely unknown. Lanosterol is an amphipathic molecule enriched in the lens. It is synthesized by lanosterol synthase (LSS) in a key cyclization reaction of a cholesterol synthesis pathway. Here we identify two distinct homozygous LSS missense mutations (W581R and G588S) in two families with extensive congenital cataracts. Both of these mutations affect highly conserved amino acid residues and impair key catalytic functions of LSS. Engineered expression of wild-type, but not mutant, LSS prevents intracellular protein aggregation of various cataract-causing mutant crystallins. Treatment by lanosterol, but not cholesterol, significantly decreased preformed protein aggregates both in vitro and in cell-transfection experiments. We further show that lanosterol treatment could reduce cataract severity and increase transparency in dissected rabbit cataractous lenses in vitro and cataract severity in vivo in dogs. Our study identifies lanosterol as a key molecule in the prevention of lens protein aggregation and points to a novel strategy for cataract prevention and treatment.

[0084] Cataracts account for over half of all cases of blindness worldwide, with the only established treatment involving surgical removal of the opacified lens. In developed nations, cataract surgeries amount to a significant portion of healthcare costs owing to the sheer

prevalence of the disease among ageing populations. In addition, there is major morbidity associated with cataracts in developing countries, where there is limited access to surgical care.

[0085] High concentrations of crystallin proteins in lens fibres contribute to lens transparency and refractive properties[2]. The crystallin superfamily is composed of a-, b- and c-crystallins, which are some of the most highly concentrated intracellular proteins in the human body. Protein aggregation is the single most important factor in cataract formation[3]. Factors that lead to protein aggregation include mutations in crystallin proteins, which are known to cause congenital cataracts, or oxidative stress, which in turn contributes to age-related cataracts. However, the precise mechanisms by which lens proteins maintain transparency or cause opacification are not completely understood.

[0086] Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase, LSS; EC 5.4.99.7) is encoded by the LSS gene. The LSS protein catalyses the conversion of (S)-2,3-oxidosqualene to lanosterol, which is a key early rate-limiting step in the biosynthesis of cholesterol, steroid hormones, and vitamin D (ref. 4). LSS was found to be expressed in the lens[5]. It was previously reported that the specific combination of hypomorphic mutations on LSS and FDFT1 (farnesyl diphosphate farnesyl transferase 1) could decrease cholesterol levels in the lens and result in cataracts in Shumiya cataract rats (SCR)[6]. Here we identify novel homozygous mutations in the LSS gene in two consanguineous families and investigate the ability of lanosterol to alleviate protein aggregation and cataract formation.

[0087] Three children with severe congenital cataract from a consanguineous family of Caucasian descent (Fig. 1a) were identified. Whole-exome sequencing was performed to an average of no less than 55-fold depth coverage on the target region (Table 1a) in order to identify the causal mutation. On average, 60,800–80,800 SNPs were detected in each exome (Table 1b). Using a consanguineous recessive model and filtering against common variants (minor allele frequency .0.5%) in public databases including dbSNP and the 1000 Genomes Project, as well as mutation function predictions (predicted by SIFT[7], Polyphen2[8], Phylop[9] and Mutationtaster[10]), we narrowed down potential candidate gene variants and identified a variant (G588S) in LSS on chromosome 21 as the most likely candidate (Table 1c). Three affected children were homozygous for the GRA transition (G588S) in LSS, (GRch37/hg19: chr21:47615645; NM_001001438.2:c.1762G.A, NM_001001438.1: p.G588S), while the unaffected father, mother and remaining child were heterozygous for the change (Fig. 1a, b). Whole-genome SNP genotyping identified three long continuous homozygous regions in this family by HomozygosityMapper[11] (chr2:q22.1–q24.1, chr2:q31.1–q32.1 and chr21:q22.3; - Fig. 6a and Table 1d). The LSS gene was located in one of the homozygous regions on

chromosome 21 (Fig. 6B). Furthermore, we screened for mutations in the LSS gene in 154 families with congenital cataracts and identified another homozygous mutation, W581R (GRch37/hg19: chr21:47615666; NM_001001438.2:c.1741T.C, NM_001001438.1: p.W581R), in a second consanguineous family (Fig. 1A, B, C). These two mutations were absent in 11,000 control chromosomes.

[0088] The amino acid residues W581 and G588 in LSS are highly conserved (Fig. 2A). We performed computational modelling analysis to investigate the effects of the W581R and G588S mutations on the 3D structure and function of LSS. The amino acid tryptophan at position 581 has been reported to contribute to the catalytic site of the cyclase activity[12]. The G588S mutant was modelled by in-place replacement followed by side-chain refinement. The S588 side-chain refinement could not resolve the van der Waals clash between the serine side chain and the backbone carbonyl of E578, which forms a key salt bridge with R639. The orientation of the E579:C584 loop needed to be distorted to accommodate the mutation. The side chain of the mutant S588 clashed into an adjacent loop, indicating that the mutation was incompatible with the normal enzymatic structure and function of LSS (Fig. 2B). Supporting the in silico results, expression of wild-type LSS in a cell transfection experiment exhibited cyclase activity and dramatically increased the amount of lanosterol production in the lipid fraction in HeLa cells, while neither the W581R nor the G588S mutant protein demonstrated any cyclase activity (Fig. 2C). In contrast, the cholesterol level was unaffected by the expression of wild-type or mutant LSS, suggesting that there may be an alternative pathway for cholesterol homeostasis. The W581R and G588S mutations did not alter subcellular localization or cause aggregates of LSS protein when compared to that of wild-type LSS, suggesting that the cataract phenotype was not due to the formation of light-scattering particles by mutant LSS proteins themselves (Fig. 7). The aggregation of crystallins, the major structural proteins in the lens, is a predominant cause of various types of cataracts[3]. To mimic protein aggregation in the cataractous lens, six known cataract-causing mutant crystallin proteins were expressed in human lens progenitor cells, human lens epithelial line B-3 (HLEB-3), or HeLa cells. These mutant crystallins formed p62-positive inclusion bodies/aggresomes in all three transfected cell lines, suggesting that aggregation is an intrinsic property of mutant crystallins (Fig. 3A and Figs 8 and 9)[13]. Co-expression of wild-type LSS and a cataract-causing mutant crystallin protein significantly reduced both the number and size of intracellular crystallin aggregates, whereas LSS mutants failed to do so alone (Fig. 3B, C and Figs 8 and 9). Western blot analysis indicated that the Y118Dmutant of aA-crystallin was released from intracellular aggregates and became more soluble with wild-type LSS (Fig. 3D and Fig. 9C). Furthermore,

addition of lanosterol, but not cholesterol, in the culture medium of cells co-expressing an LSS mutant and a mutant crystallin successfully reduced crystallin aggregation (Fig. 3C and Figs 8 and 9). This result indicated that lanosterol, but not cholesterol, could be an effective agent to release mutant crystallin proteins from aggregation.

[0089] Supporting this hypothesis, lanosterol significantly inhibited aggresome formation of both wild-type and mutated crystallin proteins in a concentration-dependent manner, while cholesterol had no effect (Fig. 3E, F and Fig. 10). Lanosterol, but not cholesterol, increased the amounts of mutant crystallins in the soluble fractions of cell lysates (Fig. 3G and Fig. 11A). Using serial live-cell imaging of cells expressing a GFP-fused Y118D mutant of aA-crystallin, we showed that addition of lanosterol could effectively diminish crystallin aggregates with a half-life of 22268 minutes (Fig. 3H), whereas addition of DMSO or cholesterol did not reduce aggresome formation (Fig. 11B). Single-particle tracking in live cells clearly showed that lanosterol has an important role in the dissociation of pre-formed intracellular protein aggregates.

[0090] To investigate whether lanosterol has a direct effect on dissolution of the aggregated proteins, the aggregates of five wild-type and nine mutant crystallins were obtained by heating wild-type and mutated crystallins in the presence of 1M guanidine chloride. Under this condition, all crystallin proteins formed amyloid-like fibrils as revealed by the enhancement of thioflavin T (ThT) fluorescence, the fibrillary structures under negatively stained transmission electron microscopy (TEM), and the low turbidity value (Fig. 4 and Fig. 11C). The morphology of the amyloid-like fibrils obtained here was similar to those crystallin proteins reported previously[14]. PBS containing liposomes formed by dipalmitoyl phosphatidylcholine (DPPC) was used to increase the solubility of sterol compounds and mimic the condition of sterols in cell membranes. Lanosterol, but not cholesterol, successfully re-dissolved the aggregated crystallin proteins from the amyloid-like fibrils in a concentration-dependent manner as indicated by the disappearance of fibrillar structures in the negatively stained TEM photographs and the decrease in ThT fluorescence intensity (Fig. 4 and Fig. 11D). As an example, the re-dissolved αA-crystallins could be identified in negatively stained TEM pictures and were around 15 nm in size (Fig. 4A)[15].

[0091] To assess the effect of lanosterol on cataract reduction in lens tissues, naturally occurring cataractous lenses from rabbits were isolated and incubated these in a 25mM lanosterol solution for 6 days and compared lens clarity before and after treatment of lanosterol. A strong trend of reduction in cataract severity, as demonstrated by an increase in lens clarity ($P<0.003$, Wilcoxon Test, Fig. 5A, B, Table 2A and Fig. 12A, B) was observed. We further

investigated the effect of lanosterol in reversing cataracts in dogs in vivo. Lanosterol treatment significantly reduced cataract severity and increased lens clarity ($P<0.009$, Wilcoxon Test, Fig. 5C, D; Table 2B and Fig. 12C).

[0092] Homozygous mutations affecting the catalytic function of LSS cause extensive congenital cataracts with severe vision loss. The critical role of lanosterol in cataract prevention is supported by the observation that a rat strain harbouring compound LSS mutations recapitulates the human cataract disease phenotype[6]. Consistent with this notion, inhibition of LSS by U18666A, an LSS inhibitor (also known as an oxidosqualene cyclase inhibitor), was found to cause cataracts[16]. Furthermore, lanosterol treatment markedly decreased protein aggregation caused by mutant crystallin proteins in cell culture, while reducing preformed cataract severity increasing lens clarity in animal models. It is conceivable that the amphipathic nature of lanosterol allows it to intercalate into and coat hydrophobic core areas of large protein aggregates, effectively allowing these aggregations to gradually become water soluble again.

[0093] In summary, lanosterol plays a key role in inhibiting lens protein aggregation and reducing cataract formation, suggesting a novel strategy for the prevention and treatment of cataracts. Cataracts are the leading cause of blindness and millions of patients every year undergo cataract surgery to remove the opacified lenses. The surgery, although very successful, is nonetheless associated with complications and morbidities. Therefore, pharmacological treatment to reverse cataracts could have large health and economic impacts. In addition, our results may have broader implications for the treatment of protein aggregation diseases, including neurodegenerative diseases and diabetes, which collectively are a significant cause of morbidity and mortality in the elderly population, by encouraging the investigation of mall-molecule approaches, such as the one demonstrated here.

Methods

[0094] Study participants. All participants had standard complete ophthalmic examinations and imaging studies. Demographic data, risk factors, and a blood sample were collected at the initial visit. We recruited a consanguineous family consisting of two adults and four children. The parents were first cousins, and three of their four children were diagnosed with retinal degeneration and cataract (Fig. 1A). We screened for LSS mutations in an additional 154 congenital cataract pedigrees and identified another family with a homozygous W581R mutation.

[0095] Exome capture and sequencing. Exome capture was carried out using Agilent SureSelect Human All Exon Kit (in solution) according to the manufacturer's protocols.

Briefly, genomic DNA samples were randomly fragmented by Covaris with a base-pair peak of 150–200 bp for the resulting fragments, and adapters were ligated to both ends of the fragments. The adapter-ligated templates were purified using Agencourt AMPure SPRI beads, and fragments with insert size ~250 bp were excised. Extracted DNA was amplified by ligation-mediated PCR, purified, and hybridized to the SureSelect Biotinylated RNA Library (BAITS) for enrichment. Hybridized fragments bound to the strepavidin beads, whereas non-hybridized fragments were washed out after 24 h. Captured ligation-mediated PCR products were subjected to the Agilent 2100 Bioanalyzer to estimate the magnitude of enrichment. Each captured library was then loaded onto the Illumina Genome Analyzer II platform, and paired-end sequencing was performed with read lengths of 90 bp, which provided at least 50× average coverage depth for each sample. Raw image files were processed by Illumina base-calling software with default parameters.

[0096] Read mapping and variant detection. Sequence reads in each individual were aligned to the human reference genome (NCBI build 37, hg19) using BWA[17] (version 0.5.9–r16). BAM files created by BWA were then processed using the GATK[18] best practice pipeline using Genome Analysis ToolKit (version GATK 2.8) for re-alignment and variation (SNV and indel) detection. Variations that passed VQSR filtering criteria were extracted for the subsequent analyses.

[0097] The consensus genotypes in the target regions were called by SOAPsnp (v1.03) and BWA (version 0.5.9–r16) with the recommended parameters. A consensus genotype with Phred-like quality of at least 20 and at least 4× coverage depth was considered to be a high-confidence genotype. The genotypes that were different from the reference were extracted as candidate SNPs, and the SNP results were filtered as follows: Phred-like SNP quality ≥20, overall depth of 4× to 200×, copy number estimate <2, and distance between two adjacent SNPs no less than 5 bp.

[0098] Functional annotation of genetic variants. Variants were functionally annotated using ANNOVAR and categorized into missense, nonsense, read-through, and splice-site mutations, which are likely to be deleterious compared with synonymous and noncoding mutations. Based on these annotations, variants were filtered first for the nonsynonymous, splice acceptor-site and donor-site and then filtered against available public databases (dbSNP129 and 1000 Genome variants databases). The variants that were found to be homozygous mutations in the three affected subjects and heterozygous mutations in the carriers (parents), but were absent in the public databases, were considered to be candidate causal variants.

[0099] Mutation screening of LSS and gene. Sanger DNA sequencing was performed to validate the G588S mutation in LSS. The 22 exons of the LSS gene were amplified by PCR and sequenced on the Genetic Analyzer 3130 (Applied Biosystems). The primers used to amplify the exons in LSS are presented in Table 3A. We screened for mutations in the LSS gene in 154 families with congenital cataracts and identified another homozygous mutation, W581R, in a second consanguineous family. These two mutations were absent in 11,000 control chromosomes, including 2,000 chromosomes from an unaffected control population in San Diego and the 1000 Genomes Project, and 8,000 chromosomes from an exome sequencing database at the University of Washington. Due to a previous report that a FDFT1 mutation modifies cataract phenotypes, we screened variants in the FDFT1 gene, identifying only one common non- synonymous variant rs4731 (GRch37/hg19: chr8:11666337; NM_001287742.1: c.134A . G, NM_001274671.1:p.K45R). The variant was excluded as the causal mutation since an unaffected daughter harboured the same homozygous change, and a relatively high frequency of general population possess this variant (minor allele frequency .4% in 1000 Genome Project data) (Table 1E).

[0100] 3D modelling of the G588S mutation. The model of the G588S mutant was built from two structures as determined by Ruf et al.[20] and deposited in the Protein Data Bank as entries 1W6K and 1W6J[12]. The X-ray coordinates were used to build a full- atom model of the enzyme, and it was refined using the Internal Coordinate Mechanics program (ICM) and its PDB conversion protocol.[21] To analyse the effect of the G588S-mutation-induced clash on lanosterol binding, we analysed all side chains involved in the pocket of the enzyme interacting with lanosterol using the 1W6K structure. The areas of contact were calculated as the differences between the solvent-accessible area of each residue with and without lanosterol and were sorted by size using the ICM program.[22]

[0101] Plasmid constructs and site-directed mutagenesis. The clone containing LSS cDNA was purchased from Thermo Scientific Inc. The coding sequence of wild- type LSS was cloned and inserted into the pcDNA3.1-N-Flag plasmid (Invitrogen). The mutants were constructed via site-directed mutagenesis by overlap extension using PCR. The common PCR primers were: NdeI forward, 59-CATATGACGG AGGGCACGTGTCT-39 and XhoI reverse, 59-CTCGAGTCAGGGGTGGCCA GCAAG-39. The primers for constructing the W581R and G588S mutants were: W581R forward, 59-TGGGAAGGCTCCCGGGGAGTTTGCT-39; reverse, 59-GTGAAGCAAACTCCCCGGGAGCCTTC-39; G588S forward, 59-GCTTCACCTACAGCACCTGGTTTG-39; G588S reverse, 59-CCAAACC

AGGTGCTGTAGGTGAAG-39. The recombinant pcDNA3.1-N-Flag plasmids containing the wild-type or mutated LSS genes were transformed into E. coli DH5a cells. The cDNA of aA-, aB-, bB2-, cC- and cD-crystallin were cloned from the total cDNA of human lens as described previously.[23-26] The mutants were constructed by site-directed mutagenesis using the primers listed in Table 3B. The amplified fragments were digested by XhoI and BamHI, and then inserted into the eukaryotic expression vector peGFP-N1 or the prokaryotic expression vector pET28a. The plasmids were obtained using the Plasmid Maxiprep kit (Vigorous) and verified by DNA sequencing. Crystallin gene constructs were made as a C-terminus eGFP fusion protein, while LSS was made as an N-terminal Flag-tagged protein.

[0102] Cell culture and transfection. HeLa cells and human lens epithelial B-3 cells (HLEB-3) were obtained from ATCC. Human lens progenitor cells were isolated from a fetal human eye.[27] The HeLa cells were cultured in DMEM medium contain- ing 10% FBS (Gibco). The HLEB-3 cells were cultured in F12 medium with 20% FBS, while human lens progenitor cells were cultured in MEM medium containing 20% FBS and 10 mg ml^{-1} FGF (Gibco). All cells were cultured at 37 uC in 5% CO_2 incubator. Cells routinely tested negative for mycoplasma contamination.

[0103] To assess the effect of LSS expression on sterol content, HeLa cells were trans- fected with wild-type LSS or LSS mutants fused with a Flag tag at the N-terminus of the coding region. The cells were harvested after 24 h transfection and the lipid fraction was extracted for LC–MS analysis. Cells transfected with the vector pcDNA3.1-N-Flag plasmids were used as a control. The expression levels of the wild-type and mutant LSS were normalized by western blot analysis using mouse anti-Flag (F1804; Sigma-Aldrich) and mouse anti-actin antibodies (BS6007M; Bioworld Technology).

[0104] To assess the effect of lanosterol on crystallin aggregation, human lens progenitor cells were co-transfected with LSS and various crystallin constructs for 4 h. Cells co-transfected with crystallin mutants and pcDNA3.1-N-Flag were used as a control. Human lens progenitor cells co-transfected with LSS and crystallin mutant constructs were cultured for 12 h before assaying for aggregates. The rescue experiments were performed after 16 h by addition of 40 mM sterols (lanosterol or cholesterol, Sigma-Aldrich) to the cell culture medium for 2 h, which was then replaced with fresh culture medium and cells cultured for 24 h. The percentage of cells with crystallin aggregates was calculated from ten randomly selected viewing fields. The values of the wild-type LSS group, mutant group, and mutant plus lanosterol group were calculated. Cells treated with 1% DMSO were used as the controls.

[0105] The impact of LSS and lanosterol on intracellular crystallin aggregation were evaluated in single-blinded observer studies. Experiments have been repeated at least three times. P values were calculated using Student's t-tests. Fluorescence microscopy. Equal amounts of the human lens progenitor cells, HLEB-3 cells or HeLa cells were seeded on glass coverslips pretreated with TC (Solarbio). After culturing for 24 h to reach 90% confluency, the cells were transfected with plasmids containing various LSS or crystallin genes or co-transfected with plasmids containing a certain crystallin gene and those containing the wild- type or mutated LSS gene. The controls were cells transfected with the plasmids containing peGFP-N1 and/or peDNA3.1-N-Flag. Both transfection and co-transfection were performed using Lipofectamine 3000 (Invitrogen) according to the instructions from the manufacturer.

[0106] The effect of wild-type or mutated LSS on the intracellular aggregation of various cataract-causing crystallin mutants was evaluated by co-expression of Flag–LSS and crystalline–GFP in the human lens progenitor cells, HLEB-3 cells or HeLa cells. The intracellular distributions of the proteins were visualized using GFP or antibody against Flag. After co-transfection for 4 h, the cells were cultured in fresh media for 24 h, and then analysed by microscopy.

[0107] The effect of lanosterol or cholesterol on the aggresome formation of various crystallins was studied by transfecting the cells with plasmids containing various crystallin genes. The cells were incubated for 24h to enable efficient protein expression and aggresome formation. The cells were then treated with 0–40 mM sterols in 1% (for human lens progenitor cells) or 2% DMSO (for HeLa cells). Cells treated with 1% or 2% DMSO were used as the control. After treatment for 2 h, the media was replaced with fresh media. After 12 h, the cells were used for microscopy analysis.

[0108] The microscopy samples were prepared by washing the slips with phosphate buffered saline (PBS) three times. The cells were fixed with 4% paraformaldehyde for 40 min followed by another three washes with PBS. The cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min and blocked with 5% normal goat serum in PBS for 1 h at 37 ^{o}C. Immunostaining was carried out by adding mouse anti-Flag antibody (1:500) or mouse anti-p62 antibody (1:200, ab56416; Abcam) in PBS buffer containing 5% normal goat serum and incubated for 1 h at 37 ^{o}C. Then the slips were washed three times with PBS, and further incubated with Alexa 649-conjugated goat anti-mouse IgG (1:250) for 1h at ambient temperature. The nuclei were counterstained with Hoechst 33342 (Invitrogen). The mounted cells were analysed using a Carl Zeiss LSM 710 con- focal microscope.

[0109] Live-cell imaging. Human lens progenitor cells were transfected with plasmids containing αA-crystallin (Y118D) mutant. After a 24 h transfection period, the cells with stable expression of αA-crystallin(Y118D) mutant were screen by incubation in culture medium containing 0.8 mg ml^{-1} G418 for 7 days. Then the obtained cells were seeded onto glass bottom cell culture dishes (In Vitro Scientific) and treated with 1% DMSO, 40mM cholesterol in 1% DMSO or 40 mM lanosterol in 1% DMSO for 4 h. Fresh culture medium was added, and the cells were analysed by serial live-cell imaging. Live-cell images were viewed with an Olympus IX81 microscope and captured with CellSens Dimension soft- ware (Olympus). Quantitative analysis of the size of aggregates was performed by measuring the fluorescence intensity of p62-positive aggregates using single-par- ticle tracking in live-cell imaging. The live-cell imaging was conducted using three biological replicates with 1–8 repetitions each.

[0110] Lipid extraction of the cells. Extraction of lipids was performed using the Bligh and Dyer method.[28] In brief, ~1 x 10^6–10^7 HeLa cells were washed 3–5 times with PBS and then scraped in 400-ml ice-cold methanol and transferred to a 1.5 ml Eppendorf tube with the addition of 200 ml chloroform. The samples were vortex- agitated for 1 min and then mixed with 300 ml of 1 M KCl. The organic and aqueous phases were separated by microcentrifugation at 20,817 x g for 5 min at 4 °C. After separation, the lower organic phase was collected. Then the residual aqueous phase was re-extracted twice using 300ml chloroform. The collected organic phases were dried using a SpeedVac sample concentrator under vacuum. The dried samples were stored at 280 °C for further LC–MS analysis.

[0111] LC–MS analysis. The dried lipid extracts were re-suspended in 100 ml methanol. The samples were vortex-agitated for 10 min, treated by 80 W ultrasonic sonica- tion for 30 min, microcentrifuged at 20,817g for 10 min, and then the supernatant was transferred to a new Eppendorf tube. The microcentrifugation treatment was repeated three times. The derived samples were analyzed by an Agilent 1290/6460 triple quadrupole LC/MS using an alternative Atmospheric Pressure Chemical Ionisation (APCI) source. The lipids were separated using an Agilent SB-C18 column. Selective ion monitoring was performed using the electron ionization mode. The highly pure lanosterol and cholesterol were used as controls. The MS determination was performed using a gas temperature of 350 °C, a gas flow rate of 4 l min^{-1}, a nebulizer of 60 p.s.i., a vaporizer of 350 °C, a capillary of 3,500 V and a corona current of 4 mA. To optimize the sensitivity and specificity, two qualifier ions were selected for the MS analysis of each compound (369.3/161.1 and 369.3/147 for cholesterol, and 409.2/191.3 and 409.2/109 for lanosterol). Western blotting. The cell lysates were prepared in RIPA buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5% sodium deoxycholate and

0.1% SDS. The supernatant and precipitation fractions were separated by centrifugation. The proteins were separated by a 12.5% SDS– PAGE and transferred to a PVDF membrane (GE Healthcare). The mouse anti- bodies against Flag (F1804; Sigma-Aldrich) or GFP (MB2005; Bioworld Technology) were used to identify the overexpressed LSS and crystallin proteins, respectively. Quantification of the western blot bands was achieved using the software GELPRO (Media Cybernetics). The presented quantitative data were calculated from three independent experiments.

[0112] Protein expression and purification. The recombinant His-tagged wild-type and mutated b- and c-crystallin proteins were overexpressed in Escherichia coli and purified using an Ni-NTA affinity column followed by gel filtration chromatography using the same protocol as described elsewhere[23,24,26,29]. The over- expression and purification of the non-tagged αA- and αB-crystallins were per- formed as described previously[30]. The purity of the proteins was estimated to be above 95% as evaluated by one homogeneous band on 12.5% SDS–PAGE, 10% native-PAGE and a single peak in the size-exclusion chromatography profile. The protein concentration was determined according to the Bradford method by using BSA as the standard[31]. All protein samples were prepared in 20 mM PBS buffer containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT.

[0113] Protein aggregation and aggregate dissociation. The aggregates of the wild-type and mutated αA- and αB-crystallin proteins were obtained by heating the protein solutions containing 1 M guanidine chloride (ultrapure, Sigma-Aldrich) at a con- centration of 5 mg/ml at 60°C for 2 h. The aggregates of the wild-type and mutated b- and c-crystallins were prepared by heating the protein solutions containing 1 M guanidine chloride at 37°C for 48 h. The formation of aggregates was confirmed by ThT fluorescence, turbidity (absorbance at 400 nm) and transmission electron microscopy (TEM) observations. The preformed aggregates were re-suspended in 20 mM PBS with a final concentration of 0.2 mg/ml (approxmately 10 mM). The re-suspended aggregates were treated with 500 mM lanosterol or cholesterol in liposomes formed by 500 mM DPPC (Sigma-Aldrich) at 37 °C. Aggregates treated by 500 mM DPPC liposome were used as a negative control. After 24 h of treatment, the protein solutions were used for ThT fluorescence, turbidity and negatively stained TEM observations. The TEM samples were prepared by depositing the protein solutions onto a freshly glow-discharged carbon-coated copper grid. Negative-staining samples were obtained by staining the grid with 1.25% uranyl acetate for 30 s. The negatively stained TEM pictures were obtained on a Hitachi

H-7650B transmission electron microscope with a voltage of 120 kV and a magnification of 48,000.

[0114] Treatment of cataractous rabbit lenses. Rabbits were euthanized by CO_2 inhalation and lenses were immediately dissected and treated with vehicle or lanosterol dissolved in vehicle to make 25 mM solutions. Lens tissues were incubated in these solutions for 6 days in the dark at room temperature. Cataracts were examined under a microscope and photographed. Degree of cataract was assessed by a blinded examiner using a previously described opacification grading system, shown below[32,33]. Improvements in lens clarity and transparency were quantified by visual inspection and grading. Lens clarity was scored by transmission of light, clarity of a grid image underneath the lens (Fig. 12), and improvement in overall clarity of a lens or improvement in clarity of localized areas of cortical cataract. Wilcoxon test was used to evaluate the treatment effect.

[0115] Cataract grading system. Grade 0: absence of opacification (gridlines clearly visible); N Grade 1: a slight degree of opacification (minimal clouding of gridlines, with gridlines still visible); N Grade 2: presence of diffuse opacification involving almost the entire lens (moderate clouding of gridlines, with main gridlines visible); N Grade 3: presence of extensive, thick opacification involving the entire lens (total clouding of gridlines, with gridlines not seen at all)

[0116] Preparation of drug-loaded nanoparticles. Lanosterol was loaded into a lipid- polymer hybrid nanoparticle through an adapted nanoprecipitation method[34]. In brief, the desired concentration of lanosterol was mixed with polycaprolactone (PCL) polymer dissolved in acetonitrile. Lecithin and 1,2-distearoyl-sn-glycero-3- phosphoethanolamine-N-carboxy(polyethylene glycol) 2000 (DSPE-PEG-COOH) were dissolved in a 4% ethanol aqueous solution at 20% of the PCL polymer weight and heated above 60uC. The lanosterol/PCL solution was then added into the preheated lipid solution under gentle stirring followed by rigorous vortexing for 3 min. The mixture solution was then stirred for 2 h to allow the nanoparticles to form and the acetonitrile to evaporate. Next, the nanoparticle solution was washed three times using an Amicon Ultra-4 centrifugal filter (Millipore) with a molecular weight cut-off of 10 kDa to remove the remaining organic solvent and free molecules. The resulting nanoparticles were then re-suspended in PBS buffer for sub- sequent use. The size, size distribution, and surface zeta potential of the drug-loaded nanoparticles were characterized by dynamic light scattering. The loading yield of lanosterol was quantified by high-performance liquid chromatography.

[0117] Treatment of cataractous lenses in dogs. To assess the effect of lanosterol treatment on cataracts in live animals, dogs were pre-medicated with intramuscular injections of acepromaxine and butorphanol. After 20 min, induction of anaesthesia was performed by application of intravenous propofol. Dogs were then immediately intubated and maintained on oxygen and 2% isoflurane at 2l min^{-1}. Lanosterol (100mg)-loaded nanoparticles were initially injected into the vitreous cavity in the test eye using a 28-gauge needle, and then were given every 3 days for the duration of the experiment. Treatment eyes or sham eyes were randomized. The control eye was given an injection with empty nanoparticle carriers as a negative control. The treatment eyes were treated with lanosterol in topical eye drops (see below for eye drop formulation). One 50-ml drop of lanosterol was administered three times daily to the test eye over 6 weeks. Degree of cataract severity was examined by slit lamp and photographed at the beginning and the end of the 6-week treatment period. Prior to examinations, pupils were dilated with 1% tropicamide and 10% phenylephrine. Degree of cataract severity was assessed by a blinded examiner and scored based on canine cataract stage, shown below[35]. Improvements in lens clarity and transparency were quantified. Wilcoxon test was used to evaluate the treatment effect.

[0118] Grading system of canine cataracts. Grade 0: absence of opacification (no cataract); N Grade 1: a slight degree of opacification (incipient stage); N Grade 2: presence of diffuse opacification involving almost the entire lens (immature stage); N Grade 3: presence of extensive, thick opacification involving the entire lens (mature stage) Topical vehicle solution. Double distilled H_2O was added to 1.1 g $(EDTA)_2Na$ combined with 0.055g alkyldimethylbenzylammonium chloride until a final volume of 1.1 1 (pH 5.66) was achieved. 25mM lanosterol in the topical vehicle solution. Double distilled H_2O was added to a mixture of 12.5 g lanosterol, 1.1 g $(EDTA)_2Na$, 0.055 g alkyldimethyl- benzylammonium chloride and 200 ml EtOH to a final volume of 1.1 l.

[0119] In one embodiment, a formulation of lanosterol eye drop solution is:

Recipe

A vehicle only solution:

Hydroxypropyl-β-Cyclodextrin	165g
Polysorbate 80	1g
EDTA2Na	1.1g
Alkyldimethylbenzylammonium chloride	0.055g
EtOH	200ml

Then add ddH2O till the final volume is 1.1L (PH 5.66)

5mM Lanosterol in a vehicle solution:

Lanosterol	2.5g
Hydroxypropyl-β-Cyclodextrin	165g
Polysorbate 80	1g
EDTA2Na	1.1g
Alkyldimethylbenzylammonium chloride	0.055g
EtOH	200ml

Then add ddH2O till the final volume is 1.1L (PH 5.66)

Table 1 Exome sequencing and variants

Table 1a. Summary of exome sequencing data production

Sample	Total effective yield(Mb)	Average sequencing depth	Mismatch rate	Coverage of target region	Fraction of target covered >= 4x	Fraction of target covered >= 10x
IV-1	3,409.20	60.16	0.20%	99.60%	99.10%	97.60%
IV-2	3,314.58	58.62	0.20%	99.60%	99.20%	97.80%
IV-3	3,327.63	57.24	0.20%	99.80%	99.20%	97.40%
III-2	3,029.40	51.89	0.21%	99.80%	99.30%	97.70%
III-1	6,877.08	54.24	0.29%	96.30%	89.40%	81.80%
IV-4	6,331.78	44.12	0.29%	96.50%	88.80%	79.80%

Table 1 b. Summary of detected variants

Sample	Total tion	Heterzygot s	Homozygot s	missense	nonsense	readthroug	synonymou	splicing	intergenic	intronic
IV-1	61,189	35,571	25,618	6,105	69	39	7,296	32	5,371	36,598
IV-2	60,829	34,698	26,131	6,074	62	41	7,211	38	5,178	36,572
IV-3	61,078	35,238	25,840	6,221	78	43	7,265	38	5,099	36,544
III-2	62,753	39,001	23,752	6,393	64	38	7,588	34	5,764	36,924
III-1	80,067	49,694	30,373	7,247	93	49	8,166	47	15,063	41,391
IV-4	80,893	48,211	32,682	7,252	85	50	8,184	50	14,547	42,414

Table 1c. Variant prioritization pipeline after exome sequencing

Filters	III-1 (carrier father)	III-2 (carrier mother)	IV-1 (affected daughter)	IV-2 (affected son)	IV-3 (affected son)	IV-4 (unaffected daughter)	Combine
Total variations	80,067	62,753	61,189	60,829	61,078	80,893	-
Missense, Nonsense, Splicing	7,389	6,495	6,213	6,177	6,342	7,387	-
Affected: 1/1; carrier: 0/1; unaffected: 0/1 or 0/0 *	5,792	4,661	3,127	3,123	3,085	5,638	9
Not in dbSNP	3,724	2,969	1,954	1,929	1,928	3,589	5
Not in 1000 Genomes Project	1,032	767	227	264	245	1,059	1
Predicted damaging	267	269	31	45	41	264	1

*Homozygous in affected child, heterozygous in carrier, no homozygous mutants in unaffected child

Table 1d. Summary of whole-gnome genotyping data

Sample	Total loci	Captured	SNP
IV-1	4,641,218	4,440,318	559,832
IV-2	4,641,218	4,446,992	605,499
IV-3	4,641,218	4,445,267	526,794
III-2	4,641,218	4,448,054	537,925
III-1	4,641,218	4,446,581	574,880
IV-4	4,641,218	4,450,657	584,347

Table 1e. Coding variants detected on gene FDFT1

Position (GRch37/hg19)	refSNP	REF	ALT	Function	III-1 (carrier father)	III-2 (carrier mother)	IV-1 (affected daughter)	IV-2 (affected son)	IV-3 (affected son)	IV-4 (unaffected daughter)
chr8:11666337	rs4731	A	G	nonsynonymous	A/G	A/G	G/G	A/G	G/G	G/G
chr8:11683653	rs904011	T	C	synonymous	C/C	C/C	C/C	C/C	C/C	C/C

Table 2 Treatment effect of lanosterol in rabbit cataract lenses and dog cataract

Table 2 a. Treatment effect of lanosterol in rabbit cataract lenses

Sample number	Before treatment	After treatment
1	3	1
2	2	0
3	2	1
4	2	0
5	3	1
6	2	1
7	2	1
8	2	0
9	1	1
10	1	0
11	2	1
12	1	1
13	2	1

Table 2 b. Treatment effect of lanosterol in dog cataract

Study eye	Treatment group		Control group	
	Before	After	Before	After
1	2	1	1	1
2	1	0	2	2
3	2	1	1	1
4	3	1		
5	1	0		
6	2	0		
7	2	1		

Table 3 Primers used for sequencing of each exon in the human LSS gene and construction of crystallin mutants

Table 3 a. Primers used for PCR-amplification and sequencing of each exon in the human LSS gene

Amplicon	Sequence (5'-3')
LSS-Exon1-F	GCCTGAGCGCCTGCCGAGGCCT
LSS-Exon1-R	GACACCTGAGGACCACCGGCCAT
LSS-Exon2-F	GTGGTCCTAGGTGCTGAGGAGA
LSS-Exon2-R	CGTGCTCCTCACGGCTCACCCCT
LSS-Exon3-F	CTTGGGCTGTATGTGAAGAGGGT
LSS-Exon3-R	CCTAGACCAGGCTGGGCCAGGAT
LSS-Exon4-F	GTTGGAGTGAGGTGCTCAGGAGGA
LSS-Exon4-R	GCAGCTGCCTGGAAACCCAAGCAT
LSS-Exon5-F	GCATTCTTAGTTTTCTGAGGAAACTC
LSS-Exon5-R	CCACTGTTTCAGCTGCAAGTGCAT
LSS-Exon6-F	CAGAGGGTGAAGCTTCCCAGCT
LSS-Exon6-R	GCTGTCACAGCCTGCACCTGAC
LSS-Exon7-F	GAAAGGGCCCAAGGTATGGATGCT
LSS-Exon7-R	GTGAGTGGACAGGTGTGGTTAGAT
LSS-Exon8-F	GAGCCAGGCCTACCAGGTGCT
LSS-Exon8-R	GCAGGGGATGAGTGCGTGAAT
LSS-Exon9-F	GCAGTGCATGGAGCTCCAGGCT
LSS-Exon9-R	CCAGGAAACCCCACTCCCAGCT
LSS-Exon10-F	GTGGATCTGGACGAGACCTTGT
LSS-Exon10-R	CACTGGGATGCAGCTGGGGCT
LSS-Exon11-F	GTGCAGGGTCTGGGTAGCAGCT
LSS-Exon11-R	GACATGATTGCAAAGGAAGCAT
LSS-Exon12-F	CTGGAGGCAGTGGCTGGGAGT
LSS-Exon12-R	GCAAGTGTGTGGCCAGCAGTGCT
LSS-Exon13-F	GGCAGGATGTGGCCAGGACCAT
LSS-Exon13-R	GCACTTCTGCCTGCAGGAGCT
LSS-Exon14-F	CCAGTCTGTCTCAGCGATGT
LSS-Exon14-R	CCAAAAACGCCAAGGGAGGAGT
LSS-Exon15-F	CTGGCTGCACCCACACCTTTGGT
LSS-Exon15-R	GCTCATCTGCAGGACACGAGGT
LSS-Exon16-F	GTTGTCAGCCCTAGTGTTGCCT
LSS-Exon16-R	CAGGTTTGTGTACCACAGTGCT
LSS-Exon17-F	GAGCTGCAGAGCCTGGGCAGCCA
LSS-Exon17-R	CCGTGTCACAGAATGATGCGT
LSS-Exon18-F	GAATTGGGATAGGTAAACTGCT
LSS-Exon18-R	CGCAGTGTGTGAGAGCAGAAACCT
LSS-Exon19-F	CTTAATGCCTGAGGCACTGGAGT
LSS-Exon19-R	CACTCATGACAGAGCATTGGGTT
LSS-Exon20-F	CAAGGCAGCCTGCTGGGGTGA
LSS-Exon20-R	CACCGGCTCACAGCTGAGTGT
LSS-Exon21-F	CTCACTGCAGCATTCCAGGGTT
LSS-Exon21-R	GTGGAAACAGCCATGCACGCT
LSS-Exon22-F	GCCAACAGCCAGGGCTCCAGTT
LSS-Exon22-R	GGTTGGAGCCCAAGACAGGGT

Table 3 b. Primers used in construction of crystallin mutants

Gene	Primer (5'-3')
αA-R116C-For	TTCCCGTGAGTTCCACTGCCGCTACCGCCTGCCGTCGCTGC
αA-R116C-Rev	CGGCAGGCGGTAGCGGCAGTGGAACTCACGGG
αA-R116H-For	TTCCCGTGAGTTCCACCACCGCTACCGCCTGCCGTCGCCAC
αA-R116H-Rev	CGGCAGGCGGTAGCGGTGGTGGAACTCACGGG
αA-Y118D-For	GAGTTCCACCGCCGCGACCGCCTGCCGTCCAACTTACGAC
αA-Y118D-Rev	CGTTGGACGGCAGGCGGTCGCGGCGGTGGAACT

αB-R120G-For	CAGGGAGTTCCACGGGAAATACCGGATAGGGGG
αB-R120G-Rev	GGATCCGGTATTTCCCGTGGAACTCCCT
βB2-V187E-For	AGGTGCAGTCCGAGCGCCGTATGTGGAG
βB2-V187E-Rev	ATACGGCGCTCGGACTGCACCT
βB2-V187M-For	AGGTGCAGTCCATGCGCCGTATGTGATG
βB2-V187M-Rev	ATACGGCGCTCGGACTGCACCT
βB2-R188H-For	TGCAGTCCGTGCACCGTATCCCGCCAC
βB2-R188H-Rev	GGATACGGTGCACGGACTGCA
γC-G129C-For	CACGTGCTGGAGTGCTGCTGGGCTGC
γC-G129C-Rev	CAGCAGCACTCCAGCACGTG
γD-W43R-For	GTGGACAGCGGCTGCCGGATGCTCTATGAGCTGGCGG
γD-W43R-Rev	GCTCATAGAGCATCCGGCAGCCGCTGTCCAC

References

1 Pascolini, D. & Mariotti, S. P. Global estimates of visual impairment: 2010. Br J Ophthalmol 96, 614-618, doi:10.1136/bjophthalmol-2011-300539 (2012).

2 Bloemendal, H. et al. Ageing and vision: structure, stability and function of lens crystallins. Prog. Biophys. Mol. Biol. 86, 407-485 (2004).3 Moreau, K. L. & King, J. A. Protein misfolding and aggregation in cataract disease and prospects for prevention. Trends Mol Med 18, 273-282, doi:S1471-4914(12)00039-1 [pii] 10.1016/j.molmed.2012.03.005 (2012).

4 Huff, M. W. & Telford, D. E. Lord of the rings--the mechanism for oxidosqualene:lanosterol cyclase becomes crystal clear. Trends Pharmacol Sci 26, 335-340, doi:S0165-6147(05)00127-6 [pii] 10.1016/j.tips.2005.05.004 (2005).

5 Diehn, J. J., Diehn, M., Marmor, M. F. & Brown, P. O. Differential gene expression in anatomical compartments of the human eye. Genome Biol 6, R74, doi:gb-2005-6-9-r74 [pii] 10.1186/gb-2005-6-9-r74 (2005).

6. Mori,M. et al. Lanosterolsynthasemutationscausecholesteroldeficiency-associated cataracts in the Shumiya cataract rat. J. Clin. Invest. 116, 395 – 404 (2006).

7 Ng, P. C. & Henikoff, S. Predicting deleterious amino acid substitutions. Genome Res 11, 863-874, doi:10.1101/gr.176601 (2001).

8. Adzhubei, I. A. et al. A method and server for predicting damaging missense mutations. Nature Methods 7, 248 – 249 (2010).

9. Pollard, K. S., Hubisz, M. J., Rosenbloom, K. R. & Siepel, A. Detection of nonneutral substitution rates on mammalian phylogenies. Genome Res. 20, 110 – 121 (2010).

10. Schwarz, J. M., Cooper, D. N., Schuelke, M. & Seelow, D. MutationTaster2: mutation prediction for the deep-sequencing age. Nature Methods 11, 361 – 362 (2014).

11. Seelow, D., Schuelke, M., Hildebrandt, F. & Nurnberg, P. HomozygosityMapper – an interactive approach to homozygosity mapping. Nucleic Acids Res. 37, W593 – W599 (2009)

12 Thoma, R. et al. Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. Nature 432, 118-122, doi:10.1038/nature02993 (2004).

13. Dobson, C. M. Protein folding and misfolding. Nature 426, 884–890 (2003).

14. Ecroyd, H. & Carver, J. A. Crystallin proteins and amyloid fibrils. Cell. Mol. Life Sci. 66, 62 – 81 (2009).

15. Braun, N. et al. Multiple molecular architectures of the eye lens chaperone aBcrystallin elucidated by a triple hybrid approach. Proc. Natl Acad. Sci. USA 108, 20491 - 20496 (2011)

16 Cenedella, R. J. et al. Direct perturbation of lens membrane structure may contribute to cataracts caused by U18666A, an oxidosqualene cyclase inhibitor. J Lipid Res 45, 1232-1241, doi:10.1194/jlr.M300469-JLR200 M300469-JLR200 [pii] (2004).

17. Li,H.&Durbin,R.Fastandaccuratelong-readalignmentwithBurrows–Wheeler transform. Bioinformatics 26, 589–595 (2010).

18. DePristo, M. A. et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nature Genet. 43, 491–498 (2011).

19. Ruf,A.etal.Themonotopicmembraneproteinhumanoxidosqualenecyclaseis active as monomer. Biochem. Biophys. Res. Commun. 315, 247–254 (2004).

20. Cardozo,T.,Totrov,M.&Abagyan,R.HomologymodelingbytheICMmethod. Proteins 23, 403–414 (1995).

21. Abagyan,R.&Argos,P.Optimalprotocolandtrajectoryvisualizationfor conformational searches of peptides and proteins. J. Mol. Biol. 225, 519–532 (1992).

22. Xu, J. et al. The congenital cataract-linked A2V mutation impairs tetramer formation and promotes aggregation of bB2-crystallin. PLoS ONE 7, e51200 (2012).

23. Wang,B.etal.AnovelCRYGDmutation(p.Trp43Arg)causingautosomaldominant congenital cataract in a Chinese family. Hum. Mutat. 32, E1939–E1947 (2011).

24. Gu,F.etal.AnovelmutationinAlphaA-crystallin(CRYAA)causedautosomal dominant congenital cataract in a large Chinese family. Hum. Mutat. 29, 769 (2008).

25. Li, X.-Q. et al. A novel mutation impairing the tertiary structure and stability of cC-crystallin (CRYGC) leads to cataract formation in humans and zebrafish lens. Hum. Mutat. 33, 391–401 (2012).

26. Nagineni,C.N.&Bhat,S.P.Humanfetallensepithelialcellsinculture:aninvitro model for the study of crystallin expression and lens differentiation. Curr. Eye Res. 8, 285–291 (1989).

27. Bligh,E.G.&Dyer,W.J.Arapidmethodoftotallipidextractionandpurification. Can. J. Biochem. Physiol. 37, 911–917 (1959).

28. Wang, S., Leng, X.-Y. & Yan, Y.-B. The benefits of being b-crystallin heteromers: bB1-crystallin protects bA3-crystallin against aggregation during co-refolding. Biochemistry 50, 10451–10461 (2011).

29. Sun,T.-X.,Das,B.K.&Liang,J.J.N.Conformationalandfunctionaldifferences between

recombinant human lens aA- and aB-crystallin. J. Biol. Chem. 272, 6220–6225 (1997).

30. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254 (1976).

31. Geraldine,P.etal.Preventionofselenite-inducedcataractogenesisbyacetyl-L- carnitine: an experimental study. Exp. Eye Res. 83, 1340–1349 (2006).

32. Makri,O.E.,Ferlemi,A.V.,Lamari,F.N.&Georgakopoulos,C.D.Saffron administration prevents selenite-induced cataractogenesis. Mol. Vis. 19, 1188–1197 (2013).

33. Zhang, L. et al. Self-assembled lipid–polymer hybrid nanoparticles: a robust drug delivery platform. ACS Nano 2, 1696–1702 (2008). La Croix, N. Cataracts: When to refer. Top. Companion Anim. Med. 23, 46–50 (2008).

34. La Croix, N. Cataracts: When to refer. Top. Companion Anim. Med. 23, 46–50 (2008).

What is claimed is:

1. Use of a composition for the preparation of medicament to treat and/or prevent vision disorders in a subject, said composition comprises a pharmaceutically acceptable ophthalmic carrier and a pharmaceutically effective amount of lanosterol.

2. The use of claim 1, wherein said subject is having or at risk of developing a vision disorder that affects the normal structure of the lens in the eye.

3. The use of claim 1, wherein said vision disorder is selected from the group consisting of cataract, congenital cataracts, cortical opacities, posterior subcapsular cataract, presbyopia nuclear sclerosis, retinal degenerative disorder, Refsum disease, Smith-Lemli-Opitz syndrome, Schnyder crystalline corneal dystrophy, drusen, age-related macular degeneration, and diabetic retinopathy.

4. The use of claim 1, wherein said lanosterol inhibits crystallin protein aggregation.

5. Use of a composition for the preparation of medicament to treat cataract or blindness/impaired vision in a subject, said composition comprises a pharmaceutically acceptable ophthalmic carrier and a pharmaceutically effective amount of lanosterol, wherein said lanosterol dissolves lens crystallin protein aggregate(s) in the eye of said subject.

6. The use of claim 4, wherein the lens crystallin protein is any of α–crystallin, β-crystallin or γ-crystallin.

7. The use of any one of claims 1-6, wherein said composition is formulated as an ophthalmic solution, an ophthalmic ointment, an ophthalmic wash, an intraocular infusion solution, a wash for anterior chamber, an internal medicine, an injection, or preservative for extracted cornea.

8. The use of any one of claims 1-6, wherein said subject is selected from the group consisting of amphibians, reptiles, avians, and mammals.

9. The use of claim 8, wherein said mammal is selected from the group consisting of rodents, cats, dogs, pigs, horses and humans.

10. A method for dissolving amyloid-like fibrils of crystallin proteins, comprising the step of contacting the amyloid-like fibrils with lanosterol in a sufficient amount and duration so as to dissolve the amyloid-like fibrils of crystalline proteins.

11. The method of claim 10, wherein the method is done in situ, in vitro or in vivo.

12. The method of claim 10, wherein the method is performed on a subject.

13. The method of claim 12, wherein the subject is human.

14. A kit for treating and/or preventing vision disorders that affect the normal structure of the eye in a subject, comprising a formulation of a pharmaceutically effective amount of lanosterol, a pharmaceutically acceptable carrier and instructions for administering said formulation such that said administration treats and/or prevents said vision disorder.

15. An ophthalmic pharmaceutical composition for treating and/or preventing vision disorders in a subject, said composition comprises a pharmaceutically acceptable ophthalmic carrier and a pharmaceutically effective amount of lanosterol.

16. The ophthalmic pharmaceutical composition of claim 15, wherein said composition is formulated as an ophthalmic solution, an ophthalmic ointment, an ophthalmic wash, an intraocular infusion solution, a wash for anterior chamber, an internal medicine, an injection, or preservative for extracted cornea.

Fig.1A

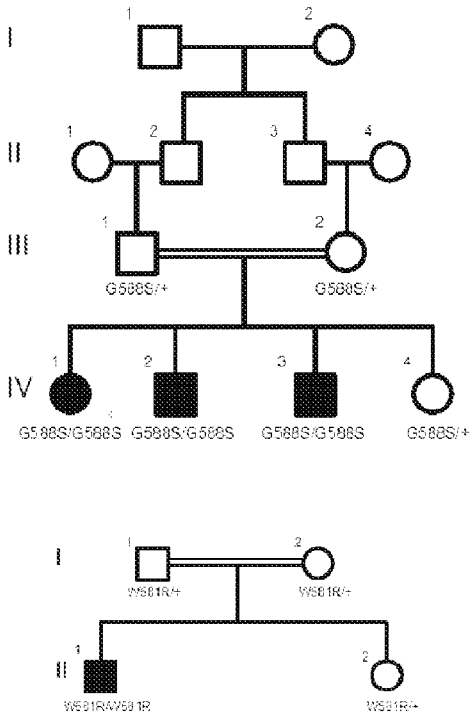


Fig.1B

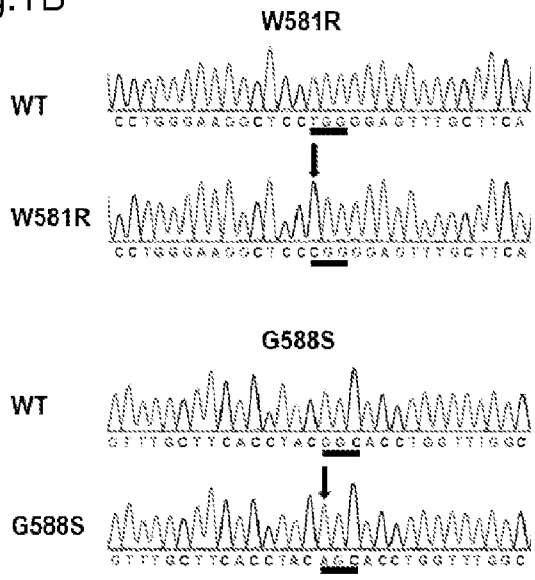


Fig.1C

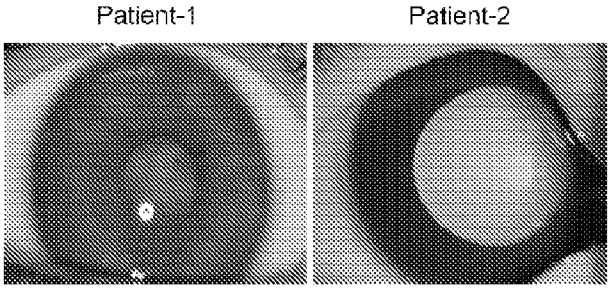


Fig.2A

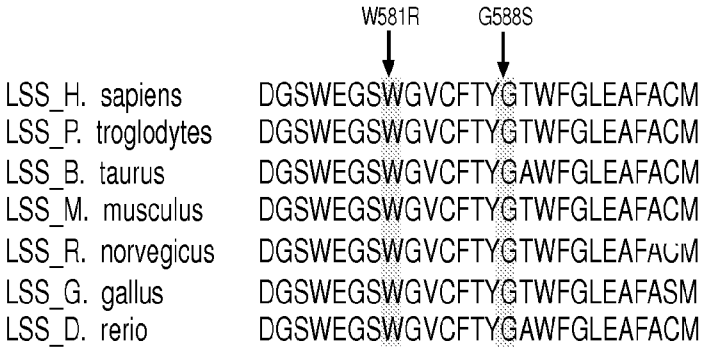


Fig.2B

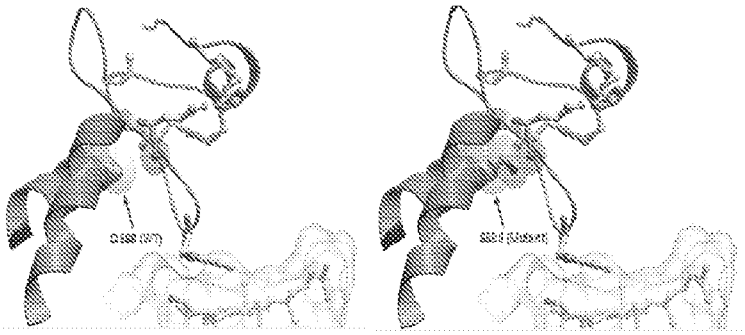


Fig.2C

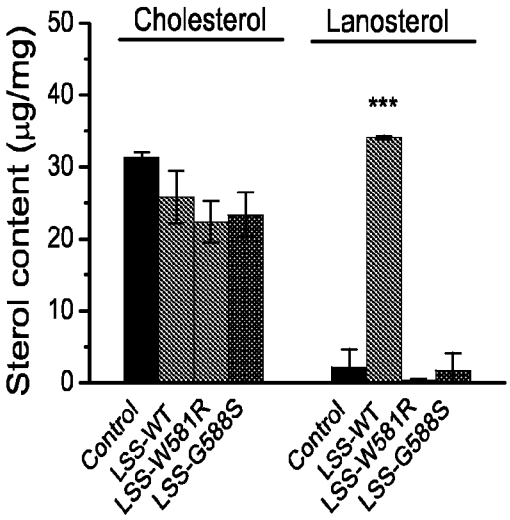


Fig.3A

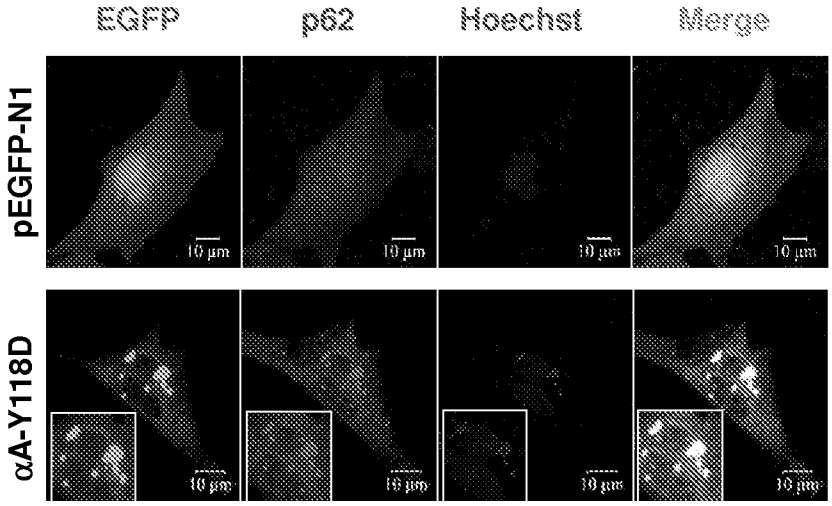

EGFP
p62
Hoechst
Merge
pEGFP-N1
αA-Y118D
10 μm

Fig.3B

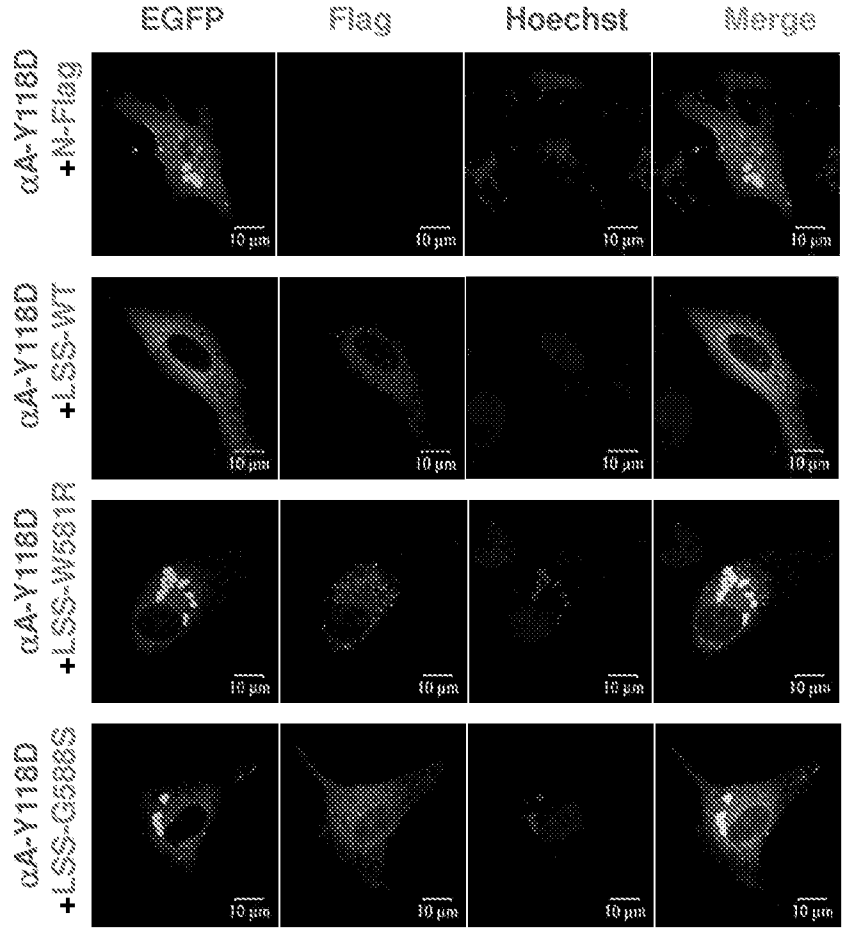

EGFP
Flag
Hoechst
Merge
αA-Y118D +N-Flag
αA-Y118D +LSS-WT
αA-Y118D +LSS-W591R
αA-Y118D +LSS-C588S
10 μm

Fig.3C

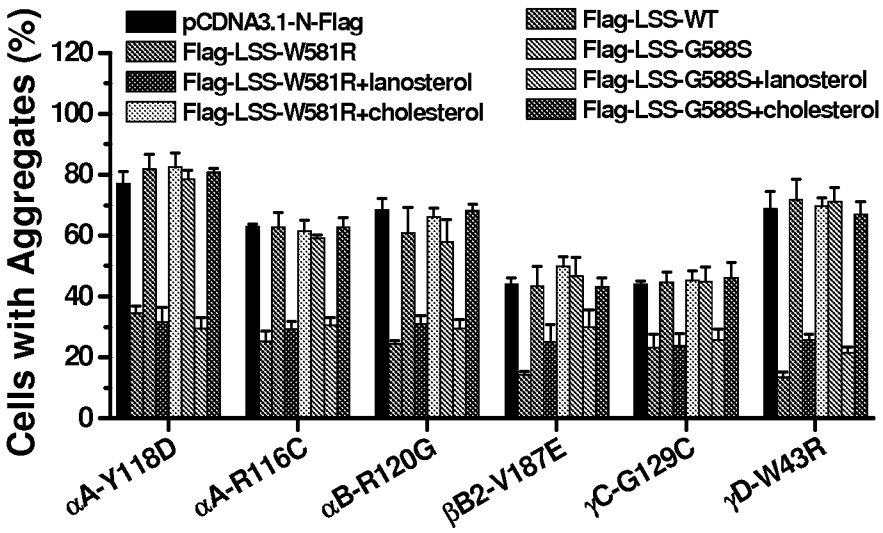

Cells with Aggregates (%)
120
100
80
60
40
20
0
pCDNA3.1-N-Flag
Flag-LSS-W581R
Flag-LSS-W581R+lanosterol
Flag-LSS-W581R+cholesterol
Flag-LSS-WT
Flag-LSS-G588S
Flag-LSS-G588S+lanosterol
Flag-LSS-G588S+cholesterol
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

Fig.3D

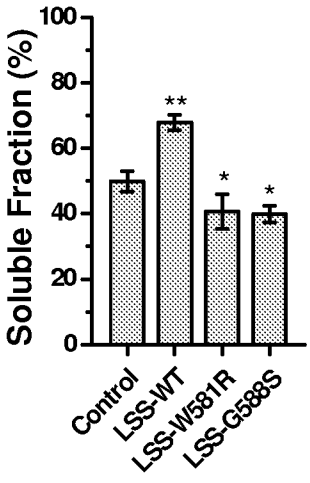

Soluble Fraction (%)
100
80
60
40
20
0
**
*
*
Control
LSS-WT
LSS-W581R
LSS-G588S

Fig.3E

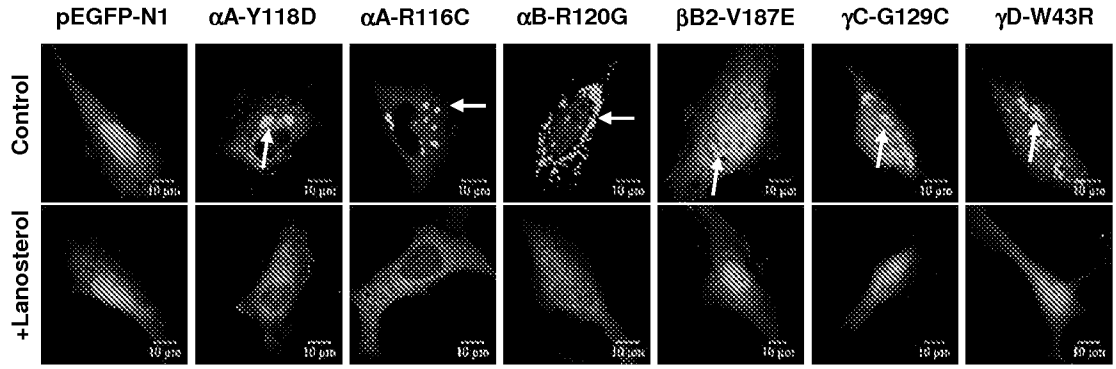


Fig.3F

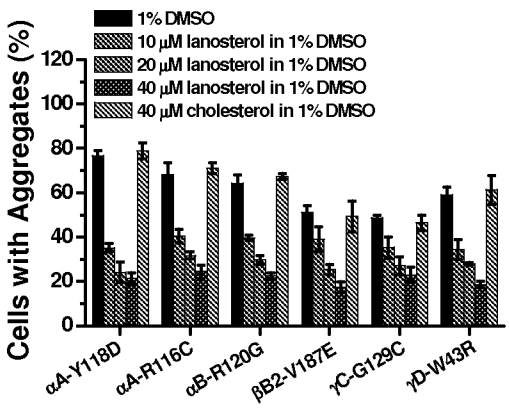


Fig.3G

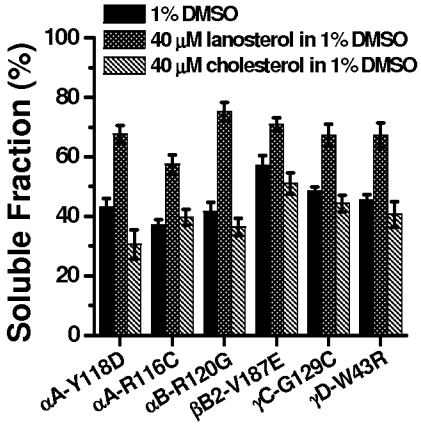


Fig.3H

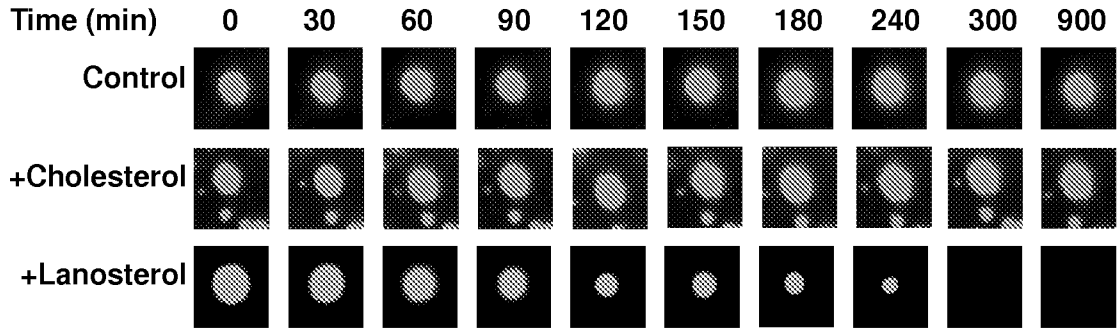

Time (min)
0
30
60
90
120
150
180
240
300
900
Control
+Cholesterol
+Lanosterol

Fig.3I

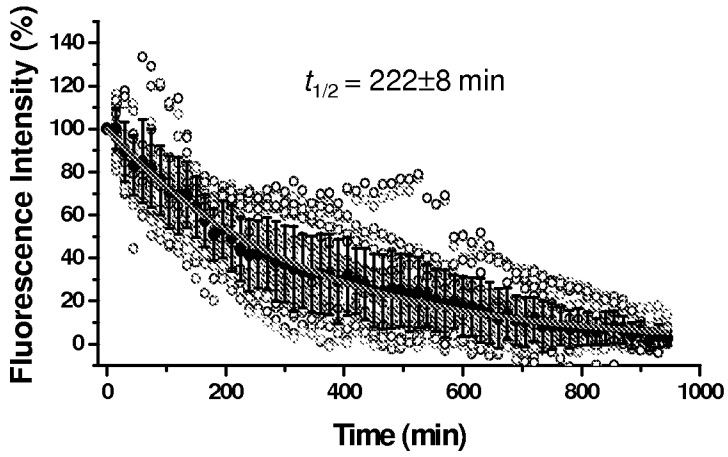

Fluorescence Intensity (%)
140
120
100
80
60
40
20
0
$t_{1/2}$ = 222±8 min
0
200
400
600
800
1000
Time (min)

Fig.4A

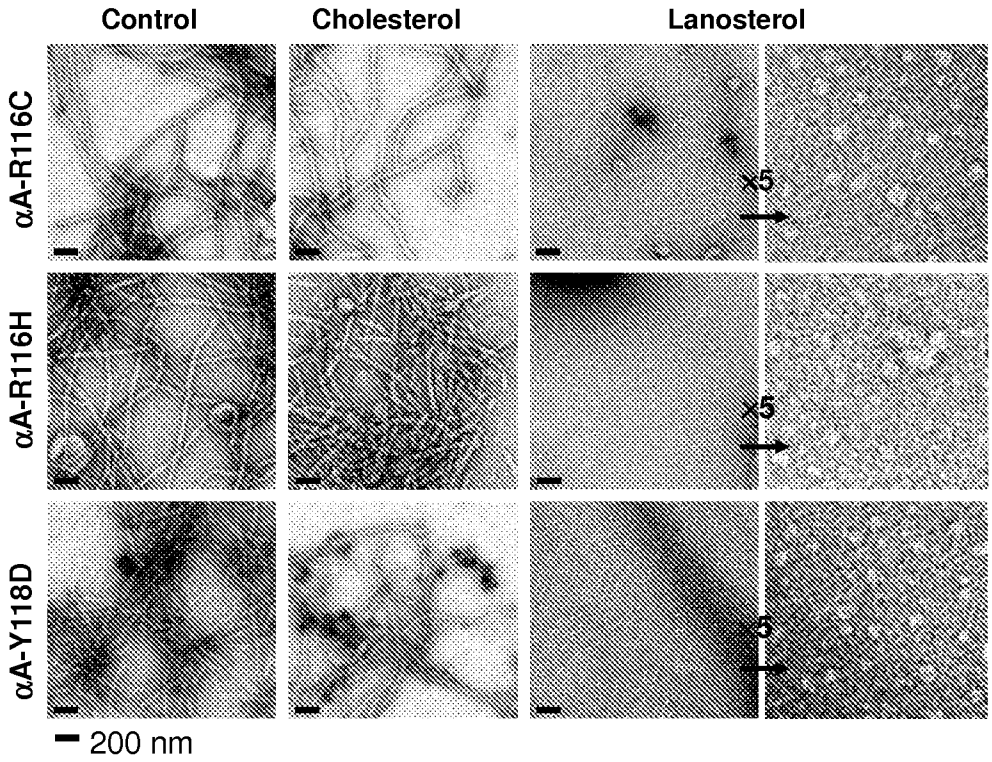


Fig.4B(i)

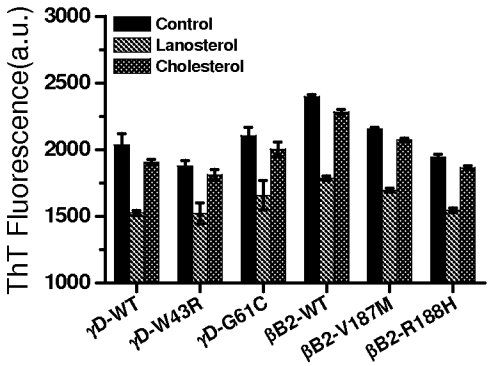


Fig.4B(ii)

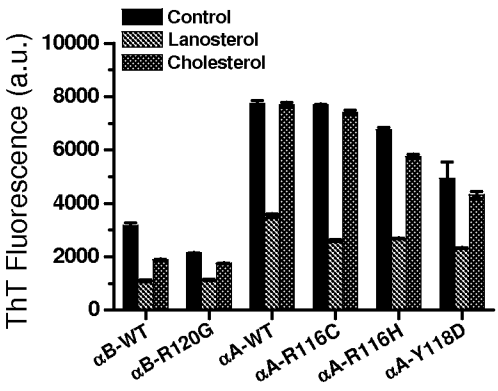


Fig.5A

Fig.5A(i)

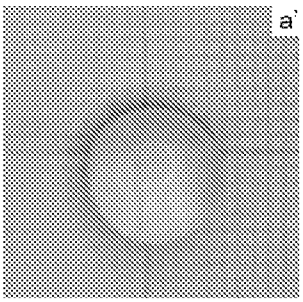


Fig.5A(ii)

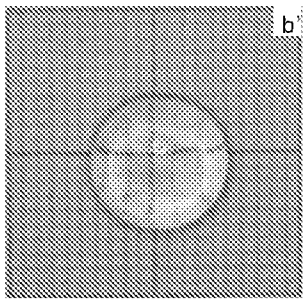


Fig.5B

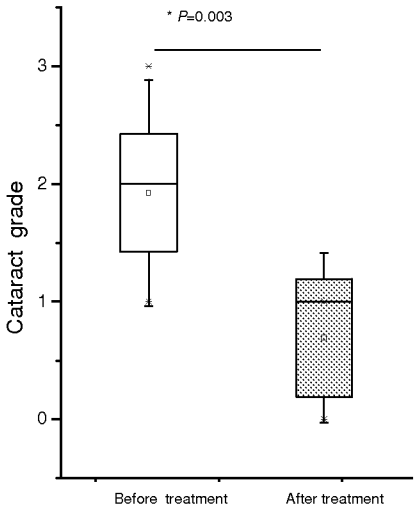


Fig. 5C

Fig.5C(i) Fig.5C(ii)

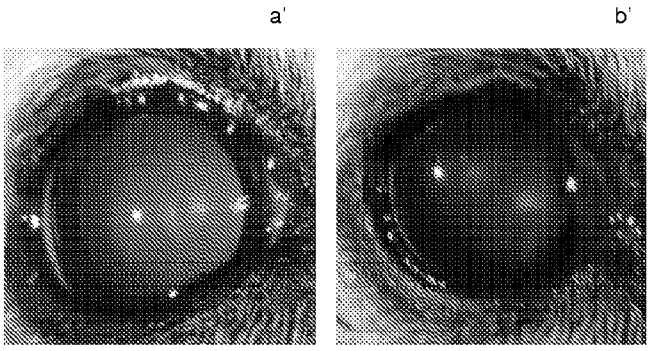


Fig. 5D

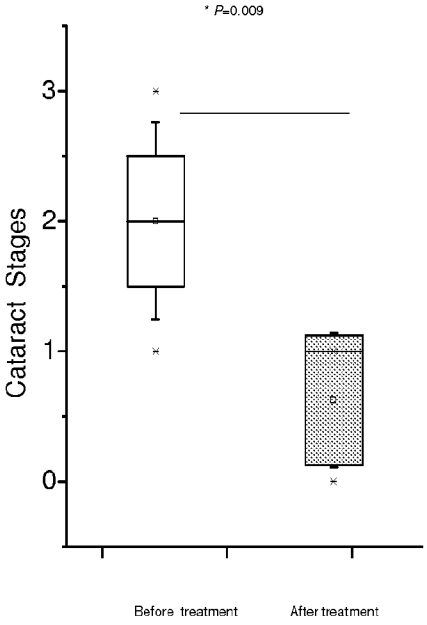


Fig. 6A

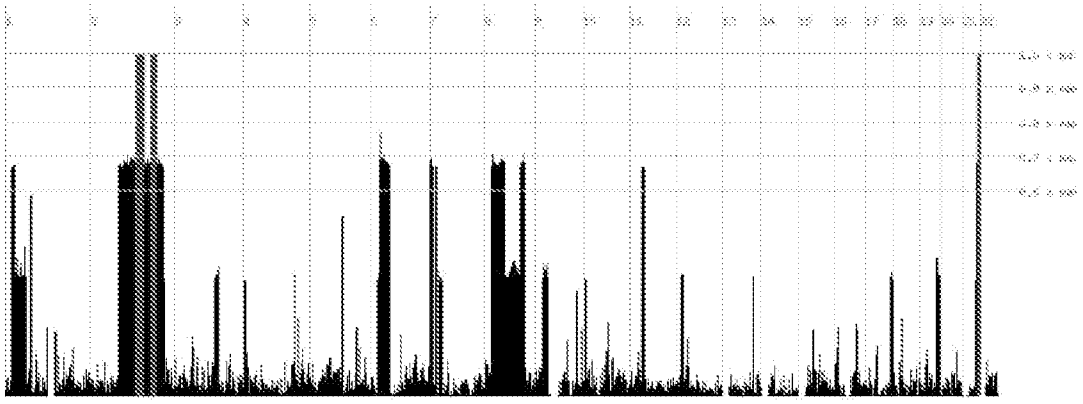


Fig. 6B

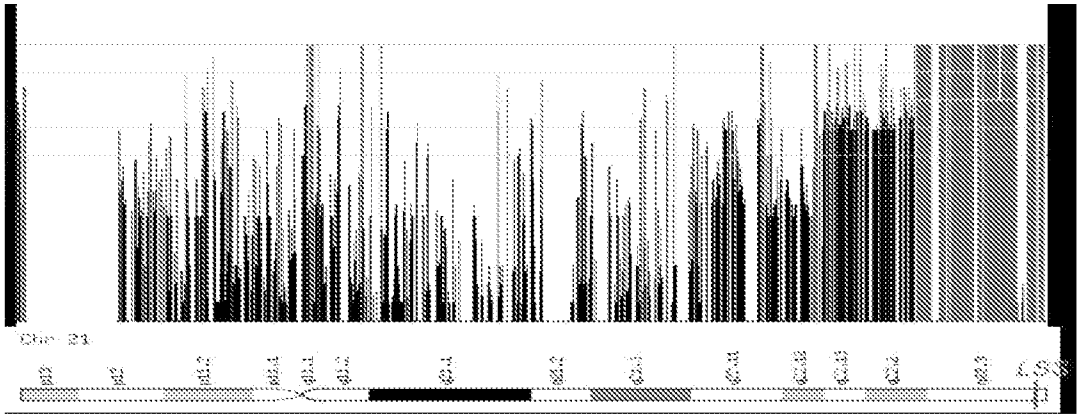


Fig. 7

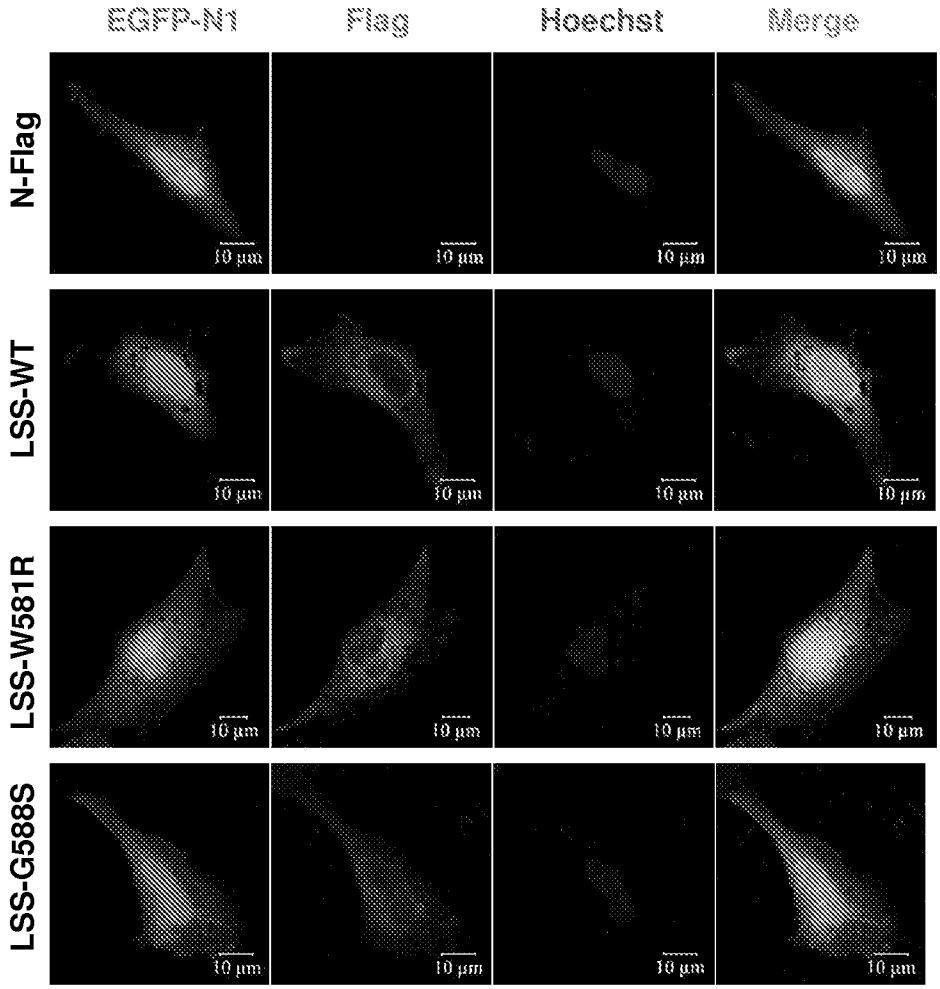

EGFP-N1
Flag
Hoechst
Merge
N-Flag
LSS-WT
LSS-W581R
LSS-G588S
10 μm

Fig. 8A

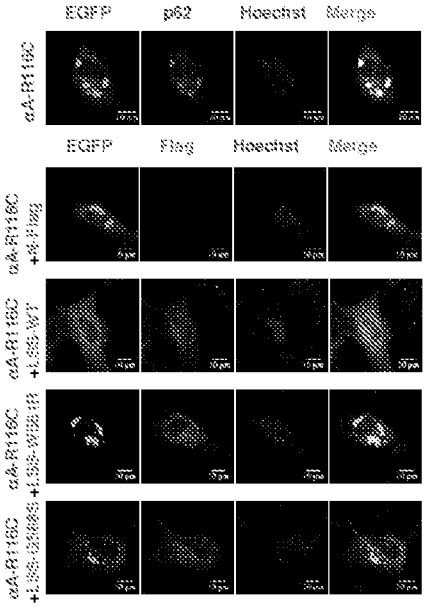


Fig. 8B

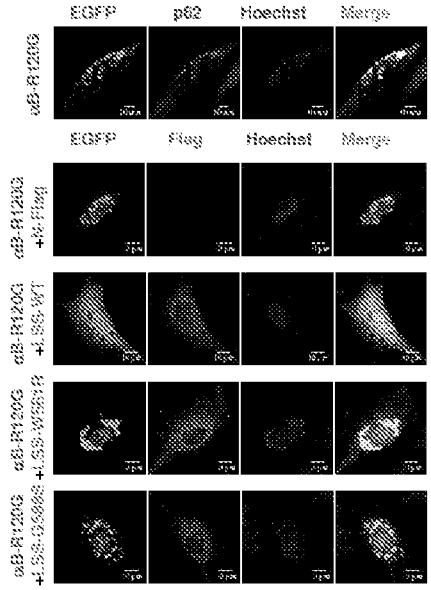


Fig. 8C

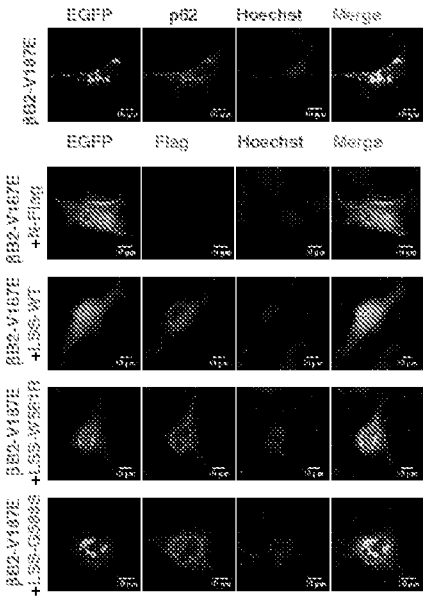


Fig. 8D

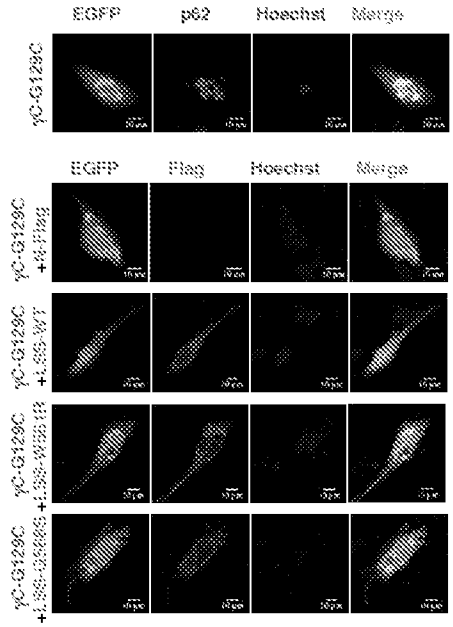

EGFP
p62
Hoechst
Merge
γC-G129C
EGFP
Flag
Hoechst
Merge
γC-G129C +N-Flag
γC-G129C +1-85-WT
γC-G129C +1-85-W43R
γC-G129C +1-85-G66S

Fig. 8E

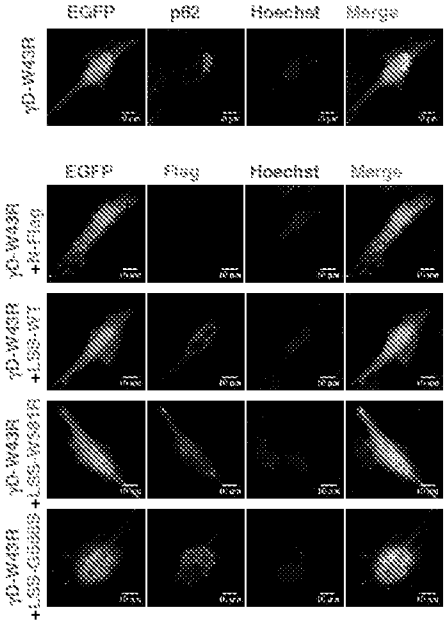

EGFP
p62
Hoechst
Merge
γD-W43R
EGFP
Flag
Hoechst
Merge
γD-W43R +N-Flag
γD-W43R +1-85-WT
γD-W43R +1-85-W43R
γD-W43R +1-85-G66S

Fig. 9A

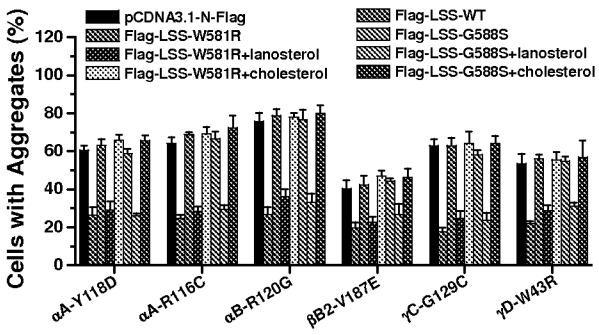


Fig. 9B

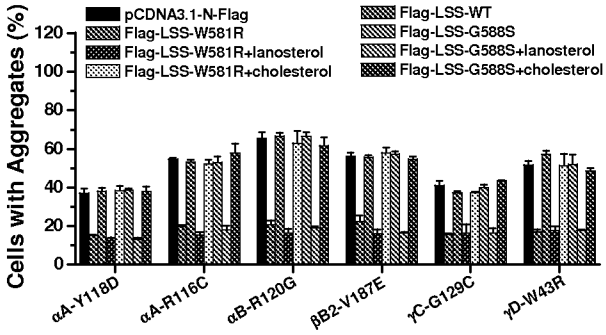


Fig. 9C

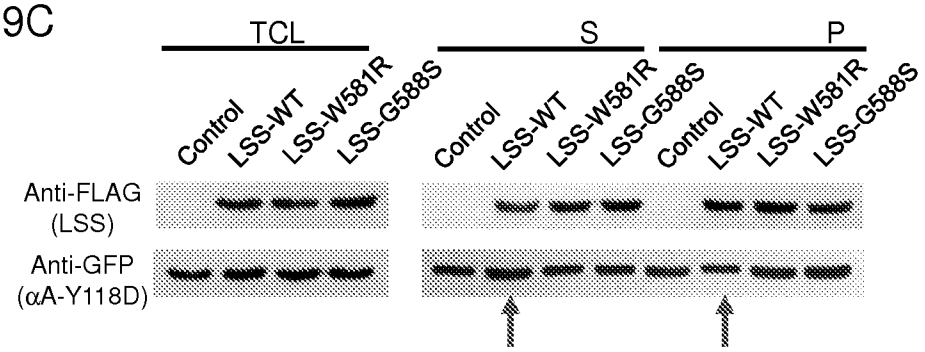


Fig. 10A

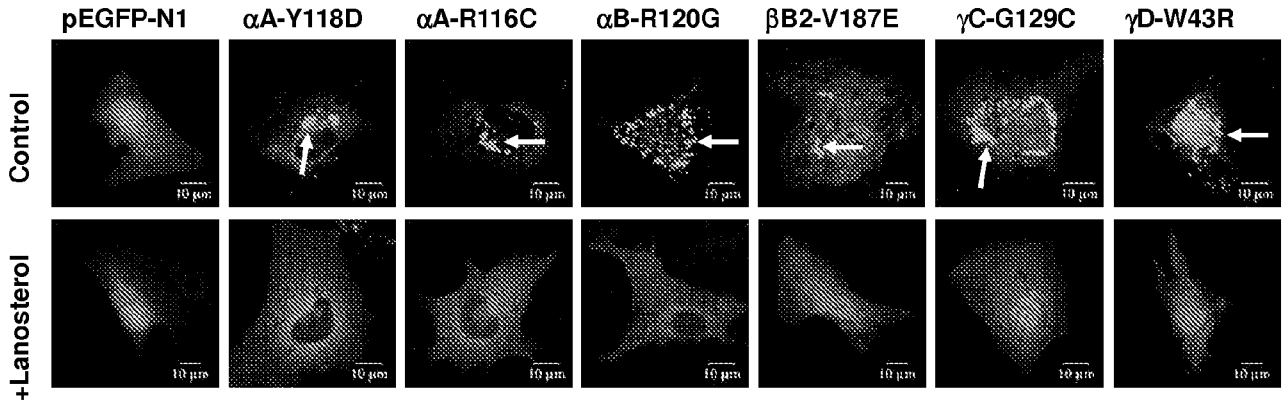


Fig. 10B

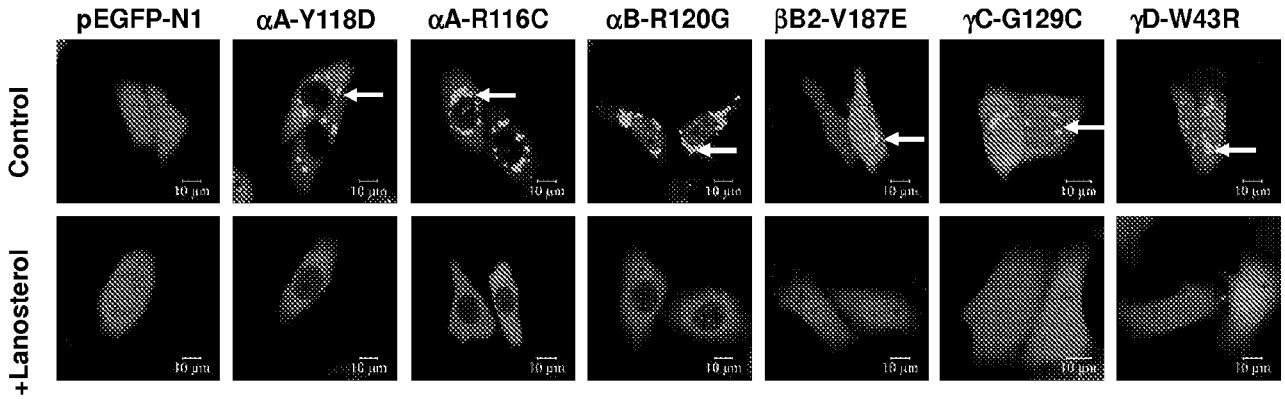


Fig. 10C

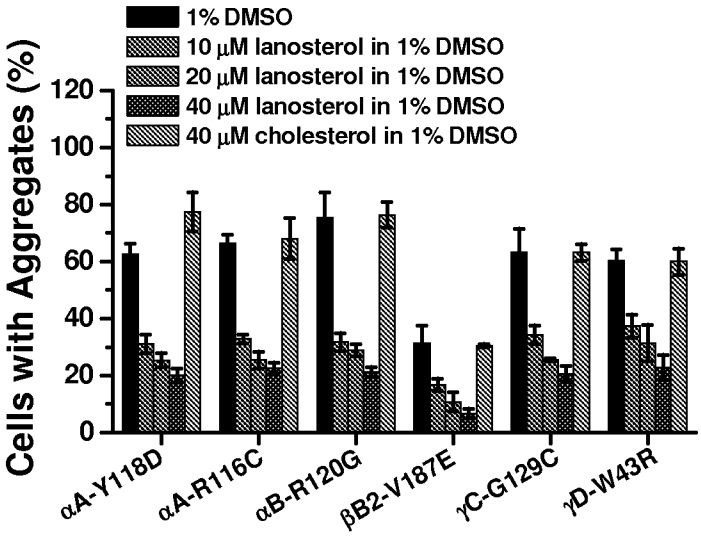


Fig. 10D

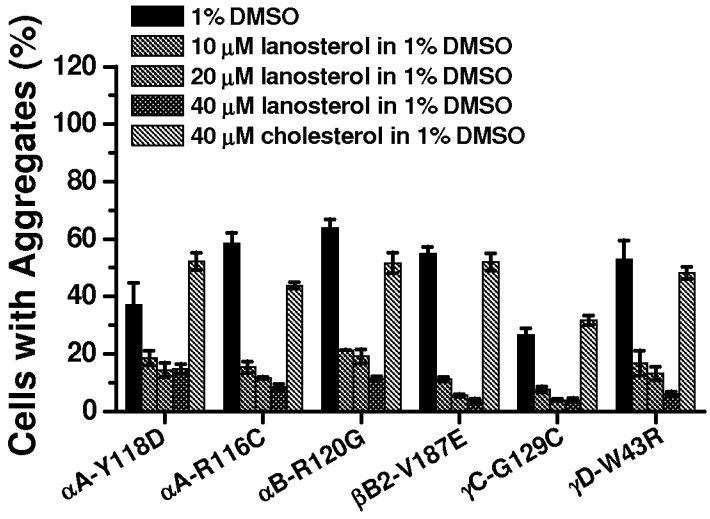


Fig.11A

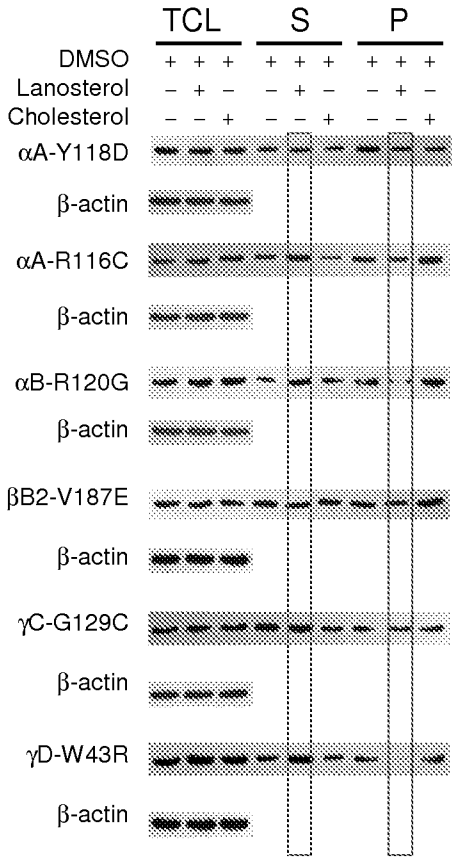

TCL
S
P
DMSO + + + + + + + + +
Lanosterol − + − − + − − + −
Cholesterol − − + − − + − − +
αA-Y118D
β-actin
αA-R116C
β-actin
αB-R120G
β-actin
βB2-V187E
β-actin
γC-G129C
β-actin
γD-W43R
β-actin

Fig. 11B

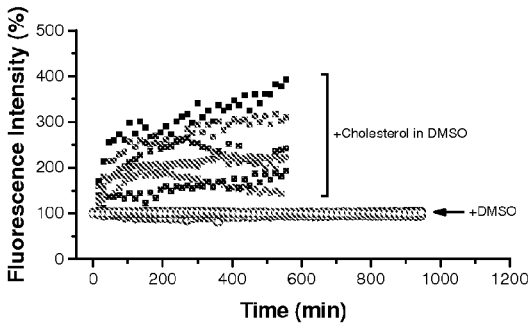


Fig. 11C (i)

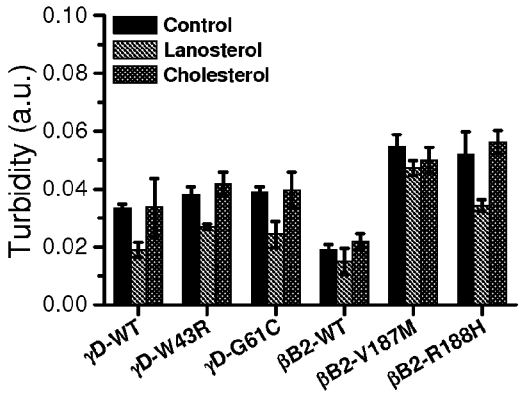


Fig. 11D

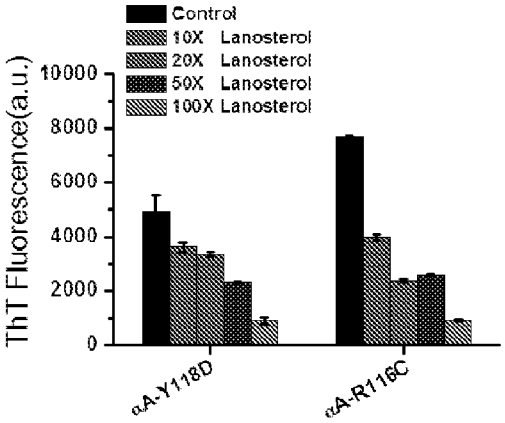


Fig. 11C (ii)

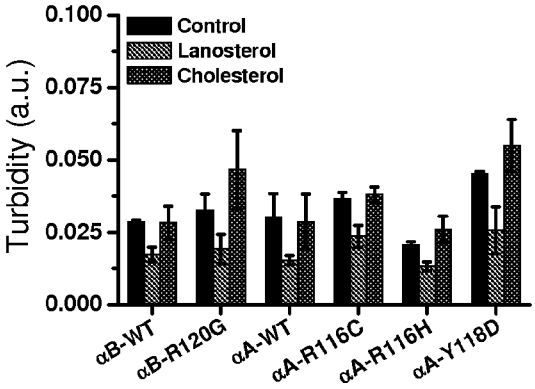


Fig. 12A

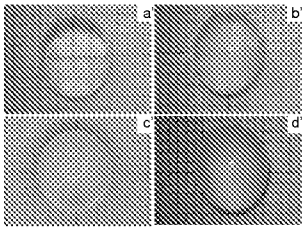


Fig. 12B

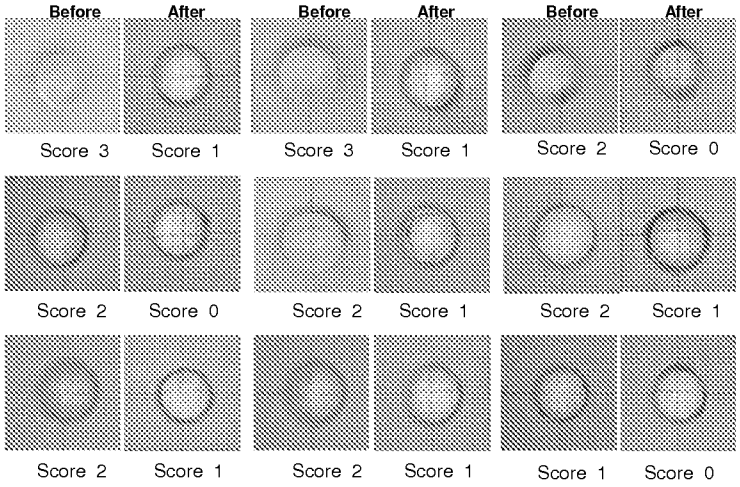
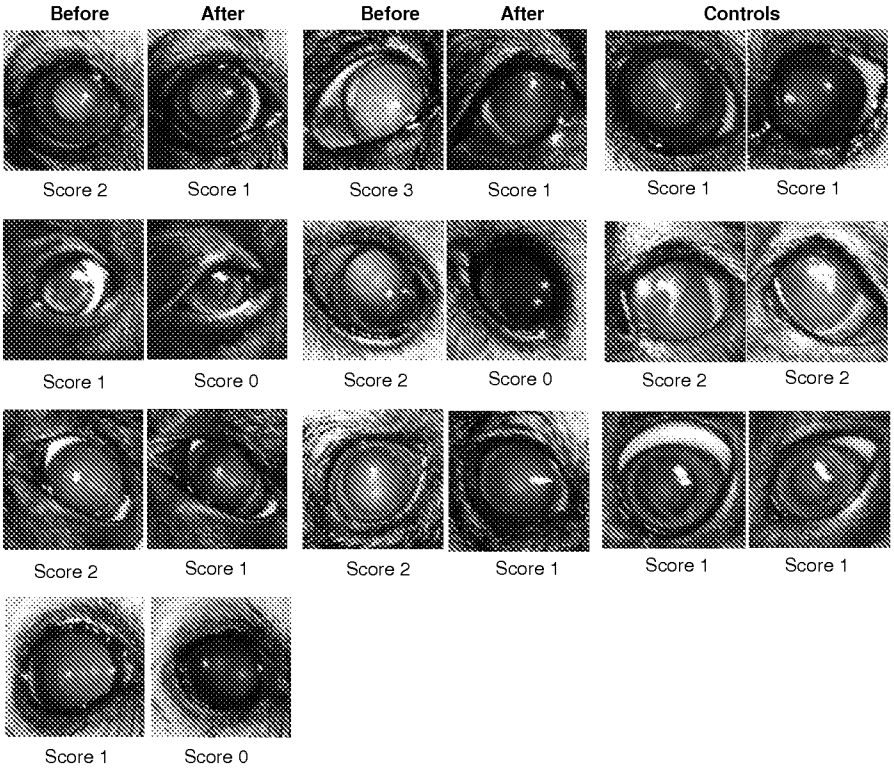


Fig. 12C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/046453

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/56 (2015.01)
CPC - A61K 31/56 (2015.10)
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)
IPC(8) - A61K 31/56, 39/395, 49/00; C40B 30/04 (2015.01)
CPC - A61K 31/56, 39/395, 49/00; C40B 30/04 (2015.10)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 31/56, 39/395, 49/00; C40B 30/04 (2015.10) (keyword delimited)
US Classes - 424/133.1; 506/9; 514/169

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google, PubMed.

Search terms used: lanosterol, lanosta-8,24-dien-3-ol, euphol, ophthalmic, cataract, cornea, retina, topical, amyloid, protein, dissolve

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2014/035451 A1 (BAUSCH & LOMB INCORPORATED et al) 06 March 2014 (06.03.2014) entire document	1, 7-9, 14-16 ---------- 2, 3
Y	US 2006/0121024 A1 (JAVITT) 08 June 2006 (08.06.2006) entire document	2, 3
T, X	ZHAO et al. Lanosterol reverses protein aggregation in cataracts," Nature, 30 July 2015 (30.07.2015), Vol. 523, Pgs. 607-611. entire document	1-16
A	US 2010/0068251 A1 (ALI et al) 18 March 2010 (18.03.2010) entire document	1-16
A	US 2011/0136773 A1 (PIANOWSKI et al) 09 June 2011 (09.06.2011) entire document	1-16
A	MEEHAN et al. "Amyloid Fibril Formation by Lens Crystallin Proteins and Its Implications for Cataract Formation*," The Journal of Biological Chemistry, 30 January 2004 (30.01.2004), Vol. 279, No. 5, Pgs. 3413-3419. entire document	1-16

☐ 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
"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
26 October 2015	25 NOV 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 107206009 A
(43)申请公布日 2017.09.26

(21)申请号 201580057679.0
(22)申请日 2015.08.24
(30)优先权数据
62/040,721 2014.08.22 US
62/194,120 2015.07.17 US
(85)PCT国际申请进入国家阶段日
2017.04.24
(86)PCT国际申请的申请数据
PCT/US2015/046453 2015.08.24
(87)PCT国际申请的公布数据
WO2016/029199 EN 2016.02.25
(71)申请人 广州康睿生物医药科技股份有限公司
地址 110000 辽宁省沈阳市浑南区17号浦江苑
申请人 加利福尼亚大学董事会
(72)发明人 张康 侯睿 蔡惠民
(74)专利代理机构 北京安信方达知识产权代理有限公司 11262
代理人 郑霞
(51)Int.Cl.
A61K 31/56(2006.01)

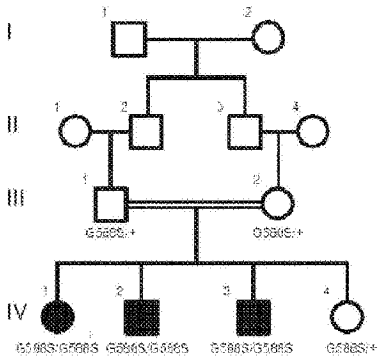
权利要求书1页 说明书27页 附图21页

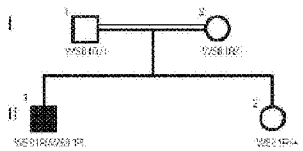
(54)发明名称

用于治疗视觉障碍的组合物和方法

(57)摘要

本发明提供了甾醇及其用于治疗视觉障碍的用途。在一个实施方案中，包含药学上有效量的羊毛甾醇的组合物用于治疗和/或预防受试者的视觉障碍。在另一个实施方案中，包含药学上有效量的羊毛甾醇的组合物用于治疗受试者的白内障或失明/视觉损伤。在又一个实施方案中，包含羊毛甾醇的组合物用于溶解晶体蛋白的淀粉样纤维。





CN 107206009 A

1.组合物在制备用于治疗和/或预防受试者的视觉障碍的药物中的用途，所述组合物包含药学上可接受的眼用载体和药学上有效量的羊毛甾醇。

2.根据权利要求1所述的用途，其中所述受试者患有视觉障碍或处于发展成视觉障碍的风险下，该视觉障碍影响眼内晶状体的正常结构。

3.根据权利要求1所述的用途，其中所述视觉障碍选自白内障、先天性白内障、皮质混浊、后囊下白内障、老视核硬化、视网膜退行性病症、雷夫叙姆病、Smith-Lemli-Opitz综合征、施奈德结晶状角膜营养不良、玻璃疣、年龄相关性黄斑变性和糖尿病视网膜病变。

4.根据权利要求1所述的用途，其中所述羊毛甾醇抑制晶体蛋白聚集。

5.组合物在制备用于治疗受试者的白内障或失明/视觉损伤的药物中的用途，所述组合物包含药学上可接受的眼用载体和药学上有效量的羊毛甾醇，其中所述羊毛甾醇溶解所述受试者的眼内的晶状体晶体蛋白聚集体。

6.根据权利要求4所述的用途，其中所述晶状体晶体蛋白为α-晶体蛋白、β-晶体蛋白或γ-晶体蛋白中的任一种。

7.根据权利要求1-6中任一项所述的用途，其中所述组合物被配制成眼用溶液、眼用软膏、眼用洗剂、眼内输注溶液、用于前房的洗剂、内服药、注射剂或用于提取的角膜的防腐剂。

8.根据权利要求1-6中任一项所述的用途，其中所述受试者选自两栖动物、爬行动物、禽类和哺乳动物。

9.根据权利要求8所述的用途，其中所述哺乳动物选自啮齿动物、猫、狗、猪、马和人。

10.一种用于溶解晶体蛋白的淀粉样纤维的方法，该方法包括使淀粉样纤维与羊毛甾醇以足够的量和持续时间接触以便溶解晶体蛋白的淀粉样纤维的步骤。

11.根据权利要求10所述的方法，其中所述方法在原位、体外或体内进行。

12.根据权利要求10所述的方法，其中所述方法对受试者进行。

13.根据权利要求12所述的方法，其中所述受试者是人。

14.一种用于治疗和/或预防影响受试者眼睛的正常结构的视觉障碍的试剂盒，该试剂盒包含药学上有效量的羊毛甾醇、药学上可接受的载体的制剂，以及关于施用所述制剂使得所述施用治疗和/或预防所述视觉障碍的说明。

15.一种用于治疗和/或预防受试者的视觉障碍的眼用药物组合物，所述组合物包含药学上可接受的眼用载体和药学上有效量的羊毛甾醇。

16.根据权利要求15所述的眼用药物组合物，其中所述组合物被配制成眼用溶液、眼用软膏、眼用洗剂、眼内输注溶液、用于前房的洗剂、内服药、注射剂或用于提取的角膜的防腐剂。

用于治疗视觉障碍的组合物和方法

相关申请的交叉引用

[0001] 本申请要求于2014年8月22日提交的美国临时申请号62/040,721和于2015年7月17日提交的美国临时申请号62/194,120的优先权,这些申请的内容通过引用而全文并入本文。

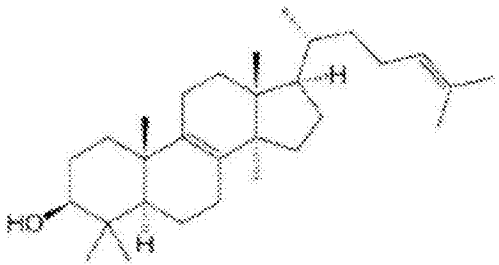
技术领域

[0002] 本公开内容总体上涉及甾醇及其用于治疗视觉障碍的用途,该视觉障碍影响患有此类视觉障碍或处于发展成此类视觉障碍的风险下的患者眼睛的晶状体的正常功能。

发明内容

[0003] 本发明提供了一种治疗或预防视觉障碍的方法,该方法包括向有需要的个体施用有效量的羊毛甾醇及其前药或药学上可接受的盐。

[0004] 本发明还提供了一种眼用药物组合物,其包含药学上可接受的眼用载体和具有以下式I的结构的羊毛甾醇:



及其前药或药学上可接受的盐。

[0005] 在所述方法的各个方面,所述视觉障碍为影响眼睛晶状体的功能、清晰度和/或结构的眼睛障碍。这样的眼睛疾病包括但不限于眼睛的白内障、眼睛的老视以及眼睛晶状体的核硬化。此外,视觉障碍还指视网膜变性,如雷夫叙姆病(Refsum disease)、Smith-Lemli-Opitz综合征(SLOS)和施奈德结晶状角膜营养不良(SCCD)、无β脂蛋白血症和家族性低β脂蛋白血症。

[0006] 在一个实施方案中,本发明提供了一种通过向受试者施用治疗或预防有效量的式I的甾醇来改善与视觉障碍相关的至少一种症状的方法。在该方法的各个方面,该组合物局部、结膜下、眼球后、眼周、视网膜下、脉络膜上或眼内施用。接受本发明甾醇的受试者可以包括但不限于哺乳动物、禽类、两栖动物、爬行动物和其他脊椎动物。在一个实施方案中,该受试者为马、猪、狗、猫、啮齿动物和/或其他伴侣宠物。在另一个实施方案中,该受试者为人。

[0007] 在一个实施方案中,本发明涉及包含眼用药学上可接受的载体中的本发明甾醇的眼用药物组合物。在一些实施方案中,该药物组合物包含眼用药学上可接受的载体中的羊毛甾醇或其衍生物。在本发明的某些实施方案中,该药学上可接受的载体为水、缓冲液或氯化钠溶液。在一些实施方案中,该药学上可接受的载体是无菌的。在其他实施方案中,该药

学上可接受的载体为软膏。在其他实施方案中，该药学上可接受的载体为凝胶。凝胶可以使用本领域公知的凝胶配制材料进行配制，该凝胶配制材料包括但不限于高粘度羧甲基纤维素、羟丙基甲基纤维素、聚氧化乙烯和卡波姆(carbomer)。在所述组合物的一些方面，该药学上可接受的眼用载体为环糊精。在一个实施方案中，该环糊精为(2-羟丙基)-β-环糊精。

[0008] 本发明的某些实施方案还涉及包含可用于治疗和/或预防与视觉障碍相关的症状的组分的试剂盒。这样的试剂盒包含含有在药学上可接受的载体中的本发明甾醇的容器，以及关于施用本发明甾醇使得与视觉障碍相关的至少一种症状得到改善或预防的说明。这样的视觉障碍包括但不限于白内障、老视和眼睛晶状体的核硬化。此外，视觉障碍还指视网膜变性，如雷夫叙姆病、Smith-Lemli-Opitz综合征(SLOS)和施奈德结晶状角膜营养不良(SCCD)、无β脂蛋白血症和家族性低β脂蛋白血症。包含在本文涉及的一些试剂盒中的容器是用于施用滴眼剂的滴管。在其他实施方案中，该容器是用于分配软膏或凝胶的管。在其他实施方案中，该容器是用于药物递送的任何合适的容器，包括但不限于注射器或适于药物的眼部递送或局部施用的其他容器。

[0009] 在其他方面，本发明提供了一种用于抑制或预防蛋白质聚集的方法。在该方法的各个方面，所述蛋白质为淀粉状蛋白形成蛋白或引起功能缺失疾病的蛋白质。在一些方面，该淀粉状蛋白形成蛋白选自Hsp27、αA-晶体蛋白、αB-晶体蛋白、βB2-晶体蛋白、βB1-晶体蛋白、γD-晶体蛋白、Hsp22、Hsp20、tau、α-突触核蛋白、IAPP、β-淀粉状蛋白、PrP、亨廷顿蛋白(Huntingtin)、降钙素、心房钠尿肽、载脂蛋白AI、血清淀粉状蛋白A、Medin、催乳素、运甲状腺素蛋白、溶菌酶、β2微球蛋白、凝溶胶蛋白(Gelsolin)、角膜上皮素(Keratoepithelin)、半胱氨酸蛋白酶抑制剂(Cystatin)、免疫球蛋白轻链AL和S-IBM。在其他方面，引起功能缺失疾病的蛋白质选自突变的β-葡糖苷酶、囊性纤维化跨膜受体、己糖胺酶A、己糖胺酶B、β-半乳糖苷酶和α-葡糖苷酶。

[0010] 根据以下详细描述，本公开内容的其他特征和优点将变得显而易见。然而，应当理解，虽然详细描述和具体实例说明了本公开内容的具体实施方案，但其仅通过示例的方式给出，因为根据该详细描述，在本公开内容的精神和范围内的各种变化和修改对本领域技术人员而言将变得显而易见。整个文件旨在作为整体的公开内容而相关联，并且应当理解，考虑了本文描述的特征的所有组合，即使特征的组合没有在该文件的相同句子或段落或章节中一起找到。除了前述内容以外，作为附加的方面，本发明还包括以任何方式范围比以上具体提及的变化方案更窄的本发明的所有实施方案。例如，如果本发明的方面被描述为“包含”特征，则也涉及到“由该特征组成”或“基本由该特征组成”的实施方案。

[0011] 在一个实施方案中，本发明公开了组合物在制备用于治疗和/或预防受试者的视觉障碍的药物中的用途，所述组合物包含药学上可接受的眼用载体和药学上有效量的羊毛甾醇。所述受试者患有视觉障碍或处于发展成视觉障碍的风险下，该视觉障碍影响眼内晶状体的正常结构。所述受试者可以选自两栖动物、爬行动物、禽类和哺乳动物；其中所述哺乳动物可以选自啮齿动物、猫、狗、猪、马和人。在另一个实施方案中，所述视觉障碍选自白内障、先天性白内障、皮质混浊(cortical opacities)、后囊下白内障、老视核硬化、视网膜退行性病症、雷夫叙姆病、Smith-Lemli-Opitz综合征、施奈德结晶状角膜营养不良、玻璃疣、年龄相关性黄斑变性和糖尿病视网膜病变，并且羊毛甾醇抑制晶体蛋白聚集。

[0012] 在又一个实施方案中，本发明公开了组合物在制备用于治疗受试者的白内障或失

明/视觉损伤的药物中的用途，所述组合物包含药学上可接受的眼用载体和药学上有效量的羊毛甾醇，其中所述羊毛甾醇溶解所述受试者眼内的晶状体晶体蛋白聚集体；其中所述晶状体晶体蛋白为α-晶体蛋白、β-晶体蛋白或γ-晶体蛋白中的任一种。上述组合物可配制成眼用溶液、眼用软膏、眼用洗剂、眼内输注溶液、用于前房的洗剂、内服药(internal medicine)、注射剂或用于提取的角膜的防腐剂。

[0013] 在又一个实施方案中，本发明公开了一种用于溶解晶体蛋白的淀粉样纤维的方法，该方法包括使淀粉样纤维与羊毛甾醇以足够的量和持续时间接触以便溶解晶体蛋白的淀粉样纤维的步骤，其中该方法可以在原位、体外或体内进行。该方法可以对选自两栖动物、爬行动物、禽类和哺乳动物的受试者进行；其中所述哺乳动物可以选自啮齿动物、猫、狗、猪、马和人。

[0014] 在另一个实施方案中，本发明公开了一种用于治疗和/或预防影响受试者眼睛的正常结构的视觉障碍的试剂盒，该试剂盒包含药学上有效量的羊毛甾醇、药学上可接受的载体的制剂，以及关于施用所述制剂使得所述施用治疗和/或预防所述视觉障碍的说明。在又一个实施方案中，本发明公开了用于治疗和/或预防受试者的视觉障碍的眼用药物组合物，所述组合物包含药学上可接受的眼用载体和药学上有效量的羊毛甾醇；其中所述组合物可配制成眼用溶液、眼用软膏、眼用洗剂、眼内输注溶液、用于前房的洗剂、内服药、注射剂或用于提取的角膜的防腐剂。

[0015] 在另一个实施方案中，本发明公开了一种用于鉴定和/或治疗处于发展成与眼内晶状体晶体蛋白聚集体的形成相关的白内障或失明/视觉损伤的风险下的受试者的方法，该方法包括：a)测定受试者的羊毛甾醇合酶活性的量；b)确定羊毛甾醇合酶活性的量是否小于没有白内障或失明/视觉损伤的对照群体的羊毛甾醇合酶活性的量，其中羊毛甾醇合酶活性的量小于对照群体的羊毛甾醇合酶活性的量指示发展成与晶状体晶体蛋白聚集体的形成相关的白内障或失明/视觉损伤的较高风险；以及c)用羊毛甾醇以有效的量和持续时间治疗受试者以便阻止或逆转受试者眼内晶状体晶体蛋白聚集体的形成，从而鉴定和治疗处于发展成与受试者眼内晶状体晶体蛋白聚集体的形成相关的白内障或失明/视觉损伤的风险下的受试者。

[0016] 在另一个实施方案中，本发明公开了一种鉴定和/或治疗处于发展成与受试者眼内晶状体晶体蛋白聚集体的形成相关的白内障或失明/视觉损伤的风险下的受试者的方法，该方法包括：a)确定羊毛甾醇合酶基因的两个等位基因是否受降低羊毛甾醇合酶表达或活性的突变的影响，其中羊毛甾醇合酶的两个等位基因中突变的存在增加了发展成与受试者眼内晶状体晶体蛋白聚集体的形成相关的白内障或失明/视觉损伤的风险；以及b)用羊毛甾醇以有效的量和持续时间治疗受试者以便阻止或逆转受试者眼内晶状体晶体蛋白聚集体的形成，从而鉴定和治疗处于发展成与受试者眼内晶状体晶体蛋白聚集体的形成相关的白内障或失明/视觉损伤的风险下的受试者。在一个实施方案中，羊毛甾醇合酶基因中的突变在密码子581处，将色氨酸(W)改为精氨酸(R)，或在密码子588处，将甘氨酸(G)改为丝氨酸(S)。

附图说明

[0017] 图1示出了LSS中引起先天性白内障的突变的鉴定。图1A，受影响的家族和白内障

表型的系谱。正方形和圆形分别表示男性和女性。1,野生型等位基因;W581R和G588S为两种突变。图1B,上图,未受影响的个体和具有纯合W581R突变的受影响的儿童(II-1)的DNA测序数据;下图,未受影响的个体和具有纯合G588S突变的受影响的儿童(IV-1)的DNA测序数据。带下划线的序列表示核酸变化。图1C,左图,在患有全白内障的第一系谱中的患者1(IV-1)的右眼的彩色照片;右图,在患有白内障的相同系谱中的患者2(IV-3)的右眼的彩色照片。

[0018] 图2显示LSS突变消除了环化酶的酶功能。图2A,LSS中的W581R和G588在以下若干物种中保守:智人(Homo sapiens)、黑猩猩(Pan troglodytes)、家牛(Bos taurus)、小家鼠(Mus musculus)、褐家鼠(Rattus norvegicus)、原鸡(Gallus gallus)和斑马鱼(Danio rerio)。图2B,LSS结构的计算机建模以及LSS W581R和G588S突变的影响。计算机建模分析鉴定了源自C584并终止于E578的环,其中在该环的末端处W581的关键侧链稳定了甾醇。该环由S-S桥和E578-R639盐桥固定。G588的酰胺氮N与来自前面的螺旋转角的C584相互作用,并且G588的Ca氢与临界E578非常接近,E578随后与同一支持螺旋的R639形成牢固的盐桥。突变G588S导致丝氨酸的侧链碰撞到所述环的E578残基,并且与该结构不相容。箭头指示突变侧链的位置。图2C,野生型蛋白(WT LSS)和LSS突变体的工程化表达对甾醇含量的影响。野生型LSS显著地增加了羊毛甾醇的产量,而W581R和G588S突变体均未表现出任何环化酶活性。每组n=3;***P<0.001。

[0019] 图3显示羊毛甾醇减少了多种晶体蛋白突变蛋白质的细胞内聚集。图3A,人晶状体祖细胞中晶体蛋白聚集体的共聚焦图像。aA-晶体蛋白的致白内障Y118D突变体形成p62阳性细胞内包涵体或聚集体(aggresomes)。绿色,eGFP-晶体蛋白;红色,p62;蓝色,细胞核。用peGFP-N1转染的细胞用作对照。图3B,LSS对晶体聚集体的抑制作用的共聚焦图像。图3C,野生型LSS(WT LSS)和羊毛甾醇而非突变LSS或胆固醇对晶体蛋白突变体聚集的抑制。图3D,通过野生型LSS而非LSS突变体的共表达引起的可溶性aA-晶体蛋白(Y118D)突变蛋白质的增加(与pcDNA3.1-N-Flag共表达的Y118D用作对照)。通过对细胞裂解物的上清液或不可溶部分的蛋白质印迹分析,使用晶体蛋白的密度测定法进行定量分析。每组n=3;代表性的蛋白质印迹分析示于图9c中;*P<0.05,**P<0.01。图3E,预先形成的晶体聚集体被羊毛甾醇再溶解的共聚焦图像。图3F,羊毛甾醇以浓度依赖性方式显著减少了多种致白内障的突变晶体蛋白引起的细胞内聚集(n=3,$P<1\times10^{-4}$)。胆固醇并不减少细胞内聚集(n=3,P>0.1)。图3G,羊毛甾醇增加了人晶状体祖细胞中多种晶体蛋白突变体的可溶部分。n=3;P<0.001。图3H,通过连续活细胞成像,DMSO、胆固醇或羊毛甾醇对人晶状体祖细胞中αA-晶体蛋白Y118D聚集体的影响。图3I,随时间推移,羊毛甾醇对细胞内晶体蛋白聚集体溶解的影响(n=22,来自3个生物重复品)。平均值±SD值显示为黑色符号。数据通过单指数衰减过程进行最佳拟合(红线)。

[0020] 图4显示羊毛甾醇使晶体蛋白的预先形成的淀粉样纤维再溶解。图4A,用脂质体媒介物、脂质体中的胆固醇或羊毛甾醇处理的aA-晶体蛋白突变蛋白质的聚集体的负染色TEM照片。右列羊毛甾醇组的图像在其右侧显示了该图像的5倍放大。图4B,根据ThT荧光,羊毛甾醇对晶体蛋白聚集体的再溶解的影响(n=3)。图4B(i),b/γ-晶体蛋白突变体;图4B(ii),a-晶体蛋白突变体。每条的数据均来自三个独立的样品。

[0021] 图5显示羊毛甾醇降低了白内障严重性并增加了清晰度。图5A,经羊毛甾醇治疗的患有白内障的兔子的晶状体的照片,其显示晶状体清晰度增加。图5A(i),左图,治疗前;图

5A(ii)，右图，治疗后。图5B，羊毛甾醇的治疗效果的定量的箱线图(n＝13)。图5C，经羊毛甾醇治疗的患有白内障的狗的晶状体的照片，其显示晶状体清晰度增加。图5C(i)，左图，治疗前；图5C(ii)，右图，治疗后。D，羊毛甾醇的治疗效果的定量的箱线图(n＝7)。示出了范围、中值(水平线)和平均值(圆圈)。十字形表示测量的最大和最小白内障等级。须触线表示标准差，而方框包含40％置信区间。

[0022] 图6A显示纯合性映射器(homozygosity mapper)将全基因组纯合性绘制为条形图。为了强调感兴趣的区域，任何高于该项目中所达到的最大分数的80％的分数均标注为红色。图6B显示纯合性分数相对于含有LSS基因的21号染色体上的物理位置作图。红色条表示具有最高分数的区域。该染色体的右侧含有LSS基因所在的长连续纯合区。

[0023] 图7示出了用Flag-LSS和eGFP共转染的细胞的代表性共聚焦图像。用野生型或突变的LSS基因和eGFP基因共转染人晶状体祖细胞4h，并在新鲜培养基中培养16h。随后使用抗-Flag抗体对LSS的细胞分布进行可视化(紫色)。eGFP的分布(绿色)用作对照。将细胞核染色并通过Hoechst 33342进行可视化(蓝色)。

[0024] 图8示出了用LSS和多种致白内障的晶体蛋白突变体共转染的细胞的代表性共聚焦图像。图8A，αA-晶体蛋白的R116C突变体。图8B，αB-晶体蛋白的R120G突变体。图8C，βB2-晶体蛋白的V187E突变体。图8D，γC-晶体蛋白的G129C突变体。图8AE，γD-晶体蛋白的W43R突变体。用野生型或突变的Flag-LSS基因和突变GFP-晶体蛋白基因共转染人晶状体祖细胞4h，并在新鲜培养基中培养16h。所有的晶体蛋白突变体都形成p62-阳性聚集体，如通过突变晶体蛋白和p62的共定位所示出的。用GFP-晶体蛋白和pcDNA3.1-N-Flag共转染的细胞用作对照。通过GFP的荧光(绿色)对多种晶体蛋白的细胞内聚集体的形成进行可视化。用抗-Flag抗体检测野生型或突变的LSS(红色)，p62用抗-p62抗体进行染色，而细胞核通过Hoechst 33342染色(蓝色)进行染色和可视化。具有聚集体的细胞的定量分析总结于图3c中。

[0025] 图9显示在HLEB-3细胞(图9A)或HeLa细胞(图9B)中野生型LSS和羊毛甾醇对晶体蛋白突变体聚集的抑制。在分析聚集体之前将用LSS和晶体蛋白突变体构建体共转染的细胞培养24h。通过向细胞培养基中添加40μM甾醇(羊毛甾醇或胆固醇)进行拯救实验(rescue experiment)2h，随后用新鲜培养基代替甾醇培养基，并将细胞培养另外12h。从十个随机选择的视野计算具有晶体蛋白聚集体的细胞的百分比。计算野生型LSS组、突变体组或突变体加羊毛甾醇组的值。与对照组相比，野生型LSS组和羊毛甾醇组的聚集体显著较低($P<1\times10^{-4}$)，而突变LSS组或胆固醇组的聚集体显示与对照组没有差异(P>0.1)。图9C，人晶状体祖细胞用野生型或突变LSS加αA-晶体蛋白(Y118D)进行共转染。与pcDNA3.1-N-Flag共表达的αA-晶体蛋白(Y118D)用作对照。在转染4h并在新鲜培养基中温育另外24h之后，将细胞裂解并离心以分离上清液和不可溶部分。分别用针对Flag和GFP的抗体检测LSS和晶体蛋白融合蛋白。红色箭头表示在含有WT-LSS的细胞的可溶部分中相对于不可溶部分中较高的晶体含量。数据根据三个独立的实验进行定量并总结于图3D中。

[0026] 图10显示当在HLEB-3或HeLa细胞中测定时，羊毛甾醇以浓度依赖性方式显著减少了由多种致白内障的突变晶体蛋白引起的细胞内聚集。图10A，用多种致白内障的晶体蛋白突变体转染的HLEB-3细胞的代表性共聚焦图像。图10B，用多种致白内障的晶体蛋白突变体转染的HeLa细胞的代表性共聚焦图像。将细胞用多种晶体蛋白构建体转染4h，并在新鲜的

培养基中培养另外24h。然后将细胞用在1％(HLEB-3细胞)或2％DMSO(HeLa细胞)中的10μM、20μM和40μM羊毛甾醇处理2h，并培养另外12h。用1％(HLEB-3细胞)或2％DMSO(HeLa细胞)处理的细胞用作对照。多种晶体蛋白的细胞内聚集体的形成通过GFP的荧光(绿色)进行可视化，并用Hoechst 33342将细胞核染色(蓝色)。箭头指示典型的细胞内聚集体。图10C，当在HLEB-3细胞中测定时，羊毛甾醇的聚集-溶解作用的浓度依赖性。图10D，当在HeLa细胞中测定时，羊毛甾醇的聚集-溶解作用的浓度依赖性。

[0027] 图11显示与对照组或突变LSS组相比，羊毛甾醇而非胆固醇处理增加了可溶部分中的致白内障的突变晶体蛋白。图11A，将人晶状体祖细胞用突变晶体蛋白基因转染4h，随后在新鲜培养基中温育另外24h。收获并裂解细胞。上清液和不可溶部分通过离心进行分离，并通过蛋白质印迹分析进行分析。分别用针对Flag和GFP标记物的抗体鉴定LSS和晶体蛋白融合蛋白。用红框突出显示羊毛甾醇处理的组。用1％DMSO处理的细胞用作对照。β-肌动蛋白用作全细胞裂解物(TCL)的内部蛋白质负荷对照。S，上清液；P，不可溶部分。图11B，通过活细胞成像中的单粒子跟踪评估的DMSO(n＝4)和胆固醇(n＝7)对人晶状体祖细胞中αA-晶体蛋白(Y118D)聚集体的大小变化的影响。图11C，通过浊度评估羊毛甾醇对晶体蛋白聚集体的溶解的影响。通过在1M盐酸胍的存在下将5mg ml^{-1}蛋白质溶液在60℃下温育2h(α-晶体蛋白)或在37℃下温育48h(β-和γ-晶体蛋白)来形成晶体蛋白聚集体。将预先形成的聚集体以0.2mg ml^{-1}的最终蛋白质浓度重悬于PBS中，并用在500μM DPPC脂质体中的500μM甾醇进行处理，并在37℃下温育24h。将仅用500μM DPPC脂质体处理的聚集体用作对照。图11D，通过ThT荧光评估的羊毛甾醇对αA-晶体蛋白突变体再溶解淀粉样纤维的浓度依赖性影响。将仅用500μM DPPC脂质体处理的聚集体用作对照。

[0028] 图12示出了白内障晶状体的分级系统。图12A，将晶状体放在网格上，并拍照。将透明度评分为0，清晰的晶状体且不存在浑浊(网格线清晰可见，a')；1，模糊的晶状体和轻度的浑浊(网格线最低限度的混浊，但网格线仍然可见，b')；2，混浊的晶状体且存在涉及几乎整个晶状体的弥漫性浑浊(网格线中度混浊，但主要的网格线可见，c')；或3，不透明的晶状体且存在涉及整个晶状体的广泛、密集的浑浊(网格线完全混浊，且网格线根本无法看到，d')。图12B，在分离出的白内障兔子晶状体中，羊毛甾醇降低了白内障的严重程度并增加了清晰度。将兔子晶状体(n＝13)切开并与羊毛甾醇一起温育6天，随后评估晶状体的清晰度和透明度。示出了显示处理前后的每个白内障兔子晶状体的成对照片，下方为分数。图12C，羊毛甾醇降低了狗的白内障严重程度并增加了晶状体的清晰度。将患有白内障的狗眼睛(n＝7)用羊毛甾醇处理6周，并评估晶状体的清晰度和透明度。示出了处理前后每个研究眼睛的成对照片，下方为分数。还呈现了单独用媒介物处理的三个对照眼睛。

具体实施方式

[0029] 现将详细参考本发明的具体实施方案，包括本发明人预期用于实施本发明的最佳方式。这些具体实施方案的实例在附图中示出。虽然结合这些具体实施方案描述了本发明，但应当理解，其并非旨在将本发明限于所述实施方案。相反，意在涵盖可被包含在如所附权利要求所限定的本发明精神和范围内的替代方案、修改和等同项。在以下描述中，阐述了具体细节以提供对本发明的彻底理解。可以在没有一些或全部这些具体细节的情况下实施本发明。另外，公知的特征可能未进行详细描述，以避免不必要地使本发明变模糊。

[0030] 本发明涉及用于治疗或预防患有视觉障碍或处于发展成视觉障碍的风险下的受试者中影响眼睛正常结构的这类视觉障碍的方法和组合物，其包括向这样的受试者施用包含药学上可接受的载体和药学上有效量的具有式I的甾醇的组合物。例如，本发明的示例性化合物包括向患者施用眼科药学上有效量的羊毛甾醇(3β-羟基-8,24-羊毛甾二烯;8,24-羊毛甾二烯-3β-醇)。

[0031] 在其他实施方案中，本公开内容描述了甾醇和使用甾醇的方法。例如，将式I的甾醇配制在包含药学上可接受的眼用载体的眼用药物组合物中以抑制晶体蛋白聚集。在某些其他实施方案中，本公开内容描述了使用式1的甾醇来抑制晶体蛋白聚集的方法。在其他实施方案中，本发明的化合物能够逆转晶体蛋白的聚集并抑制晶体蛋白的进一步聚集。治疗或预防视觉障碍的方法

[0032] 本发明提供了使用本发明预防和/或治疗患有视觉障碍或处于发展成视觉障碍的风险下的受试者中影响眼内晶状体正常结构的这类视觉障碍的眼用药物组合物和方法。如本文所述，影响眼内晶状体正常结构的视觉障碍(本文中称为短语“视觉障碍”)是指影响晶状体结构从而引起视觉功能障碍如眼睛晶状体的清晰度或硬度变化的状况。这样的状况包括白内障、老视和核硬化。另外，视觉障碍还指视网膜变性，如雷夫叙姆病、Smith-Lemli-Opitz综合征(SLOS)和施奈德结晶状角膜营养不良(SCCD)、无β脂蛋白血症和家族性低β脂蛋白血症。在某些实施方案中，本发明提供了用来减轻或逆转晶体蛋白聚集的组合物及其使用方法。在替代实施方案中，提供了用于抑制、预防和/或治疗蛋白质内或蛋白质间相互作用的破坏的组合物和方法，该相互作用形成对于晶状体透明度和折射率而言必需的宏观结构。

[0033] 如本发明中所提及的术语“白内障”意指表现出引起晶状体表面上和/或内部的混浊或不透明的症状或者诱发晶状体肿胀的症状的疾病或状况，并且该术语包括先天性白内障和后天性白内障(参见PDR Staff,“PDR of Ophthalmic Medicines 2013”,PDR Network,2012)。在一些实施方案中，所述白内障为年龄相关性白内障、糖尿病白内障、与外科手术相关的白内障、由暴露于辐射引起的白内障、由遗传疾病引起的白内障、由感染引起的白内障或由药物引起的白内障。在一些实施方案中，个体患有早发性遗传形式的白内障。这类白内障的具体实例为先天性白内障，如先天性假性白内障(congenital pseudo-cataract)、先天性膜性白内障、先天性花冠状白内障、先天性板层白内障、先天性点状白内障和先天性丝状白内障;以及后天性白内障，如老年性白内障、继发性白内障、褐变白内障(browning cataract)、并发性白内障、糖尿病白内障、外伤性白内障和可由电击、辐射、超声波、药物、全身性疾病和营养失调诱发的其他白内障。后天性白内障进一步包括为了治疗白内障插入晶状体而具有在包含该晶状体的后部中引起混浊的症状的术后白内障。

[0034] 核硬化是指通常在年老的动物中类似地导致晶状体不透明的状况。它是由新纤维形成压缩核中较老的晶状体纤维而引起的晶状体核密度的年龄相关变化。

[0035] 老视是指一种视觉状况，其中眼睛的晶状体失去其柔韧性，这使其难以聚焦于近处的物体。

[0036] 在一些实施方案中，本发明提供了一种治疗或预防视觉障碍的方法，该方法包括向有需要的个体施用有效量的包含具有结构式I的化合物的组合物。在一些实施方案中，该化合物为具有结构式I的甾醇。

[0037] 根据本发明“需要”治疗的个体为患有影响眼内晶状体正常功能的视觉障碍的个体。例如，该个体可患有年龄相关性白内障或白内障或处于发展成年龄相关性白内障或白内障的风险下。处于发展成白内障的风险下的个体包括但不限于，具有发展成白内障的家族史的个体、具有与白内障相关的突变的个体、暴露于辐射的个体、糖尿病个体等。例如，在一方面，该个体被诊断为一只眼患有白内障，且施用所述化合物以预防或减缓对侧眼中白内障的形成。类似地，根据本发明“需要”治疗的个体为可能患有老视或处于发展老视的风险下的个体。类似地，根据本发明“需要”治疗的个体为患有核硬化或处于发展成核硬化的风险下的个体。优选地，该个体为人，然而，本领域技术人员还可以鉴定患有眼病或处于眼病风险下的动物(需要治疗的动物)。可鉴定需要治疗的哺乳动物，如猫、狗、猪、马、牛和啮齿动物。此外，可鉴定需要治疗的动物，如禽类、爬行动物、两栖动物和鱼。

[0038] “治疗”视觉障碍并不需要视觉障碍的100％消除或逆转。在一些实施方案中，与不存在本发明组合物或方法时(例如，在未暴露于本发明组合物或本发明方法的化合物的生物学匹配的对照受试者或标本中)观察到的水平相比，根据本发明方法“治疗”视觉障碍将晶状体的功能障碍例如晶状体的不透明或柔韧性缺乏减轻、抑制、预防和/或逆转了例如至少约5％、至少约10％或至少约20％。在一些实施方案中，与不存在本发明方法的化合物时的晶状体功能障碍相比，功能障碍(如在晶状体上或晶状体中的白内障形成、不透明或晶体聚集)被治疗了至少约30％、至少约40％、至少约50％或至少约60％、至少约70％、至少约80％、至少约90％或更多(约100％)。晶状体功能障碍如不透明或混浊或白内障通常使用多种光学测试中的任一种进行检测，该光学测试包括但不限于视敏度测试、检眼镜检查、裂隙灯检查、角膜曲率测量、眼压测量、对比测试、眩光敏感度、波阵面映射。

[0039] 类似地，“预防”并不需要视觉障碍的100％抑制或遏制。例如，混浊或不透明的任何减少或白内障进展的减速都构成受试者的有益生物效应。同样例如，眼睛晶状体中的晶体聚集的任何减少都构成有益的生物效应。在这方面，与不存在本发明方法时(例如，在未暴露于本发明方法的化合物的生物学匹配的对照受试者或标本中)观察到的水平相比，本发明使视觉障碍减少了例如至少约5％、至少约10％或至少约20％。在一些实施方案中，视觉障碍减少了至少约30％、至少约40％、至少约50％或至少约60％、至少约70％、至少约80％、至少约90％或更多(约100％)。

[0040] 抑制、预防或逆转功能障碍并不需要100％抑制、预防、消灭或逆转。例如，对聚集的任何抑制都构成受试者中的有益生物效应。在这方面，与不存在本发明方法时(例如，在未暴露于本发明方法的化合物的生物学匹配的对照受试者或标本中)观察到的水平相比，本发明将受试者的影响眼睛晶状体正常功能的视觉障碍抑制了例如至少约5％、至少约10％或至少约20％。在一些实施方案中，视觉障碍被抑制、预防和/或逆转了至少约30％、至少约40％、至少约50％或至少约60％。在一些实施方案中，与不存在本发明方法的化合物时的淀粉状蛋白形成相比，本发明方法将淀粉状蛋白形成抑制了至少约70％、至少约80％、至少约90％或更多(约100％)。

[0041] 包含式1化合物的眼用药物组合物的“有效量”为抑制、预防或逆转个体晶状体的功能障碍的量。本发明的眼用药物组合物以治疗视觉障碍的有效量施用于有需要的受试者。如本文所用的，“治疗有效量”意指减轻视觉障碍的体征、症状或病因或任何其他所需生物系统改变中的至少一种的剂量。在预防性应用中，术语“预防有效量”意指施用于易患特

定疾病或处于特定疾病风险下的患者的剂量，其可以是与治疗有效量相同或不同的剂量。用于特定个体的组合物的有效量可取决于个体、个体状况的严重程度、施加的制剂的类型、给药频率和治疗持续时间。根据本发明，本发明的眼用药物制剂例如羊毛甾醇即使在液体滴剂中以相对较低的浓度，例如至少10^{-9}M、至少$0.5x10^{-8}$M至$1x10^{-8}$M、至少$0.5x10^{-7}$M至$1x10^{-7}$M、至少$0.5x10^{-6}$M至$1x10^{-6}$M、至少$0.5x10^{-5}$M至$1x10^{-5}$M、至少$0.5x10^{-4}$M至$1x10^{-4}$M或至少$0.5x10^{-3}$M至$1x10^{-3}$M，或在这些值之间的范围(例如，10^{-9}M至10^{-3}M)内的任何浓度施用，也可以通过每日仅一次、两次、三次或多次施加来逆转这样的视觉障碍，并且如此快速地进行。

给药途径

[0042] 如本领域技术人员将理解的，将化合物施用于受试者的最适当的方法取决于很多因素。在多个实施方案中，根据本发明的化合物局部施用于眼，例如，局部、结膜下、眼球后、眼周、视网膜下、脉络膜上或眼内施用。

[0043] 对于直接施用于眼睛特别有用的药物组合物包括配制成滴眼剂的水溶液和/或悬浮液和配制成眼用凝胶(包括凝胶形成溶液)或软膏的增稠溶液和/或悬浮液，其为眼用溶液、眼用软膏、眼用洗剂、眼内输注溶液、用于前房的洗剂、内服药、注射剂或用于提取的角膜的防腐剂。用于眼用药物递送的其他剂型包括眼用插入物(ocular insert)、玻璃体内注射剂和植入物。可注射溶液可使用细针直接注入角膜、晶状体和玻璃体或其临近组织中。该组合物还可以作为眼内灌注液施用。

[0044] 其他预期的给药途径包括但不限于以下途径中的一种或多种：口服(例如，作为片剂、胶囊或作为可摄取溶液)、经粘膜(例如，作为用于吸入的鼻腔喷雾剂或气雾剂)、经鼻、肠胃外(例如，通过可注射形式)、胃肠、脊柱内、腹膜内、肌肉内、静脉内、子宫内、皮内、颅内、气管内、阴道内、脑室内、大脑内、皮下、经皮、直肠、经颊、硬膜外和舌下。

[0045] 在一些实施方案中，用于将本发明的组合物递送至眼睛的方式是经由接触透镜。可提供用所需化合物预处理的透镜。或者，该透镜在具有用于制备镀膜透镜的组分的试剂盒中提供，该组分以用于重建的冻干粉或以浓缩的或即用型溶液的形式提供。所述组合物可以以单用途或多用途的试剂盒的形式提供。

[0046] 在一些实施方案中，用于将本发明的组合物递送至眼睛的方式是经由眼用棒(rod)(Gwon等人，Ophthalmology.1986年9月;93(9Suppl):82-5)。在一些实施方案中，用于将本发明的组合物递送至眼睛的方式是经由眼内透镜-水凝胶组件(Garty等人，Invest Ophthalmol Vis Sci,2011年8月3日;52(9):6109-16)。

剂量

[0047] 包含所述化合物的组合物以治疗有效量提供，该治疗有效量在医学上可接受的毒性水平下达到所需的生物效果。该组合物的剂量可根据给药途径和疾病的严重程度而变化。该剂量还可根据待治疗的每个患者的体重、年龄、性别和/或症状程度而调整。精确的剂量和给药途径将最终由经治医生或兽医来决定。可以理解，可能需要根据患者的年龄和体重以及待治疗的状况的严重程度对剂量作出常规改变。给药频率取决于制剂和上述参数。例如，可能期望每天施加滴眼剂至少一次，包括每天2、3、4或5次。

[0048] 普通技术人员可容易地确定最佳剂量、给药方法和重复率。最佳剂量可根据特定药物组合物的相对功效和给药方法而变化。可接受的剂量通常可根据在体外和体内动物模型中发现有效的EC50(对于测试组中的50％有效的浓度)来估算。通常，剂量为每kg体重

0.01μg-100g，并且可每天、每周、每月或每年给予一次或多次，或甚至每2至20年给予一次。本领域普通技术人员可容易地根据所测量的药物在体液或组织中的停留时间和浓度来估算给药的重复率。在成功治疗之后，可能期望使患者经受维持治疗以预防疾病状态的复发，其中本文所述的治疗组合物以在每kg体重0.01μg至100g范围内的维持剂量每日施用一次或多次到每20年施用一次。用于经由全身途径施用于人(约70kg体重)的化合物的示例性剂量为每单位剂量0.1mg至5g，例如1mg至2.5g化合物。

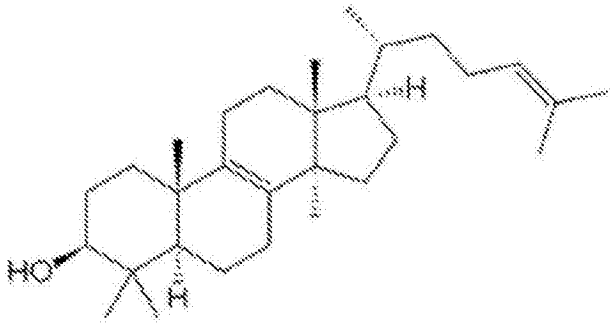
[0049] 式I化合物的优选浓度在约1μg/ml至500μg/ml的范围内，例如，约1μg/ml、约2μg/ml、约3μg/ml、约4μg/ml、约5μg/ml、约10μg/ml、约20μg/ml、约30μg/ml、约40μg/ml、约50μg/ml、约60μg/ml、约70μg/ml、约80μg/ml、约90μg/ml、约100μg/ml、约120μg/ml、约140μg/ml、约160μg/ml、约180μg/ml、约200μg/ml、约250μg/ml、约300μg/ml、约350μg/ml、约400μg/ml、约450μg/ml或约500μg/ml。抑制剂可与其他药物活性剂联合提供。

[0050] 本文所述的药物组合物可以以单剂量或多剂量的形式施用；以单独的治疗剂施用或与其他治疗剂联合施用；以及与常规疗法相组合(可依次或同时施用)。在本发明的一个实施方案中，本发明眼用制剂在人和/或动物治疗中的每日剂量为约每只眼1滴、约每只眼2滴、约每只眼3滴、约每只眼4滴、约每只眼5滴、约每只眼6滴、约每只眼7滴、约每只眼8滴、约每只眼9滴、约每只眼10滴、约每只眼11滴、约每只眼12滴或多于约每只眼12滴。在本发明的另一个实施方案中，本发明眼用制剂在人和/或动物治疗中的每日施用计划为约每天1次、约每天2次、约每天3次、约每天4次、约每天5次、约每天6次、约每天7次、约每天8次、约每天9次、约每天10次、约每天11次、约每天12次或多于约每天12次。例如，可通过外径为3mm的标准药典药用滴管将剂量标准化，该滴管在保持垂直时在25℃下递送20滴总重量为0.9至1.1克的水。

[0051] 当根据上述用药计划施用时，在人和/或动物中的治疗方案可无限期延续或直到未观察到进一步改善。或者，该治疗方案可持续1天、2天、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天、14天、15天、16天、17天、18天、19天、20天、21天、22天、23天、24天、25天、26天、27天、28天、29天、30天、31天、32天、33天、34天、35天、36天、37天、38天、39天、40天、41天、42天、43天、44天、45天、46天、47天、48天、49天、50天、60天、70天、80天、90天、100天、150天、200天、250天、300天、400天、500天、750天、1000天或多于1000天。

在治疗或预防白内障中有效的化合物

[0052] 在多个实施方案中，本发明方法或组合物的化合物为具有式I化合物的羊毛甾醇：



或其前药或药学上可接受的盐。

，

[0053] 例如，本发明方法或组合物的化合物为羊毛甾醇；其前药或药学上可接受的盐。在一个实施方案中，该化合物为羊毛甾醇。在另一个实施方案中，上述化合物的任何前药或药

学上可接受的盐均被认为在本发明的范围内。

药物组合物

[0054] 在本发明的一些实施方案中，一种或多种治疗性化合物的药物组合物可通过将这些治疗性化合物中的一种或多种配制在药学上可接受的载体中来制备。如本文所用的，“药学上或治疗上可接受的载体”是指不干扰活性成分的生物活性的有效性并且对宿主或患者无毒的载体介质。药物制剂中使用的载体类型将取决于施用该治疗性化合物的方法。制备用于多种给药途径的药物组合物的许多方法是本领域公知的。

[0055] 如本文所用的，“药学上可接受的眼用载体”是指用于将结构式1的化合物直接或间接递送至眼睛、眼睛上或眼睛附近的药学上可接受的赋形剂、载体、粘合剂和/或稀释剂。因此，本发明进一步包括包含结构式I的化合物及药学上可接受的眼用载体的组合物。

[0056] 任选地，所述组合物包含结构式I的化合物的游离酸、游离碱、盐(例如，酸或碱加成盐)、水合物或前药。如本文所用的短语“药学上可接受的盐”或“药学上可接受的酸”分别是指式I化合物的药学上可接受的有机或无机盐或酸。反荷离子可为稳定母体化合物上的电荷的任何有机或无机部分。此外，药学上可接受的盐(或酸)可在其结构中具有多于一个带电原子。多个带电原子为药学上可接受的盐(或酸)的一部分的实例可具有多个反荷离子。因此，药学上可接受的盐(酸)可具有一个或多个带电原子和/或一个或多个反荷离子。

[0057] 示例性的盐包括但不限于硫酸盐、柠檬酸盐、乙酸盐、草酸盐、氯化物、溴化物、碘化物、硝酸盐、硫酸氢盐、磷酸盐、酸式磷酸盐、异烟酸盐、乳酸盐、水杨酸盐、酸式柠檬酸盐、酒石酸盐、油酸盐、单宁酸盐、泛酸盐、酒石酸氢盐、抗坏血酸盐、琥珀酸盐、马来酸盐、龙胆酸盐(gentisinate)、延胡索酸盐、葡糖酸盐、葡糖醛酸盐、蔗糖酸盐、甲酸盐、苯甲酸盐、谷氨酸盐、甲磺酸盐、乙磺酸盐、苯磺酸盐、对甲苯磺酸盐和双羟萘酸盐(即，1,1’-亚甲基-双-(2-羟基-3-萘甲酸盐))。药学上可接受的盐可涉及包括另一种分子如乙酸根离子、琥珀酸根离子或其他反荷离子。

[0058] 所述前药为包含与载体部分共价结合的结构式I的化合物的物质。该载体部分可在体外或体内从结构式1的化合物释放，以产生结构式I的化合物。前药形式是本领域公知的，如在Sloan,K.B.,Prodrugs,M.Dekker,New York,1992以及Testa,B和Mayer,J.M.,Hydrolysis in drug and prodrug metabolism:chemistry,biochemistry,and enzymology,Wiley-VCH,Zurich,2003中所例示的。

[0059] 在本发明的一些实施方案中，通过将本发明组合物溶解在适当的溶剂中来制备药物组合物。适当的溶剂包括但不限于水、盐溶液(例如，NaCl)、缓冲溶液、软膏、凝胶或其他溶剂。在某些实施方案中，该溶剂为无菌的。

[0060] 在制备滴眼剂中使用的用于悬浮液的水溶液和稀释剂可包括蒸馏水、生理盐水等。这些药物组合物可通过以下方式配制：根据常规方法将化合物任选地与适当的药物添加剂如赋形剂、崩解剂、粘合剂、润滑剂、稀释剂、缓冲剂、防腐剂、润湿剂、乳化剂、分散剂、稳定剂和溶解助剂一起混合、稀释或溶解，并根据剂型以常规方式配制。用于悬浮液的非水溶液和稀释剂可包括食用(例如植物)油、液体石蜡、矿物油、丙二醇、对辛基十二烷醇、聚山梨醇酯、聚乙二醇、单硬脂酸铝以及类似的溶剂。

[0061] 多种添加剂可根据需要被包含在滴眼剂、眼用凝胶和/或眼用软膏中。这些添加剂可包括适于接触眼或在眼周围使用而没有过度毒性、不相容性、不稳定性、刺激性、变态反

应等的附加成分、添加剂或载体。可在适当情况下向制剂中加入添加剂如溶剂、基质、助溶剂、悬浮剂、增稠剂、乳化剂、稳定剂、缓冲剂、等渗性调节剂、pH调节剂、螯合剂、舒缓剂、防腐剂、矫味剂、调味剂、着色剂、赋形剂、粘合剂、润滑剂、表面活性剂、吸收促进剂、分散剂、防腐剂、增溶剂等。

[0062] 例如，可通过将化合物溶解在溶有表面活性剂的无菌水中并任选地添加适当的药物添加剂如防腐剂、稳定剂、缓冲剂、抗氧化剂和粘度改善剂来配制滴眼剂。

[0063] 例如，添加缓冲剂以保持pH恒定，并且该缓冲剂可包括药学上可接受的缓冲剂，如硼酸盐缓冲液、柠檬酸盐缓冲液、酒石酸盐缓冲液、磷酸盐缓冲液、乙酸盐缓冲液或Tris-HCl缓冲液(包含三(羟甲基)氨基甲烷和HCl)。例如，pH为7.4的Tris-HCl缓冲液包含3g/l的三(羟甲基)-氨基甲烷和0.76g/l的HCl。在又一个方面，缓冲液为10×磷酸盐缓冲盐水(“PBS”)或5×PBS溶液。以提供对于预期的生理条件足够的缓冲能力的量包含缓冲剂。

[0064] 其他缓冲液包括但不限于基于以下物质的缓冲液：在25℃下pK_a为7.5且pH在约6.8-8.2范围内的HEPES(N-{2-羟乙基}哌嗪-N'-{2-乙磺酸})；在25℃下pK_a为7.1且pH在约6.4-7.8范围内的BES(N,N-双{2-羟乙基}2-氨基乙磺酸)；在25℃下pK_a为7.2且pH在约6.5-7.9范围内的MOPS(3-{N-吗啉基}丙磺酸)；在25℃下pK_a为7.4且pH在约6.8-8.2范围内的TES(N-三{羟甲基}-甲基-2-氨基乙磺酸)；在25℃下pK_a为7.6且pH在约6.9-8.3范围内的MOBS(4-{N-吗啉基}丁磺酸)；在25℃下pK_a为7.52且pH在约7-8.2范围内的DIPSO(3-(N,N-双{2-羟乙基}氨基)-2-羟基丙烷))；在25℃下pK_a为7.61且pH在约7-8.2范围内的TAPS({(2-羟基-3{三(羟甲基)甲基氨基}-1-丙磺酸))；在25℃下pK_a为8.4且pH在约7.7-9.1范围内的TAPS({(2-羟基-1,1-双(羟甲基)乙基)氨基}-1-丙磺酸))；在25℃下pK_a为8.9且pH在约8.2-9.6范围内的TABS(N-三(羟甲基)甲基-4-氨基丁磺酸)；在25℃下pK_a为9.0且pH在约8.3-9.7范围内的AMPSO(N-(1,1-二甲基-2-羟乙基)-3-氨基-2-羟基丙磺酸)；在25℃下pK_a为9.5且pH在约8.6-10.0范围内的CHES(2-环己基氨基)乙磺酸)；在25℃下pK_a为9.6且pH在约8.9-10.3范围内的CAPSO(3-(环己基氨基)-2-羟基-1-丙磺酸)；以及在25℃下pK_a为10.4且pH在约9.7-11.1范围内的CAPS(3-(环己基氨基)-1-丙磺酸)。

[0065] 除了缓冲液之外，还可以向滴眼剂中添加等渗剂以制备与泪液等渗的制剂。等渗剂包括但不限于糖类，如右旋糖、葡萄糖、蔗糖和果糖；糖醇，如甘露糖醇和山梨糖醇；多元醇，如甘油、聚乙二醇和丙二醇；以及盐，如氯化钠、柠檬酸钠、苯扎氯铵、盐酸麻黄碱(phedrine chloride)、氯化钾、盐酸普鲁卡因(procaine chloride)、氯霉素(chloramphenicol)和琥珀酸钠。以使滴眼剂的渗透压等于泪液渗透压的量添加等渗剂。

[0066] 可以添加防腐剂以维持滴眼剂和/或眼用软膏的完整性。防腐剂的实例包括但不限于山梨酸、苯扎氯铵、苄十二烷基二甲基铵溴化物(benzododecinium bromide)、对羟基苯甲酸酯、氯丁醇、苄醇、苯乙醇、依地酸二钠、山梨酸、聚季铵盐-1或本领域技术人员已知的其他试剂。

[0067] 在一些实施方案中，使用增稠剂来增加眼用制剂如滴眼剂、眼用凝胶和/或眼用软膏的粘度。可以使用的增稠剂包括但不限于甘油、聚乙二醇、羧甲基纤维素和羧基乙烯基聚合物。

[0068] 除了上述物质以外，在一些实施方案中，还期望使用附加的试剂，包括但不限于稳定剂，如亚硫酸钠、碳酸钠和丙二醇；抗氧化剂，如抗坏血酸、抗坏血酸钠、丁羟甲苯(BHT)、

丁羟茴醚(BHA)、生育酚、硫代硫酸钠;和/或螯合剂,如乙二胺四乙酸(EDTA)、乙二醇-双-(2-氨基乙基)-N,N,N,N-四乙酸(EGTA)和柠檬酸钠。

[0069] 滴眼剂、眼用凝胶和/或眼用软膏可通过无菌操作来制备,或者可替代地在制备的合适阶段进行灭菌。例如,无菌药物组合物可以通过无菌地混合无菌成分来制备。或者,该无菌药物组合物可通过先将成分混合随后将最终制剂灭菌来制备。灭菌方法可包括但不限于热灭菌、辐射和过滤。

[0070] 眼用软膏(眼膏)可通过将活性成分混合至用于制备眼膏的基质中,随后采用本领域已知的任何方法配制成药物制剂来无菌制备。用于眼膏的典型基质的示例为凡士林、jelene 50、plastibase以及聚乙二醇。此外,可以添加表面活性剂以增加亲水性。

[0071] 可使用多种用于活性剂的控制释放的有效方法。参见,例如,Wagh V.D.,Inamdar B.,Samanta M.K.,Polymers used in ocular dosage form and drug delivery systems.Asian J Pharm 2,2008,12-17以及其中引用的参考文献,其内容通过引用并入本文。特别考虑使用聚合物(例如,纤维素衍生物,如羟丙基甲基纤维素(HPMC)和羟丙基纤维素(HPC)、聚(丙烯酸)(PAA)、聚丙烯酸酯、环糊精和天然树胶、聚原酸酯(POE)以及粘膜粘附聚合物);半固体,如凝胶、膜和其他插入物;树脂,如离子交换树脂;离子电渗递送;以及胶粒,如微球和纳米颗粒。

[0072] 本发明的化合物还可以与其他治疗剂联合提供。在一些实施方案中,本发明的化合物可以与其他活性剂共同配制,该活性剂包括但不限于抗感染剂、抗生素、抗病毒剂、抗真菌剂、抗原生动物剂、消炎药、抗过敏剂(包括抗组胺药)、人工泪液血管收缩剂、血管扩张剂、局部麻醉剂、镇痛剂、眼压降低剂、免疫调节剂、抗氧化剂、维生素和矿物质、酶抑制剂或可替代的蛋白酶和肽酶、细胞因子抑制剂等。

[0073] 在多个实施方案中,本发明的化合物还可以与眼部治疗剂联合提供,该眼部治疗剂选自安贺拉(Acular)(酮咯酸氨丁三醇眼用溶液)0.5%、Acuvail(酮咯酸氨丁三醇)、AK-Con-A(萘甲唑啉眼药)、Akten(盐酸利多卡因)、Alamast、Alphagan(溴莫尼定)、Alrex、Astepro(盐酸氮䓬斯汀鼻喷雾剂)、AzaSite(阿奇霉素)、Bepreve(苯磺酸贝他斯汀眼用溶液)、Besivance(贝西沙星眼用悬浮液)、Betaxon、BSS无菌灌洗液、Cosopt、Durezol(二氟泼尼酯)、Eylea(阿柏西普)、Lotemax、Lucentis(雷珠单抗)、Lumigan(比马前列素眼用溶液)、Macugen(哌加他尼)、Ocuflox(氧氟沙星眼用溶液)0.3%、OcuHist、Ozurdex(地塞米松)、Quixin(左氧氟沙星)、Rescula(乌诺前列酮异丙基眼用溶液)0.15%、Restasis(环孢菌素眼用乳液)、Salagen片剂、Travatan(曲伏前列素眼用溶液)、Valcyte(盐酸缬更昔洛韦)、三氟胸苷(Viroptic)、Vistide(西多福韦)、Visudyne(注射用维替泊芬)、Vitrasert植入物、福米韦生注射剂、ZADITOR、Zioptan(他氟前列素眼用溶液)、Zirgan(更昔洛韦眼用凝胶)、Zymaxid(加替沙星眼用溶液)、阿托品、氟比洛芬、毒扁豆碱(Physostimine)、派立明(Azopt)、庆大霉素、匹鲁卡品(Proparacaine)、杆菌肽、羟丙甲纤维素眼液(Goniosol)、多粘菌素B、聚维酮碘(Betadine)、短杆菌肽、泼尼松龙、倍他洛尔、Humorsol、丙美卡因、倍他洛尔眼液(Betoptic)、Hylartin、Propine、布林佐胺、高渗NaCl、Puralube、BSS、吲哚菁绿(Indocycanine Green)、玫瑰红(Rose Bengal)、卡巴胆碱、伊曲康唑、透明质酸钠、头孢唑啉、拉坦前列素、舒洛芬、潇莱威(Celluvisc)、甘露糖醇、土霉素、氯霉素、醋甲唑胺、噻吗洛尔、Ciloxan、咪康唑、妥布霉素、环丙沙星、Miostat、曲安西龙、Cosopt、Muro 128、三氟尿

苷、Demecarium、新霉素、托吡卡胺、地塞米松、甲醋唑胺(Neptazane)、Trusopt、地匹福林、Ocuflox、阿糖腺苷、多佐胺、氧氟沙星、Vira-A、肾上腺素、氧四环素、三氟胸苷、荧光素、苯肾上腺素和适利达(Xalatan)。试剂盒

[0074] 本发明的一些实施方案涉及用于预防和/或减轻与眼病相关的一种或多种症状的试剂盒。该试剂盒可包含含有一种或多种本文所述的治疗性化合物的一个或多个容器。该化合物可以作为制备的药物组合物存在于容器中，或者可替代地，该化合物可以是未经配制的。在这样的实施方案中，该试剂盒可以在容器中包含与药学上可接受的载体分离的未配制的化合物。在使用之前，将该化合物用该药学上可接受的载体稀释或与之混合。

[0075] 本文提供的试剂盒的一些实施方案还包含描述用于以某种方式施用所述药物组合物的方法的说明，与眼病相关的一种或多种症状包括但不限于视网膜变性、老视、白内障和/或眼晶状体的核硬化。在一些实施方案中，该说明还描述了用于将包含在试剂盒中的治疗性化合物与眼用药学上可接受的载体混合的程序。

[0076] 在本发明的一些实施方案中，包含本文所述治疗性化合物的容器是用于眼部施用的容器。在某些实施方案中，该容器是用于施用滴眼剂的滴管。在其他实施方案中，该容器是用于施用眼用凝胶或眼用软膏的管。

[0077] 本发明的一些实施方案通过以下实施例进一步阐明，该实施例不应被解释为限制性的。本领域技术人员将会理解，下面的实施例中公开的技术代表本发明人发现在本文描述的本发明实施方案的实施中运作良好的技术，并因此可被认为构成用于实施这些实施方案的优选方式。然而，根据本公开内容，本领域技术人员将理解，在不脱离本发明的精神和范围的情况下，可以在本文公开的具体实施方案中作出许多改变，并且仍可获得同样或相似的结果。

装置

[0078] 本发明的一些实施方案涉及用于将本发明甾醇施用于受试者的装置。在一些实施方案中，该装置包含含有配制在药学上可接受的载体中的本发明甾醇的内部部分、腔体或储器。在这样的实施方案中，该药学上可接受的载体包括但不限于溶液、凝胶和软膏。在某些实施方案中，该内部部分、腔体或储器含有本文所述的一种或多种含有本发明甾醇的药物制剂。

[0079] 在一些实施方案中，本文考虑的装置还包含与该装置的内部部分、腔体或储器耦合的涂抹器。该涂抹器可以为允许将含有本发明甾醇的药物制剂从内部部分、腔体或储器递送至眼睛的圆柱形、圆锥形或任何其他形状。在优选的实施方案中，该涂抹器为锥形圆筒，其中宽端与内部部分、腔体或储器耦合，并且锥形端形成含有本发明甾醇的药物制剂到眼睛的通路的出口。

[0080] 除非另有定义，本文使用的所有技术和科学术语均具有与本发明所属领域的普通技术人员通常所理解的含义相同的含义。虽然与本文所述的那些类似或等同的任何方法、装置和材料可用于本发明的实施或测试，但现在描述的是优选的方法、装置和材料。

[0081] 本文提及的所有出版物均通过引用而全文并入本文，以用于描述和公开可能与当前描述的发明关联使用的出版物中描述的方法的目的。上文和整个文本中讨论的出版物仅提供其在本申请的申请日之前的公开内容。本文中的任何内容都不应被解释为承认发明人无权因在先发明而早于此公开内容。

[0082] 以下实施例旨在说明本发明，而非以任何方式、形状或形式明确地或隐含地限制本发明。虽然它们是可以使用的典型代表，但也可以替代地使用本领域技术人员已知的其他程序、方法或技术。

实施例1

[0083] 人晶状体主要由组装成对于晶状体透明度和折射率而言必需的高度有序、相互作用的宏观结构的晶体蛋白组成。对蛋白质内或蛋白质间相互作用的任何破坏都将改变此精细结构，使疏水性表面暴露，并伴有后续的蛋白质聚集和白内障形成。在世界范围内白内障是失明的最常见病因，其影响数千万人，并且当前唯一的治疗是手术去除白内障晶状体。晶状体蛋白质阻止聚集和保持晶状体透明度的确切机制在很大程度上都是未知的。羊毛甾醇是在晶状体中富集的两性分子。它由羊毛甾醇合酶(LSS)在胆固醇合成途径的关键环化反应中合成。这里我们鉴定了患有广泛先天性白内障的两个家庭的两种不同的纯合LSS错义突变(W581R和G588S)。这两种突变均影响高度保守的氨基酸残基并削弱LSS的关键催化功能。野生型而非突变的LSS的工程化表达阻止多种致白内障的突变晶体蛋白的细胞内蛋白质聚集。羊毛甾醇而非胆固醇治疗显著减少了在体外和细胞转染实验中预先形成的蛋白质聚集体。我们进一步证明了羊毛甾醇治疗既可以在体外降低切开的兔子白内障晶状体的白内障严重程度并增加透明度，又可以在体内降低狗的白内障严重程度。我们的研究将羊毛甾醇鉴定为在阻止晶状体蛋白质聚集中的关键分子，并提出了用于白内障预防和治疗的新策略。

[0084] 白内障占全世界所有失明病例的半数以上，且唯一确定的治疗包括手术去除不透明的晶状体。在发达国家，由于该疾病在老年人群中的高患病率，使得白内障手术占医疗保健费用的很大一部分。此外，在发展中国家中也有与白内障相关的高发病率，其中手术治疗的机会是有限的。

[0085] 晶状体纤维中高浓度的晶体蛋白有助于晶状体的透明度和折射性能[2]。晶体蛋白超家族由a-、b-和c-晶体蛋白组成，它们是人体内最高度浓缩的细胞内蛋白质中的一些。蛋白质聚集是白内障形成中的一个最重要的因素[3]。导致蛋白质聚集的因素包括晶体蛋白中的突变，已知这些突变导致先天性白内障或氧化应激，这转而促成年龄相关性白内障。然而，晶状体蛋白质保持透明度或引起浑浊的确切机制尚未完全了解。

[0086] 羊毛甾醇合酶(2,3-氧代角鲨烯-羊毛甾醇环化酶，LSS;EC 5.4.99.7)由LSS基因编码。LSS蛋白催化(S)-2,3-氧代角鲨烯转化为羊毛甾醇，这是胆固醇、类固醇激素和维生素D的生物合成中的关键早期限速步骤(参考文献4)。发现LSS在晶状体中表达[5]。据先前报道，LSS和FDFT1(法呢基二磷酸酯法呢基转移酶1)上的亚效等位基因突变的特定组合可以降低晶状体中的胆固醇水平并在Shumiya白内障大鼠(SCR)中导致白内障[6]。这里我们在两个血缘家庭中鉴定LSS基因中的新型纯合突变，并研究羊毛甾醇减轻蛋白质聚集和白内障形成的能力。

[0087] 鉴定了来自高加索人血统的血缘家庭的患有重度先天性白内障的三名儿童(图1a)。进行全外显子组测序至目标区域上平均不低于55倍深度覆盖(表1a)，以便鉴定致病突变。平均在每个外显子组中检测到60,800-80,800个SNP(表1b)。使用血缘隐性模型和针对包括dbSNP和1000基因组计划(1000Genomes Project)在内的公共数据库中的共同变体(次等位基因频率为0.5％)过滤以及突变功能预测(通过SIFT[7]、Polyphen2[8]、Phylop[9]和

Mutationtaster[10]预测)，我们缩小了潜在的候选基因变体，并将21号染色体上的LSS中的变体(G588S)鉴定为最可能的候选物(表1c)。三名受影响的儿童对于LSS中的GRA转换(G588S)是纯合的，(GRch37/hg19:chr21:47615645;NM_001001438.2:c.1762G.A,NM_001001438.1:p.G588S)，而未受影响的父亲、母亲和其余儿童对于该变化是杂合的(图1a，图1b)。全基因组SNP基因分型通过HomozygosityMapper[11]鉴定了三个长的连续纯合区(chr2:q22.1-q24.1、chr2:q31.1-q32.1和chr21:q22.3;图6a和表1d)。LSS基因位于21号染色体上的一个纯合区中(图6B)。此外，我们在154个患有先天性白内障的家庭中针对LSS基因中的突变进行筛选，并在第二个血缘家庭中鉴定了另一个纯合突变，W581R(GRch37/hg19:chr21:47615666;NM_001001438.2:c.1741T.C,NM_001001438.1:p.W581R)(图1A，图1B，图1C)。这两种突变在11,000个对照染色体中是不存在的。

[0088] LSS中的氨基酸残基W581和G588是高度保守的(图2A)。我们进行了计算建模分析，以研究W581R和G588S突变对LSS的3D结构和功能的影响。已报道位置581处的氨基酸色氨酸对环化酶活性的催化位点有贡献[12]。G588S突变体通过原位替换及随后的侧链精修(refinement)来建模。S588侧链精修不能解决丝氨酸侧链与E578的骨架羰基之间的范德华力碰撞，该骨架羰基与R639形成关键的盐桥。E579:C584环的取向需要扭曲以适应该突变。突变S588的侧链碰撞到相邻的环，表明该突变与LSS的正常酶结构和功能不相容(图2B)。该计算机模拟结果得到以下证据的支持：细胞转染实验中野生型LSS的表达显示出环化酶活性，并显著增加了HeLa细胞的脂质部分中羊毛甾醇的产生量，而W581R和G588S突变蛋白质均未显示任何环化酶活性(图2C)。相反，胆固醇水平未受野生型或突变LSS的表达的影响，这表明可能存在胆固醇体内平衡的替代途径。与野生型LSS相比，W581R和G588S突变没有改变亚细胞定位或导致LSS蛋白的聚集体，这表明白内障表型并非由于通过突变LSS蛋白自身形成光散射粒子而产生(图7)。晶体蛋白——晶状体中主要的结构蛋白质——的聚集是多种类型的白内障的主要病因[3]。为了模拟白内障晶状体中的蛋白质聚集，在人晶状体祖细胞、人晶状体上皮细胞系B-3(HLEB-3)或HeLa细胞中表达六种已知的致白内障突变晶体蛋白。在全部三种转染的细胞系中，这些突变晶体蛋白形成p62-阳性包涵体/聚集体，这表明聚集是突变晶体蛋白的固有特性(图3A以及图8和图9)[13]。野生型LSS和致白内障的突变晶体蛋白的共表达显著减小了细胞内晶体蛋白聚集体的数目和大小，而LSS突变体不能单独实现这一点(图3B、图3C以及图8和图9)。蛋白质印迹分析表明，aA-晶体蛋白的Y118D突变体从细胞内聚集体中释放，并且具有野生型LSS的变得更易溶(图3D和图9C)。此外，在共表达LSS突变体和突变晶体蛋白的细胞的培养基中添加羊毛甾醇而非胆固醇成功地减少了晶体蛋白聚集(图3C以及图8和图9)。该结果表明，羊毛甾醇而非胆固醇可能是从聚集释放突变晶体蛋白的有效药剂。

[0089] 该假设得到以下证据的支持：羊毛甾醇以浓度依赖性方式显著抑制了野生型和突变的晶体蛋白的聚集体形成，而胆固醇没有效果(图3E、图3F和图10)。羊毛甾醇而非胆固醇增加了细胞裂解物的可溶部分中突变晶体蛋白的量(图3G和图11A)。利用表达aA-晶体蛋白的GFP-融合Y118D突变体的细胞的连续活细胞成像，我们证明了添加羊毛甾醇可以有效减少半衰期为22268分钟的晶体蛋白聚集体(图3H)，而添加DMSO或胆固醇没有减少聚集体形成(图11B)。活细胞中的单粒子跟踪清楚地显示，羊毛甾醇在分解预先形成的细胞内蛋白质聚集体中具有重要作用。

[0090] 为了研究羊毛甾醇是否对聚集的蛋白质的分解具有直接作用，通过在1M盐酸胍的存在下加热野生型和突变的晶体蛋白获得了五种野生型晶体蛋白和九种突变晶体蛋白的聚集体。在该条件下，如由硫代黄素T(ThT)荧光的增强、在负染色的透射电子显微镜(TEM)下的纤维结构以及低浊度值所显示的(图4和图11C)，所有晶体蛋白均形成了淀粉样纤维。在此获得的淀粉样纤维的形态类似于先前报道的那些晶体蛋白[14]。由二棕榈酰磷脂酰胆碱(DPPC)形成的含PBS的脂质体用来增加甾醇化合物的溶解度并模拟甾醇在细胞膜中的状态。如由负染色的TEM照片中纤维结构的消失和ThT荧光强度的减小所示的(图4和图11D)，羊毛甾醇而非胆固醇成功地以浓度依赖性方式再溶解了来自淀粉样纤维的聚集的晶体蛋白。作为一个实例，再溶解的αA-晶体蛋白可以在负染色的TEM图片中得到确认，并且大小为约15nm(图4A)[15]。

[0091] 为了评估羊毛甾醇对减少晶状体组织中的白内障的作用，从兔子中分离出天然存在的白内障晶状体，并将其在25mM羊毛甾醇溶液中温育6天，并比较羊毛甾醇治疗前后的晶状体清晰度。如由晶状体清晰度的增加所示的($P<0.003$，Wilcoxon检验，图5A，图5B，表2A以及图12A、图12B)，观察到强烈的白内障严重程度降低趋势。我们进一步研究了羊毛甾醇在体内逆转狗的白内障的作用。羊毛甾醇治疗显著降低了白内障严重程度，并增加了晶状体清晰度($P<0.009$，Wilcoxon检验，图5C，图5D；表2B和图12C)。

[0092] 影响LSS的催化功能的纯合突变导致伴有严重视觉丧失的广泛先天性白内障。羊毛甾醇在白内障预防中的关键作用得到以下观察结果的支持：携带复合LSS突变的大鼠品系概括了人白内障疾病表型[6]。与此见解相一致的是，发现LSS抑制剂(也称为氧代角鲨烯环化酶抑制剂)U18666A对LSS的抑制导致白内障[16]。此外，羊毛甾醇治疗显著地减少了在细胞培养物中由突变晶体蛋白引起的蛋白质聚集，同时在动物模型中降低了预先形成的白内障严重程度，从而增加了晶状体清晰度。可以想象，羊毛甾醇的两性性质允许其嵌入并包覆大蛋白质聚集体的疏水核区域，从而有效地使这些聚集再次逐渐变为水溶性的。

[0093] 总之，羊毛甾醇在抑制晶状体蛋白质聚集和减少白内障形成方面发挥关键作用，这提出了预防和治疗白内障的新策略。白内障是失明的主要病因，并且每年有数百万患者接受白内障手术以去除不透明的晶状体。该手术虽然非常成功，但伴随有并发症和发病率。因此，用于逆转白内障的药理学治疗可具有巨大的健康和经济影响。此外，通过鼓励研究小分子方法，如本文所示的方法，我们的结果对于治疗蛋白质聚集疾病可具有更广泛的意义，该蛋白质聚集疾病包括神经变性疾病和糖尿病，它们共同成为老年人群中发病率和死亡率的重要病因。

方法

[0094] 研究参与者。所有参与者均进行标准的全面眼科检查和影像学研究。在首次访视时收集人口数据、风险因素和血液样品。我们招募了一个由两名成人和四名儿童组成的血缘家庭。父母是嫡堂表亲，并且他们四个孩子中的三个被诊断为患有视网膜变性和白内障(图1A)。我们在另外154个先天性白内障系谱中针对LSS突变进行筛选，并鉴定了另一个具有纯合W581R突变的家庭。

[0095] 外显子组捕获和测序。使用Agilent SureSelect Human All Exon试剂盒(溶液中)根据制造商的方案进行外显子组捕获。简而言之，通过Covaris将基因组DNA样品进行随机片段化，其中所得片段的碱基对峰为150-200bp，并且衔接子与片段的两端连接。使用

Agencourt AMPure SPRI珠子对衔接子连接的模板进行纯化，并且切下插入片段大小约为250bp的片段。提取的DNA通过连接介导的PCR进行扩增，纯化，并与SureSelect Biotinylated RNA文库(BAITS)杂交以供富集。杂交的片段与链霉亲和素珠子结合，而非杂交的片段在24h后被洗出。使捕获的连接介导的PCR产物在Agilent 2100生物分析仪上分析以估算富集的量级。随后将每个捕获的文库加载到Illumina Genome Analyzer II平台上，并以读长90bp进行配对末端测序，这为每个样品提供了至少50×的平均覆盖深度。由采用默认参数的Illumina碱基判定软件处理原始图像文件。

[0096] 读序作图和变体检测。使用BWA[17](0.5.9-r16版)将每个个体的序列读序与人参考基因组(NCBI版本37，hg19)进行比对。随后利用基因组分析工具箱(Genome Analysis ToolKit)(GATK 2.8版)用GATK[18]最佳实践管线(best practice pipeline)处理由BWA创建的BAM文件，以进行再比对和变异(SNV和插入缺失)检测。提取通过VQSR过滤标准的变异以供后续分析。

[0097] 使用推荐的参数通过SOAPsnp(v1.03)和BWA(0.5.9-r16版)对目标区域中的共有基因型进行判定。Phred样质量至少为20且覆盖深度至少为4×的共有基因型被认为是高可信度基因型。提取与参考不同的基因型作为候选SNP，并且如下过滤SNP结果：Phred样SNP质量≥20，总深度为4×至200×，拷贝数估计值<2，并且两个邻近SNP之间的距离不小于5bp。

[0098] 遗传变体的功能注释。变体使用ANNOVAR进行功能注释并分类为错义突变、无义突变、连读突变和剪接位点突变，它们与同义突变和非编码突变相比很可能是有害的。基于这些注释，首先针对非同义、剪接受体位点和供体位点对变体进行过滤，随后针对可获得的公共数据库(dbSNP129和1000基因组变体数据库)进行过滤。被发现在所述三位受影响的受试者中是纯合突变且在携带者(父母)中是杂合突变但在公共数据库中不存在的变体，被认为是候选致病变体。

[0099] LSS和基因的突变筛选。进行Sanger DNA测序以验证LSS中的G588S突变。将LSS基因中的22个外显子通过PCR进行扩增，并在遗传分析仪(Genetic Analyzer)3130(Applied Biosystems)上进行测序。用来扩增LSS中的外显子的引物示于表3A中。我们在154个患有先天性白内障的家庭中针对LSS基因中的突变进行了筛选，并在第二个血缘家庭中鉴定了另一个纯合突变W581R。这两种突变在11,000个对照染色体(包括来自San Diego的未受影响的对照人群和1000基因组计划的2,000个染色体，以及来自华盛顿大学外显子组测序数据库的8,000个染色体)中不存在。由于先前报道FDFT1突变修饰白内障表型，因此我们筛选了FDFT1基因中的变体，鉴定了仅一个共同的非同义变体rs4731(GRch37/hg19:chr8:11666337;NM_001287742.1:c.134A.G,NM_001274671.1:p.K45R)。因为未受影响的女儿携带相同的纯合变化，并且相对较高频率的一般人群具有该变体(在1000基因组计划数据中次等位基因频率为.4%)(表1E)，所以该变体作为致病突变被排除。

[0100] G588S突变的3D建模。由Ruf等人[20]确定并且以条目1W6K和1W6J[12]登录在蛋白质数据库中的两个结构来构建G588S突变体的模型。使用X射线坐标构建该酶的全原子模型，并且使用内坐标力学程序(Internal Coordinate Mechanics program)(ICM)及其PDB转换方案[21]将该模型精细化。为了分析G588S突变诱导的碰撞对羊毛甾醇结合的影响，我们使用1W6K结构分析了与羊毛甾醇相互作用的酶的口袋所涉及的所有侧链。将接触面积计算为具有羊毛甾醇与不具有羊毛甾醇的每个残基的溶剂可及面积之间的差，并且使用ICM程序[22]按

大小对接触面积进行排序。

[0101] 质粒构建体和定点诱变。含有LSS cDNA的克隆购自Thermo Scientific Inc.。将野生型LSS的编码序列克隆并插入pcDNA3.1-N-Flag质粒(Invitrogen)中。通过使用PCR的重叠延伸经由定点诱变来构建突变体。共同PCR引物为:NdeI正向,59-CATATGACGG AGGGCACGTGTCT-39,以及XhoI反向,59-CTCGAGTCAGGGGTGGCCA GCAAG-39。用于构建W581R和G588S突变体的引物为:W581R正向,59-TGGGAAGGCTCCCGGGGAGTTTGCT-39;反向,59-GTGAAGCAAACTCCCCGGGAGCCTTC-39;G588S正向,59-GCTTCACCTACAGCACCTGGTTTG-39;G588S反向,59-CCAAACCAGGTGCTGTAGGTGAAG-39。将含有野生型或突变的LSS基因的重组pcDNA3.1-N-Flag质粒转化到大肠杆菌(E.coli)DH5a细胞中。如先前所述[23-26],从人晶状体的总cDNA克隆aA-、aB-、bB2-、cC-和cD-晶体蛋白的cDNA。使用表3B中列出的引物通过定点诱变构建突变体。扩增的片段用XhoI和BamHI消化,随后插入真核表达载体peGFP-N1或原核表达载体pET28a中。使用Plasmid Maxiprep试剂盒(Vigorous)获得质粒,并通过DNA测序进行验证。将晶体蛋白基因构建体制备为C-末端eGFP融合蛋白,而将LSS制备为N-末端Flag标记的蛋白质。

[0102] 细胞培养和转染。HeLa细胞和人晶状体上皮B-3细胞(HLEB-3)从ATCC获得。从人胎儿眼中分离出人晶状体祖细胞[27]。将HeLa细胞在含有10%FBS的DMEM培养基(Gibco)中培养。将HLEB-3细胞在含有20%FBS的F12培养基中培养,而将人晶状体祖细胞在含有20%FBS和10mg ml^{-1}FGF的MEM培养基(Gibco)中培养。所有细胞均在37℃下在5%CO_2培养箱中培养。细胞经常规测试为支原体污染阴性。

[0103] 为了评估LSS表达对甾醇含量的影响,将HeLa细胞用在编码区的N-末端处与Flag标记物融合的野生型LSS或LSS突变体进行转染。在24h转染后收获细胞并提取脂质部分以供LC-MS分析。用载体pcDNA3.1-N-Flag质粒转染的细胞用作对照。通过使用小鼠抗-Flag(F1804;Sigma-Aldrich)和小鼠抗肌动蛋白抗体(BS6007M;Bioworld Technology)的蛋白质印迹分析对野生型和突变LSS的表达水平进行归一化。

[0104] 为了评估羊毛甾醇对晶体蛋白聚集的影响,将人晶状体祖细胞用LSS和多个晶体蛋白构建体共转染4h。用晶体蛋白突变体和pcDNA3.1-N-Flag共转染的细胞用作对照。在分析聚集体之前将用LSS和晶体蛋白突变体构建体共转染的人晶状体祖细胞培养12h。在16h后通过向细胞培养基中添加40mM甾醇(羊毛甾醇或胆固醇,Sigma-Aldrich)进行拯救实验2h,随后将培养基替换为新鲜培养基并培养细胞24h。从十个随机选择的视野计算具有晶体蛋白聚集体的细胞的百分比。计算野生型LSS组、突变体组和突变体加羊毛甾醇组的值。用1%DMSO处理的细胞用作对照。

[0105] 在单盲观察者研究中评估LSS和羊毛甾醇对细胞内晶体蛋白聚集的影响。实验重复至少三次。使用Student t检验计算P值。荧光显微镜检查。将等量的人晶状体祖细胞、HLEB-3细胞或HeLa细胞接种在用TC(Solarbio)预处理的玻璃盖玻片上。在培养24小时达到90%汇合后,将细胞用含有不同LSS或晶体蛋白基因的质粒进行转染,或用含有某个晶体蛋白基因的质粒和含有野生型或突变的LSS基因的质粒进行共转染。对照是用含有peGFP-N1和/或peDNA3.1-N-Flag的质粒转染的细胞。使用Lipofectamine 3000(Invitrogen)根据制造商的说明进行转染和共转染。

[0106] 根据Flag-LSS和晶体蛋白-GFP在人晶状体祖细胞、HLEB-3细胞或HeLa细胞中的共

表达来评估野生型或突变的LSS对多种致白内障的晶体蛋白突变体的细胞内聚集的影响。使用GFP或抗Flag抗体对蛋白质的细胞内分布进行可视化。在共转染4h之后，将细胞在新鲜培养基中培养24h，随后通过显微镜检查进行分析。

[0107] 通过用含有不同晶体蛋白基因的质粒转染细胞来研究羊毛甾醇或胆固醇对多种晶体蛋白的聚集体形成的影响。将细胞温育24h以实现高效的蛋白质表达和聚集体形成。随后用在1％(对于人晶状体祖细胞)或2％DMSO(对于HeLa细胞)中的0-40mM甾醇处理细胞。用1％或2％DMSO处理的细胞用作对照。在处理2h之后，将培养基替换为新鲜培养基。在12h之后，将细胞用于显微镜分析。

[0108] 如下制备显微镜检查样品：将玻片用磷酸盐缓冲盐水(PBS)洗涤三次。将细胞用4％低聚甲醛固定40min，接着用PBS另外洗涤三次。将细胞用在PBS中的0.1％Triton X-100(Sigma)透化处理10min，并用在PBS中的5％正常山羊血清在37℃下封闭1h。如下进行免疫染色：添加在含有5％正常山羊血清的PBS缓冲液中的小鼠抗-Flag抗体(1:500)或小鼠抗-p62抗体(1:200，ab56416;Abcam)，并在37℃下温育1h。随后将玻片用PBS洗涤三次，并进一步与Alexa 649缀合的山羊抗小鼠IgG(1:250)一起在环境温度下温育1h。用Hoechst 33342(Invitrogen)对细胞核进行复染。使用Carl Zeiss LSM 710共聚焦显微镜分析所固定的细胞。

[0109] 活细胞成像。将人晶状体祖细胞用含有αA-晶体蛋白(Y118D)突变体的质粒进行转染。在24h转染期之后，通过在含有0.8mg ml^{-1}G418的培养基中温育7天来筛选具有αA-晶体蛋白(Y118D)突变体的稳定表达的细胞。随后，将获得的细胞接种到玻璃底细胞培养皿(In Vitro Scientific)上，并用1％DMSO、在1％DMSO中的40mM胆固醇或在1％DMSO中的40mM羊毛甾醇处理4h。添加新鲜培养基，并通过连续活细胞成像来分析细胞。用Olympus IX81显微镜观察活细胞图像并用CellSens Dimension软件(Olympus)捕获图像。通过在活细胞成像中使用单粒子跟踪测量p62-阳性聚集体的荧光密度进行聚集体大小的定量分析。使用三个生物重复品(每个重复1-8次)进行活细胞成像。

[0110] 细胞的脂质提取。使用Bligh和Dyer的方法[28]进行脂质的提取。简而言之，将约1×10^{6}-10^{7}个HeLa细胞用PBS洗涤3-5次，随后在400-ml冰冷的甲醇中刮下，并转移至添加有200ml氯仿的1.5ml Eppendorf管中。将样品涡旋搅拌1min，随后与300ml的1M KCl混合。通过在4℃下以20,817×g微量离心5min来分离有机相和水相。分离后，收集下部的有机相。随后使用300ml氯仿将剩余的水相再萃取两次。使用SpeedVac样品浓缩器在真空下干燥收集的有机相。将干燥的样品在280℃下储存以供进一步的LC-MS分析。

[0111] LC-MS分析。将干燥的脂质提取物重悬于100ml甲醇中。将样品涡旋搅拌10min，通过80W超声波降解法处理30min，在20,817g下微量离心10min，随后将上清液转移至新的Eppendorf管中。微量离心处理重复三次。使用替代的气压化学电离(APCI)源，通过Agilent1290/6460三级四极(triple quadrupole)LC/MS分析得到的样品。使用Agilent SB-C18柱分离脂质。使用电子电离模式进行选择性离子监测。使用高纯度的羊毛甾醇和胆固醇作为对照。使用350℃的气体温度、41 min^{-1}的气体流速、60p.s.i.的喷雾器、350℃的汽化器、3,500V的毛细管和4mA的电晕电流进行MS测定。为了优化灵敏度和特异性，针对每种化合物的MS分析选择两种限定离子(qualifier ion)(对于胆固醇为369.3/161.1和369.3/147，而对于羊毛甾醇为409.2/191.3和409.2/109)。蛋白质印迹法。在含有50mM Tris(pH 8.0)、150mM NaCl、1％Triton X-100、1mM EDTA、0.5％脱氧胆酸钠和0.1％SDS的RIPA缓冲

液中制备细胞裂解物。通过离心分离上清液和沉淀部分。通过12.5％SDS-PAGE分离蛋白质并将该蛋白质转移至PVDF膜(GE Healthcare)。分别使用小鼠抗Flag抗体(F1804;Sigma-Aldrich)或抗GFP抗体(MB2005;Bioworld Technology)鉴定超表达的LSS和晶体蛋白。使用软件GELPRO(Media Cybernetics)来实现对蛋白质印迹条带的定量。从三个独立实验计算所呈现的定量数据。

[0112] 蛋白质表达和纯化。使His标记的重组野生型和突变b-和c-晶体蛋白在大肠杆菌中超表达，并使用Ni-NTA亲和柱及随后的凝胶过滤色谱法，采用如别处所述[23,24,26,29]的相同方案进行纯化。如先前所述[30]进行非标记的αA-和αB-晶体蛋白的超表达和纯化。如通过12.5％SDS-PAGE、10％非变性PAGE上的一个均质条带和尺寸排阻色谱图中的单峰所评估的，蛋白质的纯度被估计为95％以上。通过使用BSA作为标准，根据Bradford法测定蛋白质浓度[31]。在含有150mM NaCl、1mM EDTA和1mM DTT的20mM PBS缓冲液中制备所有蛋白质样品。

[0113] 蛋白质聚集和聚集体分解。通过在60℃下加热含有1M盐酸胍(超纯，Sigma-Aldrich)的浓度为5mg/ml的蛋白质溶液2h获得野生型和突变的αA-和αB-晶体蛋白的聚集体。通过在37℃下加热含有1M盐酸胍的蛋白质溶液48h制备野生型和突变的b-和c-晶体蛋白的聚集体。聚集体的形成通过ThT荧光、浊度(400nm下的吸光度)和透射电子显微镜(TEM)观察来确认。将预先形成的聚集体以0.2mg/ml(约10mM)的最终浓度重悬于20mM PBS中。在37℃下用在由500mM DPPC(Sigma-Aldrich)形成的脂质体中的500mM羊毛甾醇或胆固醇处理重悬的聚集体。用500mM DPPC脂质体处理的聚集体用作阴性对照。在处理24h之后，将蛋白质溶液用于ThT荧光、浊度和负染色的TEM观察。通过将蛋白质溶液沉积到新鲜辉光放电的碳涂覆的铜网格上来制备TEM样品。通过用1.25％乙酸铀酰对网格染色30s获得负染色样品。在电压为120kV且放大倍数为48,000的Hitachi H-7650B透射电子显微镜上得到负染色的TEM图片。

[0114] 白内障兔子晶状体的处理。通过吸入CO_2对兔子施以安乐死且立即切开晶状体，并用媒介物或溶解于媒介物中的羊毛甾醇处理以制成25mM溶液。将晶状体组织在室温下、黑暗中在这些溶液中温育6天。在显微镜下检查白内障并拍照。白内障的程度由不知情的检查者使用先前描述的浑浊分级系统进行评估，如下所示[32,33]。通过目测和分级对晶状体清晰度和透明度的改善进行定量。通过透光性、晶状体下面的网格图像的清晰度(图12)以及晶状体总体清晰度的改善或皮质性白内障局部区域的清晰度的改善来对晶状体清晰度进行评分。使用Wilcoxon检验来评估治疗效果。

[0115] 白内障分级系统。0级：不存在浑浊(网格线清晰可见)；N 1级：轻微程度的浑浊(网格线最低程度的混浊，但网格线仍然可见)；N 2级：存在涉及几乎整个晶状体的弥漫性浑浊(网格线中度混浊，但主要的网格线可见)；N 3级：存在涉及整个晶状体的广泛、密集的浑浊(网格线完全混浊，且网格线根本不可见)。

[0116] 载药纳米颗粒的制备。通过修改的纳米沉淀法[34]将羊毛甾醇负载到脂质-聚合物混合纳米颗粒中。简而言之，将所需浓度的羊毛甾醇与溶解在乙腈中的聚己内酯(PCL)聚合物混合。以该PCL聚合物重量的20％，将卵磷脂和1,2-二硬脂酰-sn-甘油基-3-磷酸乙醇胺-N-羧基(聚乙二醇)2000(DSPE-PEG-COOH)溶解在4％乙醇水溶液中，并加热至60℃以上。随后在轻柔搅拌下将羊毛甾醇/PCL溶液添加至预热的脂质溶液中，接着剧烈涡旋3min。随后将该混合溶液搅拌2h以使纳米颗粒形成并使乙腈蒸发。然后，使用截留分子量为10kDa的Amicon

Ultra-4离心过滤器(Millipore)将纳米颗粒溶液洗涤三次，以去除剩余的有机溶剂和游离分子。随后将所得纳米颗粒重悬于PBS缓冲液中以供后续使用。通过动态光散射表征载药纳米颗粒的大小、大小分布和表面ζ电位。通过高效液相色谱法对羊毛甾醇的负载量进行定量。

[0117] 狗的白内障晶状体的治疗。为了评估羊毛甾醇治疗对活动物的白内障的效果，采用肌肉内注射乙酰丙嗪(acepromaxine)和布托啡诺对狗进行预先药物治疗。20min之后，通过静脉内施用异丙酚进行麻醉诱导。随后立即给狗插管并保持21 min^{-1}的氧气和2％异氟烷。首先使用28号针头将负载羊毛甾醇(100mg)的纳米颗粒注射到测试眼的玻璃体腔中，随后在实验期间每3天给予一次。治疗眼或假治疗眼是随机的。向对照眼给予具有空纳米颗粒载体的注射剂作为阴性对照。采用在局部滴眼剂中的羊毛甾醇(参见以下滴眼剂配方)对治疗眼进行治疗。在6周内，每日三次将一滴50-ml羊毛甾醇施用于测试眼。通过裂隙灯检查白内障严重程度并在6周治疗期开始和结束时拍照。在检查之前，用1％托品酰胺和10％苯肾上腺素散瞳。白内障严重程度由不知情的检查者来评估，并根据如下所示的犬白内障阶段进行评分[35]。对晶状体清晰度和透明度的改善进行定量。使用Wilcoxon检验评估治疗效果。

[0118] 犬白内障的分级系统。0级：不存在浑浊(无白内障)；N 1级：轻微程度的浑浊(初期)；N 2级：存在涉及几乎整个晶状体的弥漫性浑浊(未成熟期)；N 3级：存在涉及整个晶状体的广泛、密集的浑浊(成熟期)局部媒介物溶液。将双蒸H_2O添加至与0.055g烷基二甲基苄基氯化铵组合的1.1g(EDTA)$_2$Na中，直到最终体积达到1.11(pH 5.66)。局部媒介物溶液中的25mM羊毛甾醇。将双蒸H_2O添加到12.5g羊毛甾醇、1.1g(EDTA)$_2$Na、0.055g烷基二甲基苄基氯化铵和200ml EtOH的混合物中至最终体积为1.11。

[0119] 在一个实施方案中，羊毛甾醇滴眼剂溶液的配方为：

配方

仅含媒介物的溶液：

羟丙基-β-环糊精	165g
聚山梨醇酯80	1g
EDTA2Na	1.1g
烷基二甲基苄基氯化铵	0.055g
EtOH	200ml

随后添加ddH2O直到最终体积为1.1L(PH 5.66)

在媒介物中含有5mM羊毛甾醇的溶液：

羊毛甾醇	2.5g
羟丙基-β-环糊精	165g
聚山梨醇酯80	1g
EDTA2Na	1.1g
烷基二甲基苄基氯化铵	0.055g
EtOH	200ml

随后添加ddH2O直到最终体积为1.1L(PH 5.66)

表1外显子组测序和变体

表1a.外显子组测序数据生成的总结

样品	总有效产量(Mb)	平均测序深度	错配率	目标区域的覆盖率	目标覆盖>= 4x 的分数	目标覆盖>= 10x 的分数
IV-1	3,409.20	60.16	0.20%	99.60%	99.10%	97.60%
IV-2	3,314.58	58.62	0.20%	99.60%	99.20%	97.80%
IV-3	3,327.63	57.24	0.20%	99.80%	99.20%	97.40%
III-2	3,029.40	51.89	0.21%	99.80%	99.30%	97.70%
III-1	6,877.08	54.24	0.29%	96.30%	89.40%	81.80%
IV-4	6,331.78	44.12	0.29%	96.50%	88.80%	79.80%

表1b.检测的变体的总结

样品	总变异	杂合	纯合	错义	无义	连接	同义	剪接	基因间	基因内
IV-1	61,189	35,571	25,618	6,105	69	39	7,296	32	5,371	36,598
IV-2	60,829	34,698	26,131	6,074	62	41	7,211	38	5,178	36,572
IV-3	61,078	35,238	25,840	6,221	78	43	7,265	38	5,099	36,544
III-2	62,753	39,001	23,752	6,393	64	38	7,588	34	5,764	36,924
III-1	80,067	49,694	30,373	7,247	93	49	8,166	47	15,063	41,391
IV-4	80,893	48,211	32,682	7,252	85	50	8,184	50	14,547	42,414

表1c.外显子组测序后的变体优先化管线

过滤器	III-1(携带者父亲)	III-2(携带者母亲)	IV-1(受影响的女儿)	IV-2(受影响的儿子)	IV-3(受影响的儿子)	IV-4(未受影响的女儿)	组合
总变异	80,067	62,753	61,189	60,829	61,078	80,893	-
错义、无义、剪接	7,389	6,495	6,213	6,177	6,342	7,387	-
受影响的：1/1；携带者：0/1；未受影响的：0/1 或 0/0*	5,792	4,661	3,127	3,123	3,085	5,638	9
不在 dbSNP 中	3,724	2,969	1,954	1,929	1,928	3,589	5
不在 1000 基因组计划中	1,032	767	227	264	245	1,059	1
预测的损伤	267	269	31	45	41	264	1

*在受影响的儿童中为纯合的，在携带者中为杂合的，在未受影响的儿童中没有纯合突变体

表1d.全基因组基因分型数据的总结

样品	全部基因座	捕获的	SNP
IV-1	4,641,218	4,440,318	559,832
IV-2	4,641,218	4,446,992	605,499
IV-3	4,641,218	4,445,267	526,794
III-2	4,641,218	4,448,054	537,925
III-1	4,641,218	4,446,581	574,880
IV-4	4,641,218	4,450,657	584,347

表1e.在基因FDFT1上检测到的编码变体

位置(GRch37/hg19)	refSNP	REF	ALT	功能	III-1(携带者父亲)	III-2(携带者母亲)	IV-1(受影响的女儿)	IV-2(受影响的儿子)	IV-3(受影响的儿子)	IV-4(未受影响的女儿)
chr8:11666337	rs4731	A	G	非同义	A/G	A/G	G/G	A/G	G/G	G/G
chr8:11683653	rs904011	T	C	同义	C/C	C/C	C/C	C/C	C/C	C/C

表2羊毛甾醇在兔子白内障晶状体和狗白内障中的治疗效果

表2a.羊毛甾醇在兔子白内障晶状体中的治疗效果

样品编号	治疗前	治疗后
1	3	1
2	2	0
3	2	1
4	2	0
5	3	1
6	2	1
7	2	1
8	2	0
9	1	1
10	1	0
11	2	1
12	1	1
13	2	1

表2b.羊毛甾醇在狗白内障中的治疗效果

	治疗组		对照组	
研究眼睛	前	后	前	后
1	2	1	1	1
2	1	0	2	2
3	2	1	1	1
4	3	1		
5	1	0		
6	2	0		
7	2	1		

表3用于对人LSS基因中的每个外显子进行测序和构建晶体蛋白突变体的引物

表3a.用于PCR扩增和对人LSS基因中的每个外显子进行测序的引物

扩增子	序列(5'-3')
LSS-外显子 1-F	GCCTGAGCGCCTGCCGAGGCCT
LSS-外显子 1-R	GACACCTGAGGACCACCGGCCAT
LSS-外显子 2-F	GTGGTCCTAGGTGCTGAGGAGA
LSS-外显子 2-R	CGTGCTCCTCACGGCTCACCCCT
LSS-外显子 3-F	CTTGGGCTGTATGTGAAGAGGGT
LSS 外显子 3 R	CCTAGACCAGGCTGGGCCAGGAT
LSS-外显子 4-F	GTTGGAGTGAGGTGCTCAGGAGGA
LSS-外显子 4-R	GCAGCTGCCTGGAAACCCAAGCAT
LSS-外显子 5-F	GCATTCTTAGTTTTCTGAGGAAACTC
LSS-外显子 5-R	CCACTGTTTCAG CTG CAAGTG CAT
LSS-外显子 6-F	CAGAGGGTGAAGCTTCCCAGCT
LSS 外显子 6-R	GCTGTCACAGCCTGCACCTGAC
LSS-外显子 7-F	GAAAGGGCCCAAGGTATGGATGCT
LSS-外显子 7-R	GTGAGTGGACAGGTGTGGTTAGAT
LSS-外显子 8-F	GAGCCAGGCCTACCAGGTGCT
LSS-外显子 8-R	GCAGGGGATGAGTGCGTGAAT
LSS-外显子 9-F	GCAGTGCATGGAGCTCCAGGCT
LSS-外显子 9-R	CCAGGAAACCCCACTCCCAGCT
LSS-外显子 10-F	GTGGATCTGGACGAGACCTTGT
LSS-外显子 10-R	CACTGGGATGCAGCTGGGGCT
LSS 外显子 11 F	GTGCAGGGTCTGGGTAGCAGCT
LSS-外显子 11-R	GACATGATTGCAAAGGAAGCAT
LSS-外显子 12-F	CTGGAGGCAGTGGCTGGGAGT
LSS-外显子 12-R	GCAAGTGTGTGGCCAGCAGTGCT
LSS-外显子 13-F	GGCAGGATGTGGCCAGGACCAT
LSS-外显子 13-R	GCACTTCTGCCTGCAGGAGCT
LSS 外显子 14 F	CCAGTCTGTCTCAGCGATGT
LSS-外显子 14-R	CCAAAAACGCCAAGGGAGGAGT
LSS-外显子 15-F	CTGGCTGCACCCACACCTTTGGT
LSS-外显子 15-R	GCTCATCTGCAGGACACGAGGT
LSS-外显子 16-F	GTTGTCAGCCCTAGTGTTGCCT
LSS-外显子 16-R	CAGGTTTGTGTACCACAGTGCT
LSS 外显子 17 F	GAGCTGCAGAGCCTGGGCAGCCA
LSS-外显子 17-R	CCGTGTCACAGAATGATGCGT
LSS-外显子 18-F	GAATTGGGATAGGTAAACTGCT
LSS 外显子 18 R	CGCAGTGTGTGAGAGCAGAAACCT
LSS-外显子 19-F	CTTAATGCCTGAGGCACTGGAGT
LSS-外显子 19-R	CACTCATGACAGAGCATTGGGTT
LSS 外显子 20 F	CAAGGCAGCCTGCTGGGGTGA
LSS-外显子 20-R	CACCGGCTCACAGCTGAGTGT
LSS-外显子 21-F	CTCACTGCAGCATTCCAGGGTT
LSS 外显子 21 R	GTGGAAACAGCCATGCACGCT
LSS 外显子 22-F	GCCAACAGCCAGGGCTCCAGTT
LSS-外显子 22-R	GGTTGGAGCCCAAGACAGGGT

表3b.用于构建晶体蛋白突变体的引物

基因	引物(5'-3')
αA-R116C-For	TTCCCGTGAGTTCCACTGCCGCTACCGCCTGCCGTCGCTGC
αA-R116C-Rev	CGGCAGGCGGTAGCGGCAGTGGAACTCACGGG
αA-R116H-For	TTCCCGTGAGTTCCACCACCGCTACCGCCTGCCGTCGCCAC
αA-R116H-Rev	CGGCAGGCGGTAGCGGTGGTGGAACTCACGGG
αA-Y118D-For	GAGTTCCACCGCCGCGACCGCCTGCCGTCCAACTTACGAC
αA-Y118D-Rev	CGTTGGACGGCAGGCGGTCGCGGCGGTGGAACT
αB-R120G-For	CAGGGAGTTCCACGGGAAATACCGGATAGGGGG
αB-R120G-Rev	GGATCCGGTATTTCCCGTGGAACTCCCT
βB2-V187E-For	AGGTGCAGTCCGAGCGCCGTATGTGGAG
βB2-V187E-Rev	ATACGGCGCTCGGACTGCACCT
βB2-V187M-For	AGGTGCAGTCCATGCGCCGTATGTGATG
βB2-V187M-Rev	ATACGGCGCTCGGACTGCACCT
βB2-R188H-For	TGCAGTCCGTGCACCGTATCCCGCCAC
βB2-R188H-Rev	GGATACGGTGCACGGACTGCA
γC-G129C-For	CACGTGCTGGAGTGCTGCTGGGCTGC
γC-G129C-Rev	CAGCAGCACTCCAGCACGTG
γD-W43R-For	GTGGACAGCGGCTGCCGGATGCTCTATGAGCTGGCGG
γD-W43R-Rev	GCTCATAGAGCATCCGGCAGCCGCTGTCCAC

参考文献

1.Pascolini,D.和Mariotti,S.P.Global estimates of visual impairment: 2010.Br J Ophthalmol96,614-618,doi:10.1136/bjophthalmol-2011-300539(2012).

2.Bloemendal,H等人.Ageing and vision:structure,stability and function of lens crystallins.Prog.Biophys.Mol.Biol.86,407-485(2004).

3.Moreau,K.L.&King,J.A.Protein misfolding and aggregation in cataract disease and prospects for prevention.Trends Mol Med 18,273-282,doi:S1471-4914(12)00039-1[pii]10.1016/j.molmed.2012.03.005(2012).

4.Huff,M.W.&Telford,D.E.Lord of the rings--the mechanism for

oxidosqualene:lanosterol cyclase becomes crystal clear.Trends Pharmacol Sci 26,335-340,doi:S0165-6147(05)00127-6[pii]10.1016/j.tips.2005.05.004(2005).

5.Diehn,J.J.,Diehn,M.,Marmor,M.F.&Brown,P.O.Differential gene expression in anatomical compartments of the human eye.Genome Biol 6,R74,doi:gb-2005-6-9-r74[pii]10.1186/gb-2005-6-9-r74(2005).

6.Mori,M等人.Lanosterol synthase mutations cause cholesterol deficiency-associated cataracts in the Shumiya cataract rat.J.Clin.Invest.116,395-404(2006).

7.Ng,P.C.&Henikoff,S.Predicting deleterious amino acid substitutions.Genome Res 11,863-874,doi:10.1101/gr.176601(2001).

8.Adzhubei,I.A等人.A method and server for predicting damaging missense mutations.Nature Methods 7,248-249(2010).

9.Pollard,K.S.,Hubisz,M.J.,Rosenbloom,K.R.&Siepel,A.Detection of nonneutral substitution rates on mammalian phylogenies.Genome Res.20,110-121(2010).

10.Schwarz,J.M.,Cooper,D.N.,Schuelke,M.&Seelow,D.MutationTaster2:mutation prediction for the deep-sequencing age.Nature Methods 11,361-362(2014).

11.Seelow,D.,Schuelke,M.,Hildebrandt,F.&Nurnberg,P.HomozygosityMapper-an interactive approach to homozygosity mapping.Nucleic Acids Res.37,W593-W599(2009)

12.Thoma,R等人.Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase.Nature 432,118-122,doi:10.1038/nature02993(2004).

13.Dobson,C.M.Protein folding and misfolding.Nature 426,884-890(2003).

14.Ecroyd,H.&Carver,J.A.Crystallin proteins and amyloid fibrils.Cell.Mol.Life Sci.66,62-81(2009).

15.Braun,N等人.Multiple molecular architectures of the eye lens chaperone aBcrystallin elucidated by a triple hybrid approach.Proc.Natl Acad.Sci.USA 108,20491-20496(2011)

16.Cenedella,R.J等人.Direct perturbation of lens membrane structure may contribute to cataracts caused by U18666A,an oxidosqualene cyclase inhibitor.J Lipid Res 45,1232-1241,doi:10.1194/jlr.M300469-JLR200M300469-JLR200[pii](2004).

17.Li,H.&Durbin,R.Fast and accurate long-read alignment with Burrows-Wheeler transform.Bioinformatics 26,589-595(2010).

18.DePristo,M.A等人.A framework for variation discovery and genotyping using next-generation DNA sequencing data.Nature Genet.43,491-498(2011).

19.Ruf,A等人.The monotopic membrane protein human oxidosqualene cyclase is active as monomer.Biochem.Biophys.Res.Commun.315,247-254(2004).

20.Cardozo,T.,Totrov,M.&Abagyan,R.Homology modeling by the ICM method.Proteins23,403-414(1995).

21.Abagyan,R.&Argos,P.Optimal protocol and trajectory visualization for conformational searches of peptides and proteins.J.Mol.Biol.225,519-532(1992).

22.Xu,J等人.The congenital cataract-linked A2V mutation impairs tetramer formation and promotes aggregation of bB2-crystallin.PLoS ONE 7,e51200(2012).

23.Wang,B等人.A novel CRYGD mutation(p.Trp43Arg)causing autosomal dominant congenital cataract in a Chinese family.Hum.Mutat.32,E1939-E1947(2011).

24.Gu,F等人.A novel mutationin AlphaA-crystallin(CRYAA)caused autosomal dominant congenital cataract in a large Chinese family.Hum.Mutat.29,769(2008).

25.Li,X.-Q等人.A novel mutation impairing the tertiary structure and stability of cC-crystallin(CRYGC)leads to cataract formation in humans and zebrafish lens.Hum.Mutat.33,391-401(2012).

26.Nagineni,C.N.&Bhat,S.P.Human fetal lens epithelial cells in culture:an in vitro model for the study of crystallin expression and lens differentiation.Curr.Eye Res.8,285-291(1989).

27.Bligh,E.G.&Dyer,W.J.A rapid method of total lipid extraction and purification.Can.J.Biochem.Physiol.37,911-917(1959).

28.Wang,S.,Leng,X.-Y.&Yan,Y.-B.The benefits of being b-crystallin heteromers:bB1-crystallin protects bA3-crystallin against aggregation during co-refolding.Biochemistry50,10451-10461(2011).

29.Sun,T.-X.,Das,B.K.&Liang,J.J.N.Conformational and functional differences between recombinant human lens aA-and aB-crystallin.J.Biol.Chem.272,6220-6225(1997).

30.Bradford,M.M.A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.Anal.Biochem.72,248-254(1976).

31.Geraldine,P等人.Prevention of selenite-induced cataractogenesis by acetyl-L-carnitine:an experimental study.Exp.Eye Res.83,1340-1349(2006).

32.Makri,O.E.,Ferlemi,A.V.,Lamari,F.N.&Georgakopoulos,C.D.Saffron administration prevents selenite-induced cataractogenesis.Mol.Vis.19,1188-1197(2013).

33.Zhang,L等人.Self-assembled lipid-polymer hybrid nanoparticles:a robust drug delivery platform.ACS Nano 2,1696-1702(2008).La Croix,N.Cataracts:When to refer.Top.Companion Anim Med.23,46-50(2008).

34.La Croix,N.Cataracts:When to refer.Top.Companion Anim Med.23,46-50(2008).

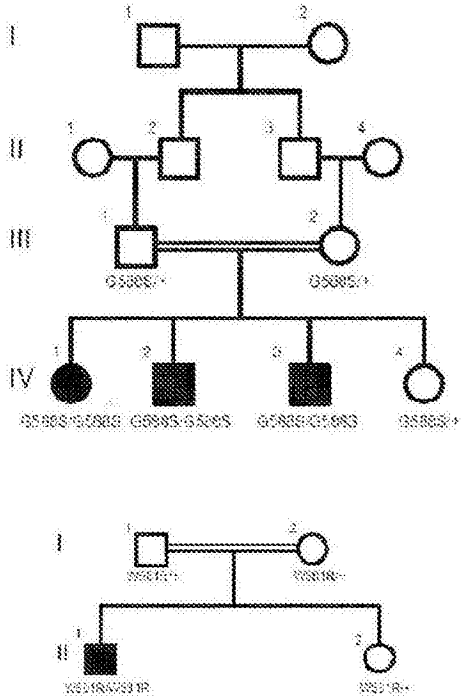


图1A

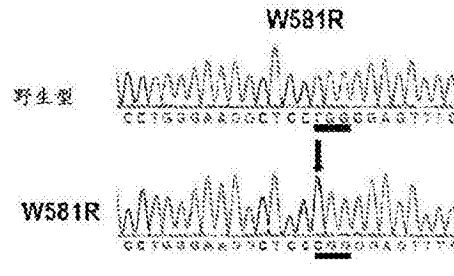


图1B

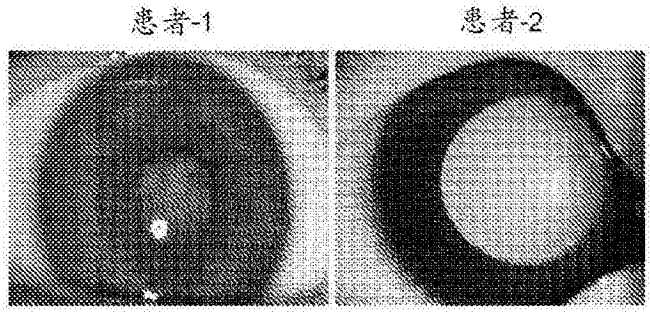

患者-1
患者-2

图1C

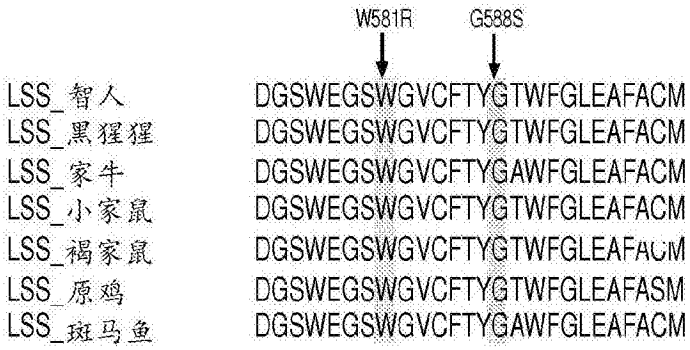

W581R
G588S
LSS_智人 DGSWEGSWGVCFTYGTWFGLEAFACM
LSS_黑猩猩 DGSWEGSWGVCFTYGTWFGLEAFACM
LSS_家牛 DGSWEGSWGVCFTYGAWFGLEAFACM
LSS_小家鼠 DGSWEGSWGVCFTYGTWFGLEAFACM
LSS_褐家鼠 DGSWEGSWGVCFTYGTWFGLEAFACM
LSS_原鸡 DGSWEGSWGVCFTYGTWFGLEAFASM
LSS_斑马鱼 DGSWEGSWGVCFTYGAWFGLEAFACM

图2A

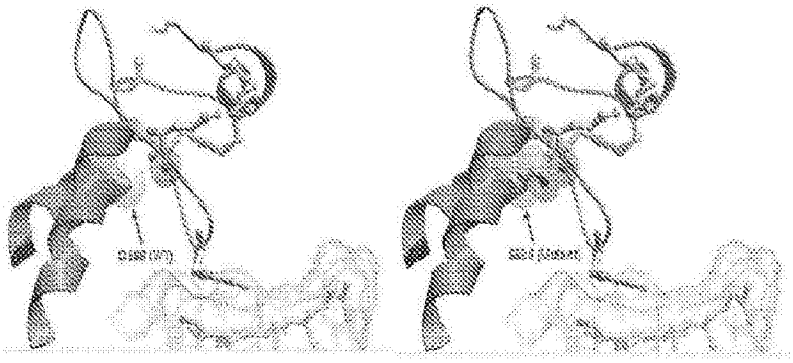


图2B

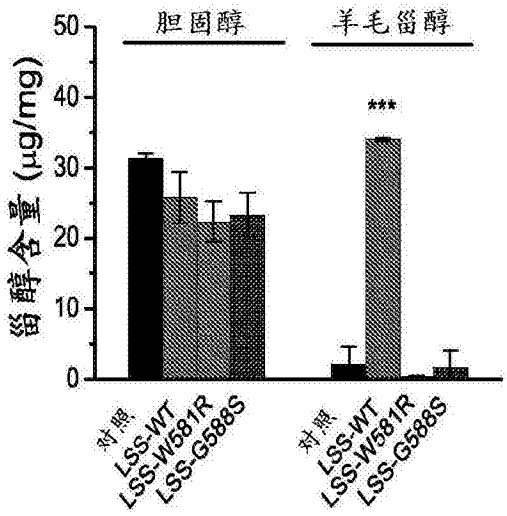

胆固醇
羊毛甾醇
甾醇含量 (μg/mg)
50
40
30
20
10
0
***
对照
LSS-WT
LSS-W581R
LSS-G588S
对照
LSS-WT
LSS-W581R
LSS-G588S

图2C

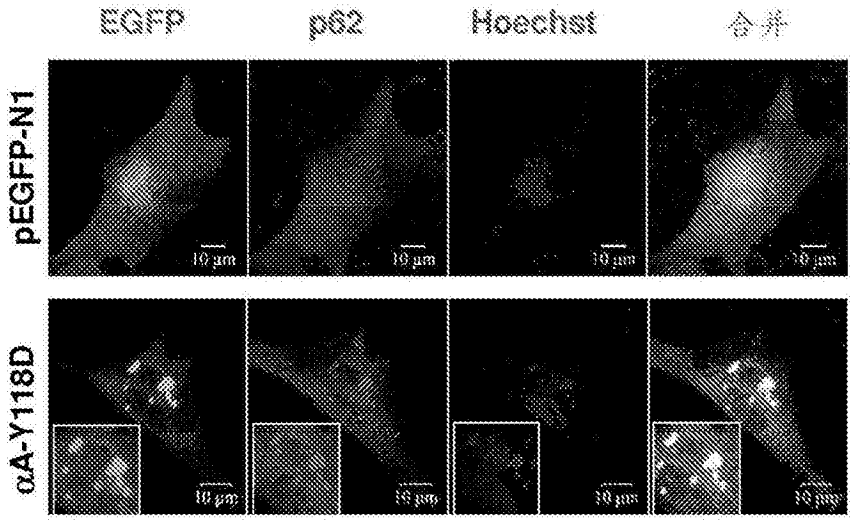

EGFP
p62
Hoechst
合并
pEGFP-N1
αA-Y118D
10 μm

图3A

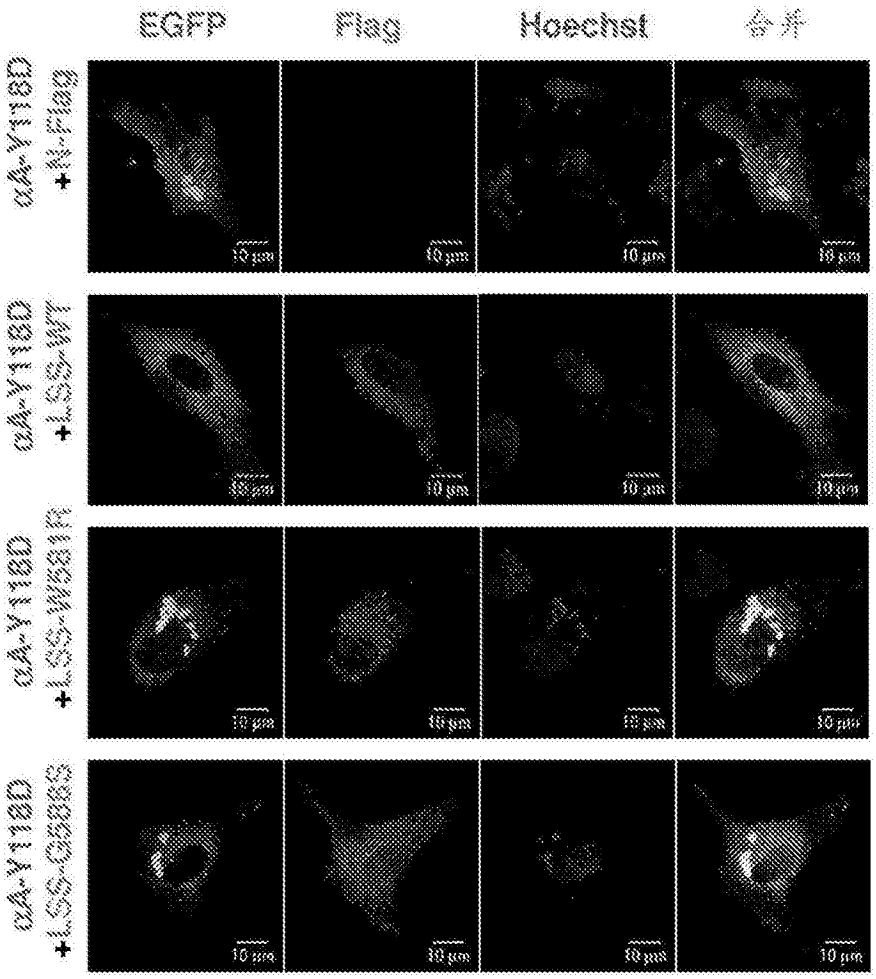

EGFP
Flag
Hoechst
合并
αA-Y118D +N-Flag
αA-Y118D +LSS-WT
αA-Y118D +LSS-W581R
αA-Y118D +LSS-G588S
10 μm

图3B

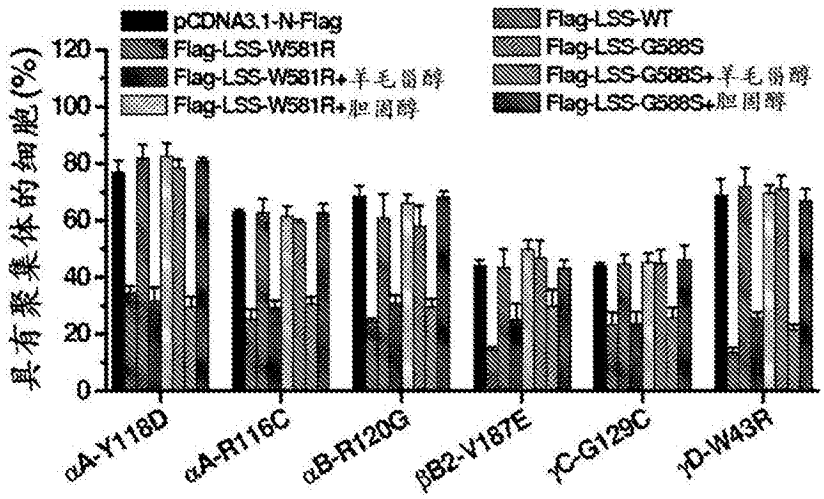

pCDNA3.1-N-Flag
Flag-LSS-W581R
Flag-LSS-W581R+羊毛甾醇
Flag-LSS-W581R+胆固醇
Flag-LSS-WT
Flag-LSS-G588S
Flag-LSS-G588S+羊毛甾醇
Flag-LSS-G588S+胆固醇
具有聚集体的细胞(%)
0
20
40
60
80
100
120
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图3C

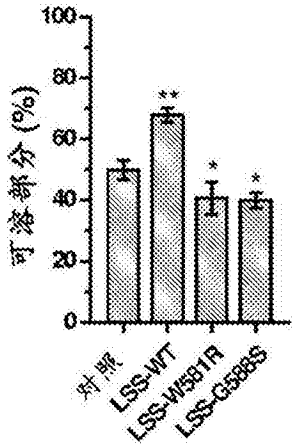

可溶部分 (%)
100
80
60
40
20
0
**
*
*
对照
LSS-WT
LSS-W581R
LSS-G588S

图3D

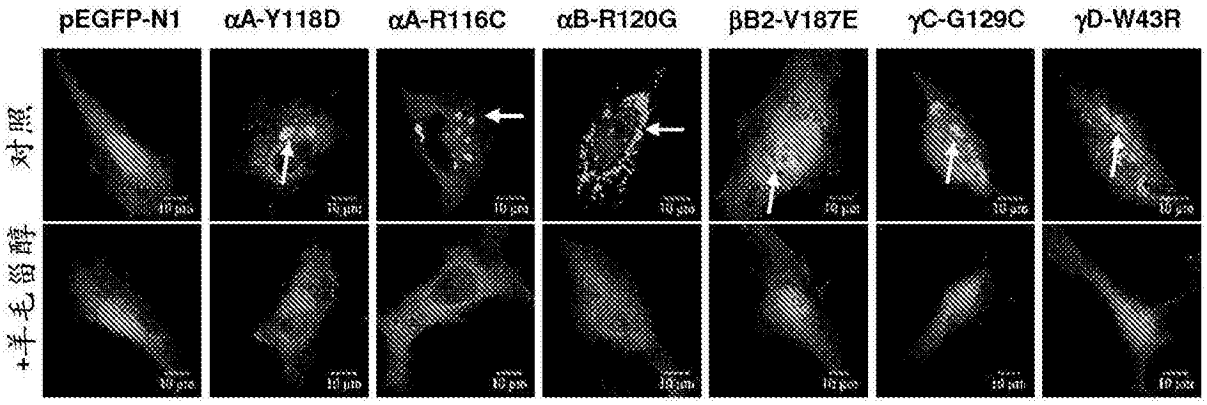

pEGFP-N1
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R
对照
+羊毛甾醇

图3E

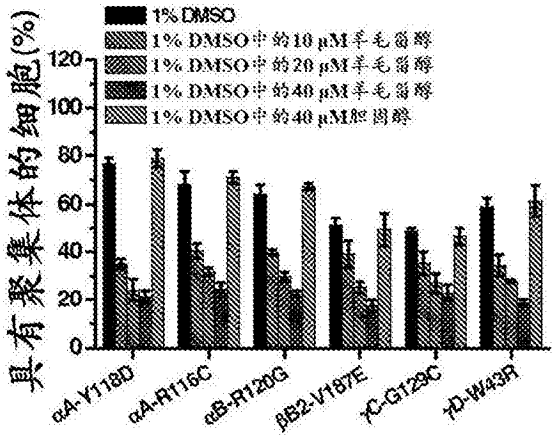

1% DMSO
1% DMSO中的10 μM羊毛甾醇
1% DMSO中的20 μM羊毛甾醇
1% DMSO中的40 μM羊毛甾醇
1% DMSO中的40 μM胆固醇
具有聚集体的细胞(%)
120
100
80
60
40
20
0
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图3F

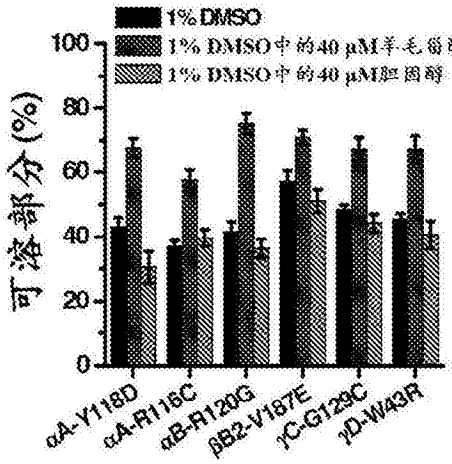

1% DMSO
1% DMSO中的40 μM羊毛甾醇
1% DMSO中的40 μM胆固醇
可溶部分(%)
100
80
60
40
20
0
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图3G

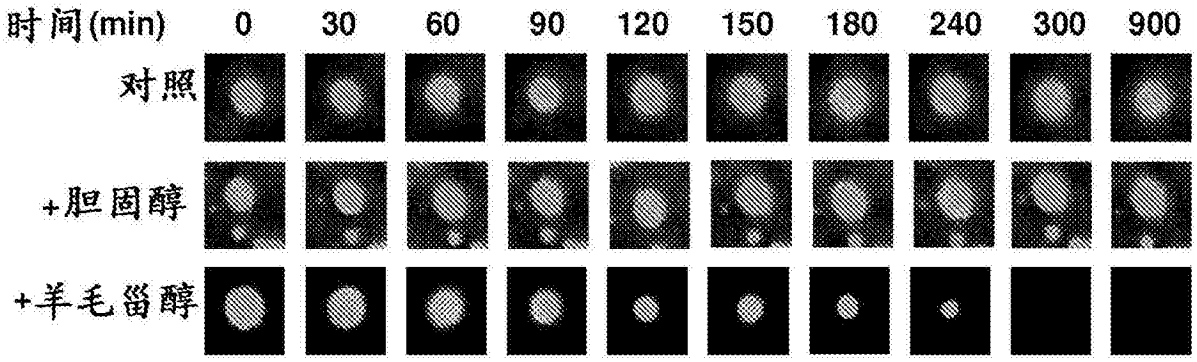

时间(min)
0
30
60
90
120
150
180
240
300
900
对照
+胆固醇
+羊毛甾醇

图3H

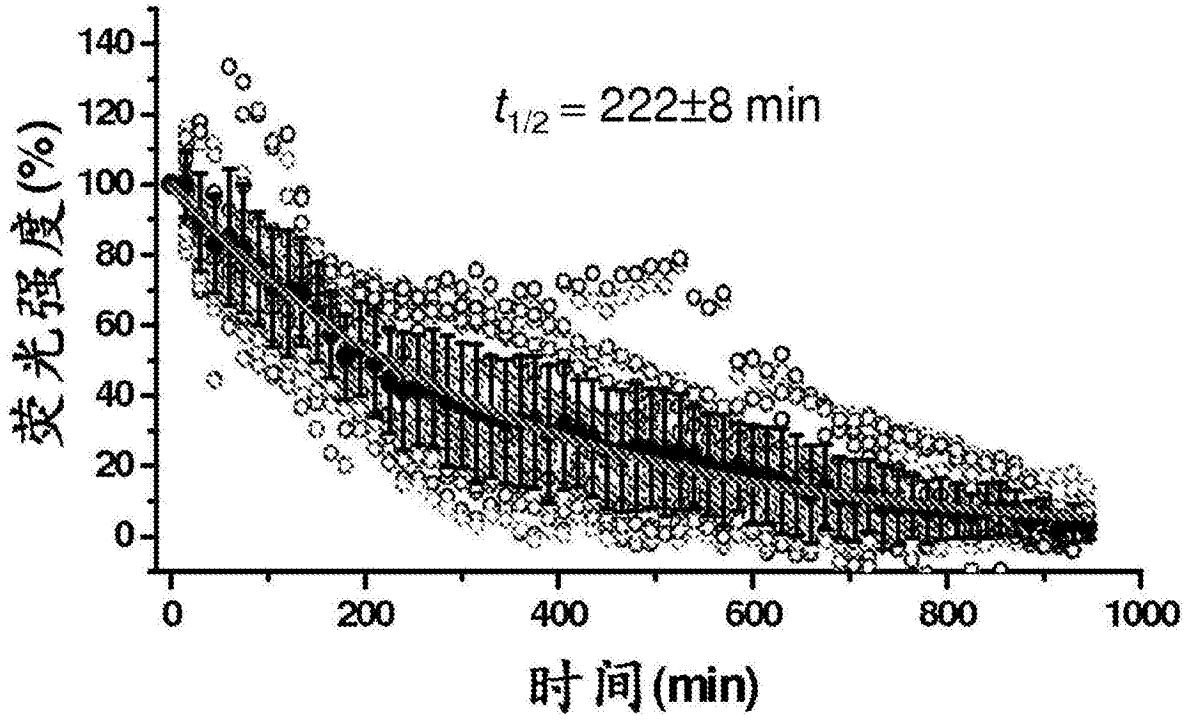

$t_{1/2}$ = 222±8 min
荧光强度(%)
时间(min)
0
20
40
60
80
100
120
140
0
200
400
600
800
1000

图3I

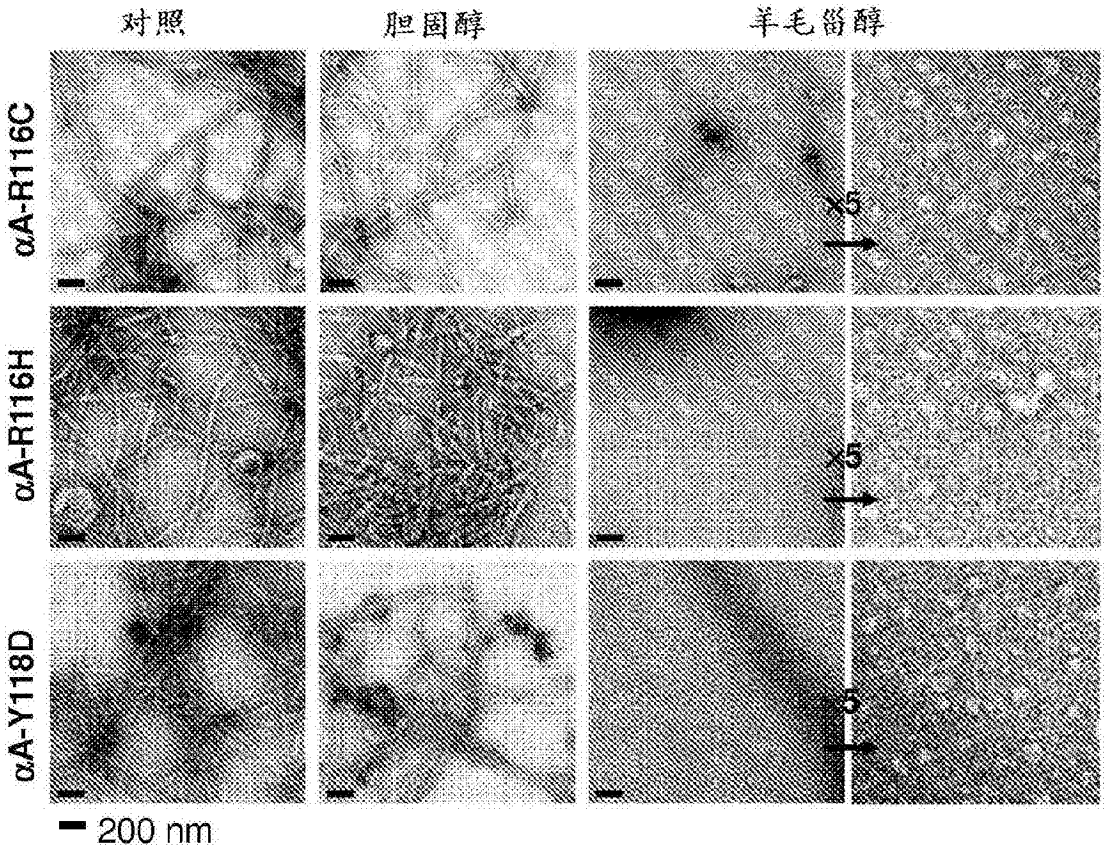

对照
胆固醇
羊毛甾醇
αA-R116C
αA-R116H
αA-Y118D
×5
×5
×5
200 nm

图4A

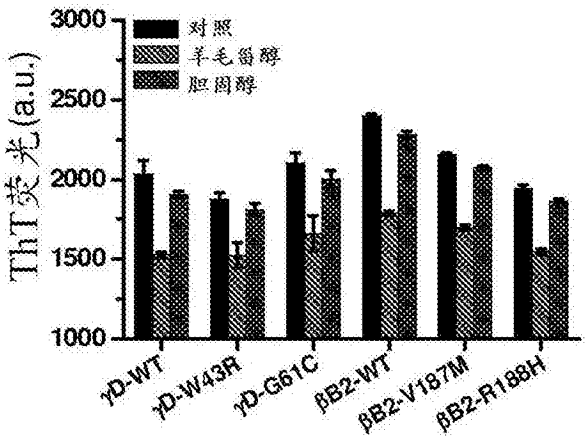


图4B(i)

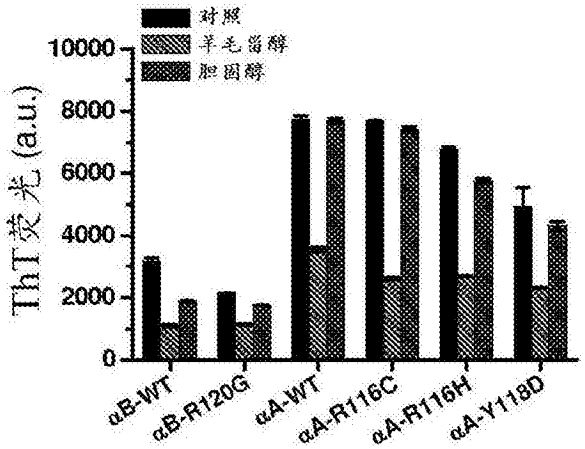


图4B(ii)

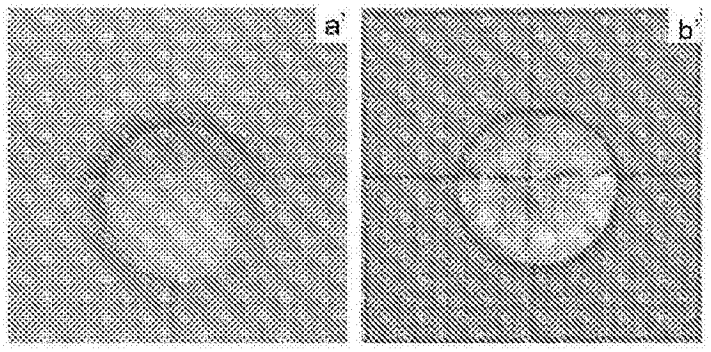


图 5A(i) 图 5A(ii)

图5A

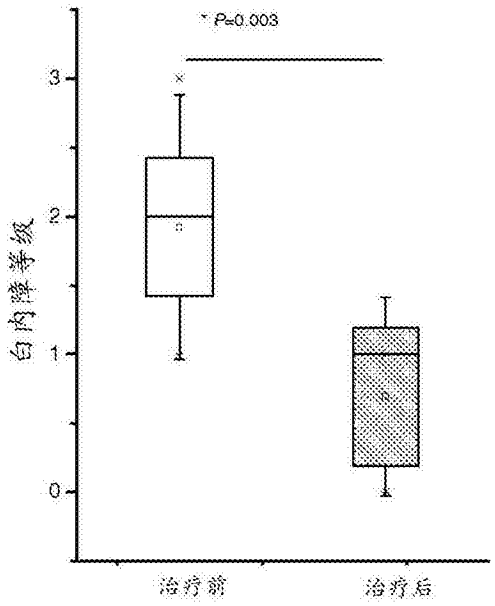


图5B

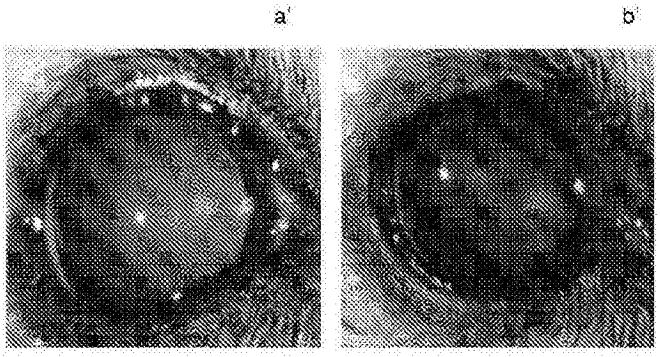


图 5C(i) 图 5C(ii)

图5C

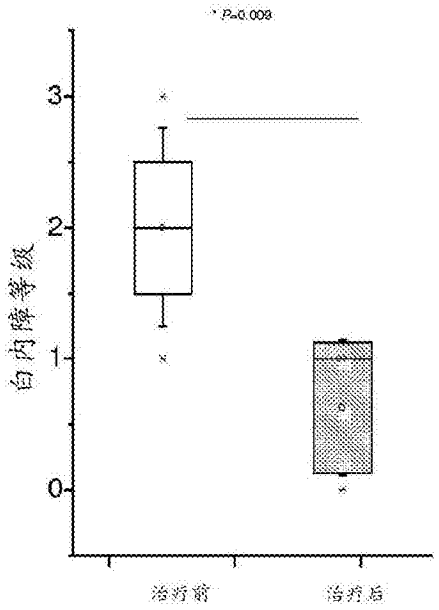


图5D

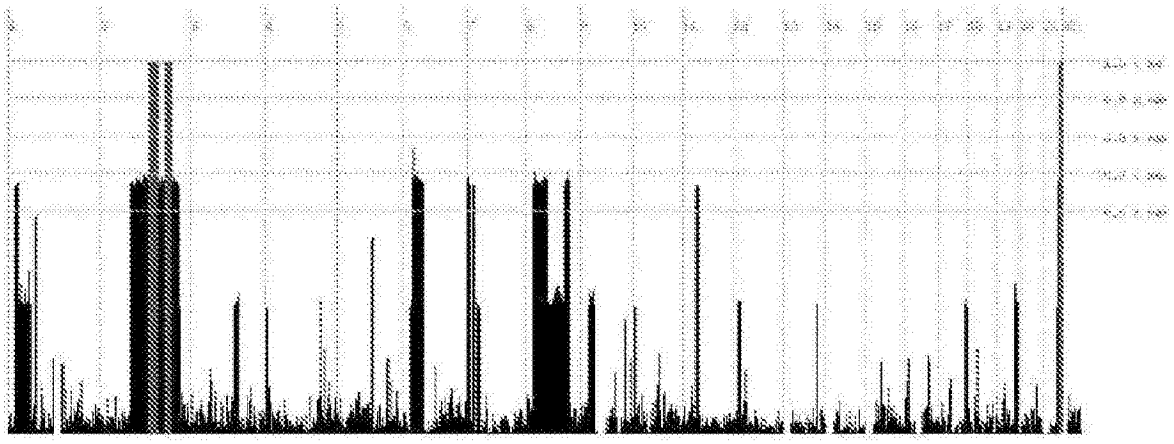


图6A

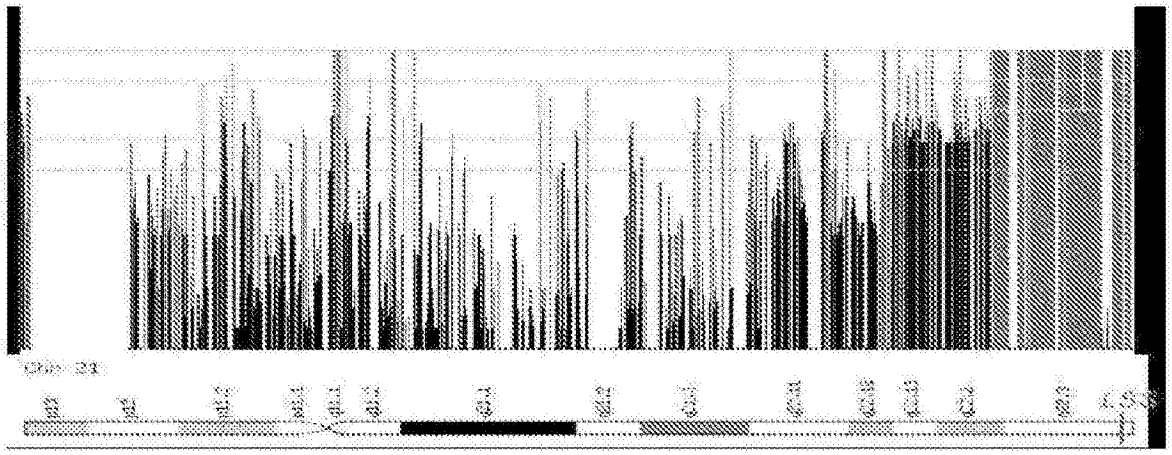


图6B

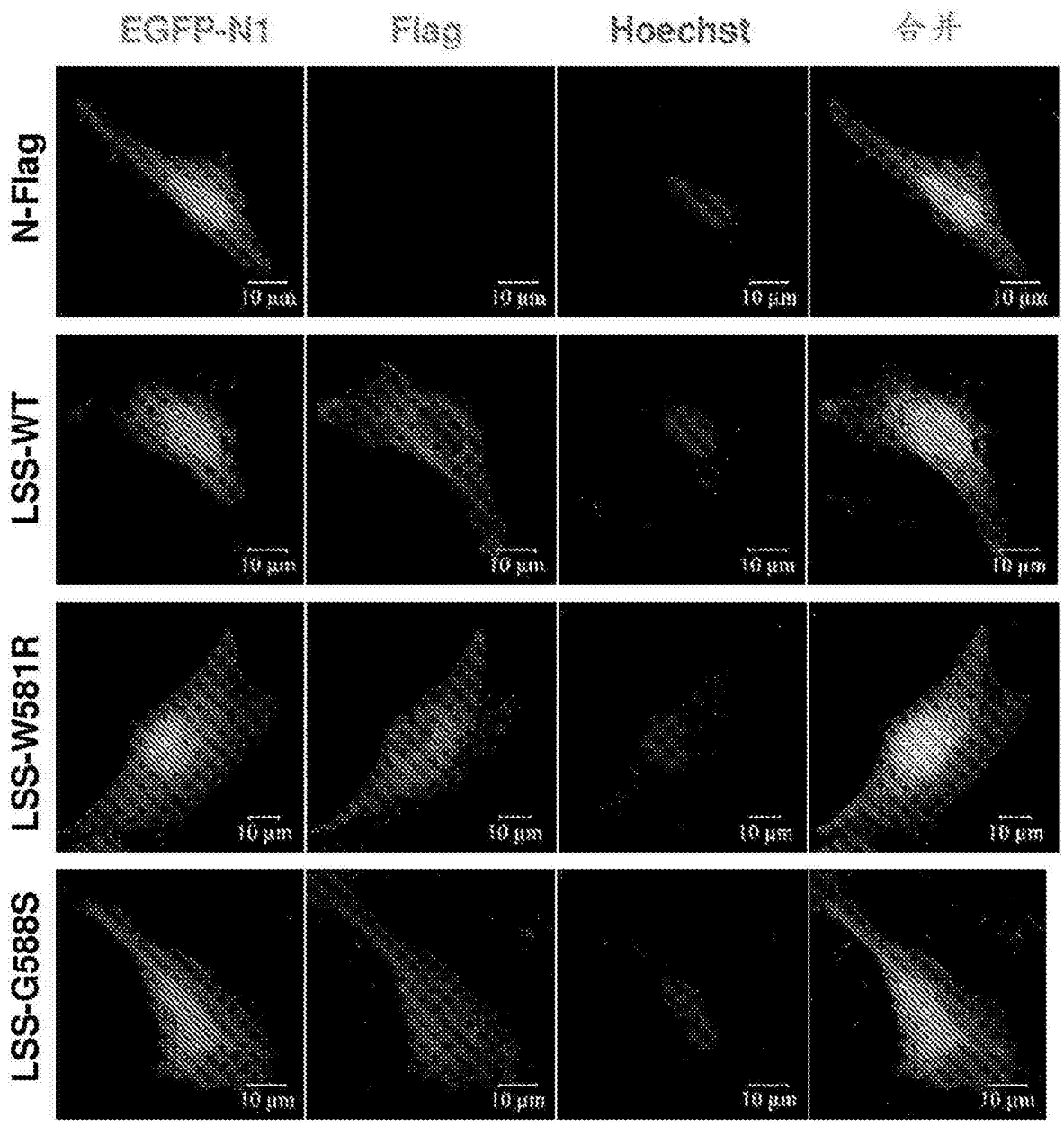


图7

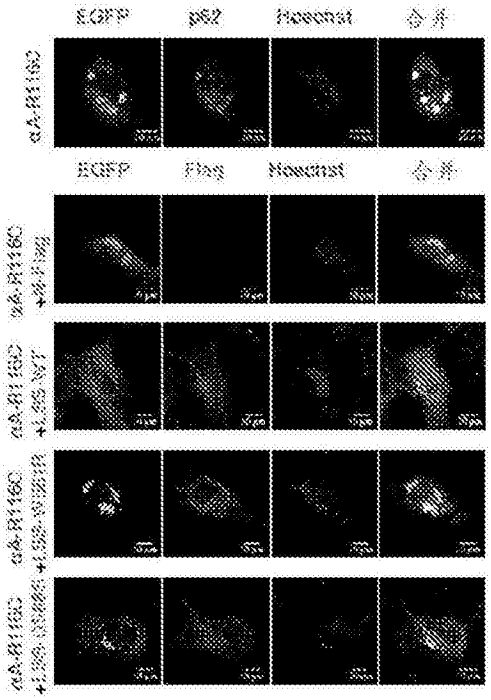

EGFP
p62
Hoechst
合并
αA-R116C
EGFP
Flag
Hoechst
合并
αA-R116C
+αA-Flag
αA-R116C
αA-R116C

图8A

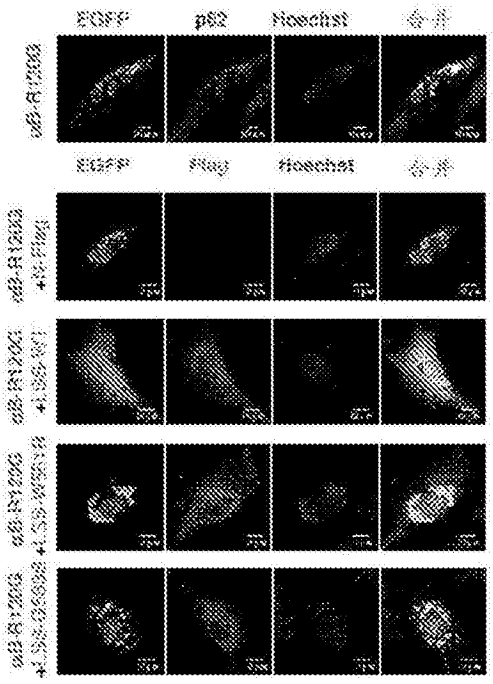

EGFP
p62
Hoechst
合并
αB-R120G
EGFP
Flag
Hoechst
合并
αB-R120G
αB-R120G
αB-R120G

图8B

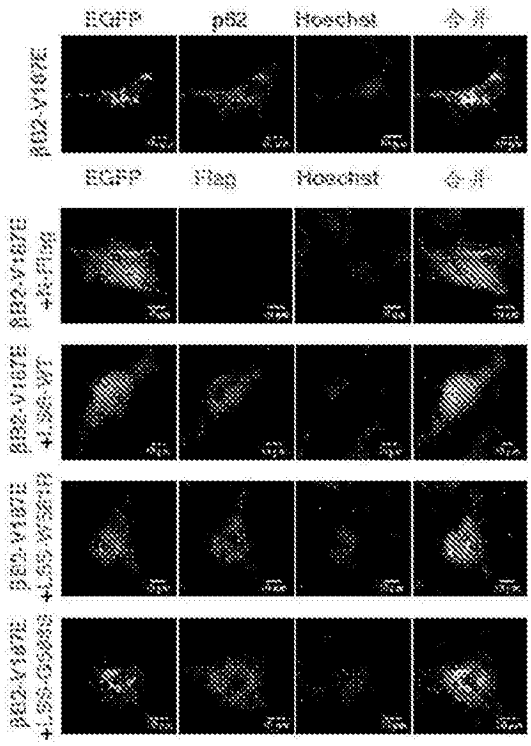

EGFP
p62
Hoechst
合并
EGFP
Flag
Hoechst
合并

图8C

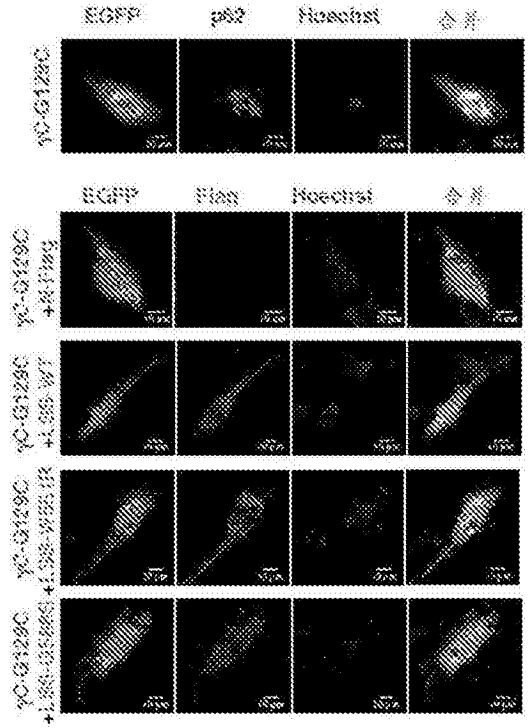

EGFP
p62
Hoechst
合并
EGFP
Flag
Hoechst
合并

图8D

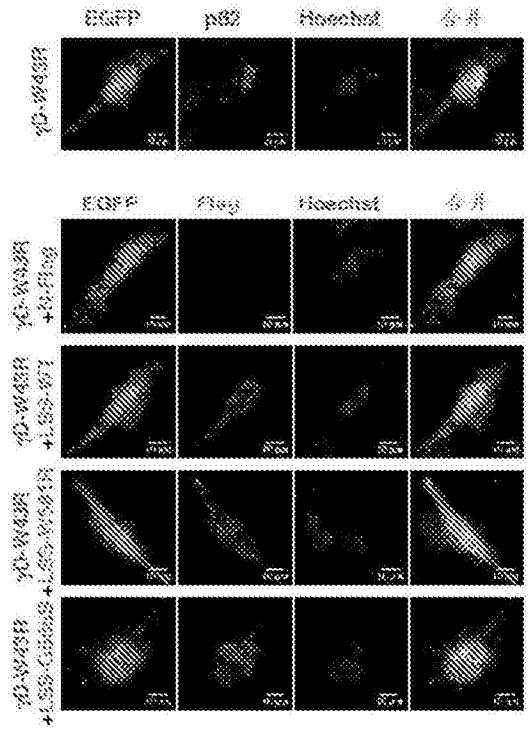

EGFP
pB2
Hoechst
合并
EGFP
Flag
Hoechst
合并

图8E

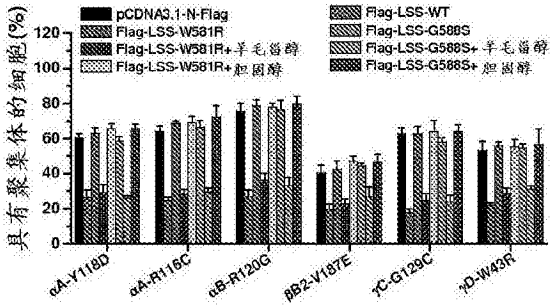

pCDNA3.1-N-Flag
Flag-LSS-W581R
Flag-LSS-W581R+羊毛甾醇
Flag-LSS-W581R+胆固醇
Flag-LSS-WT
Flag-LSS-G588S
Flag-LSS-G588S+羊毛甾醇
Flag-LSS-G588S+胆固醇
具有聚集体的细胞(%)
0
20
40
60
80
100
120
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图9A

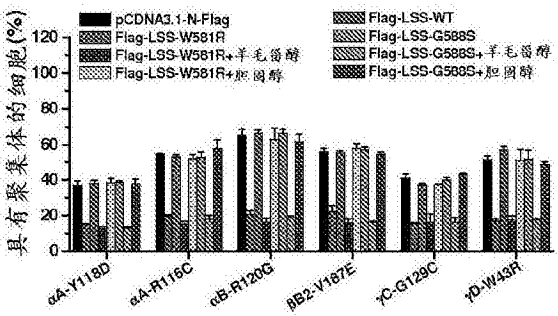

pCDNA3.1-N-Flag
Flag-LSS-W581R
Flag-LSS-W581R+羊毛甾醇
Flag-LSS-W581R+胆固醇
Flag-LSS-WT
Flag-LSS-G588S
Flag-LSS-G588S+羊毛甾醇
Flag-LSS-G588S+胆固醇
具有聚集体的细胞(%)
0
20
40
60
80
100
120
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图9B

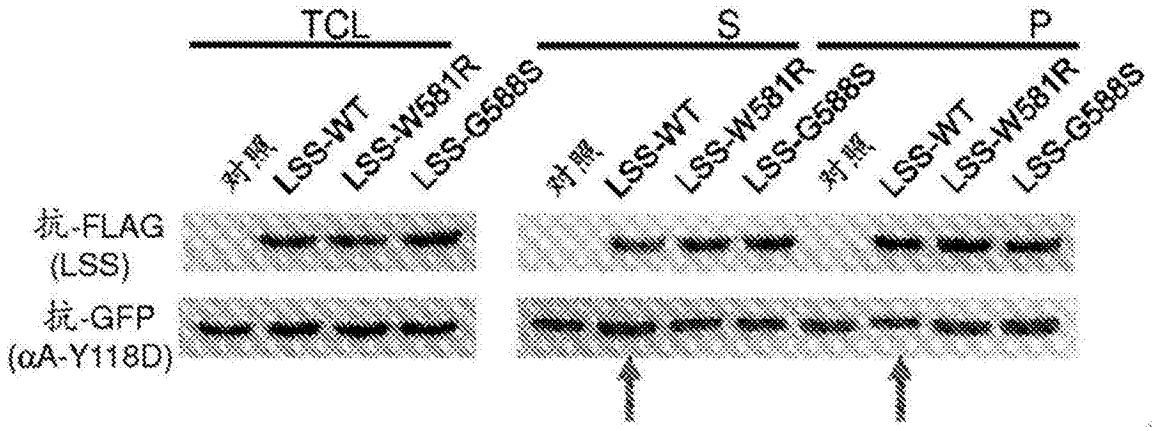


图9C

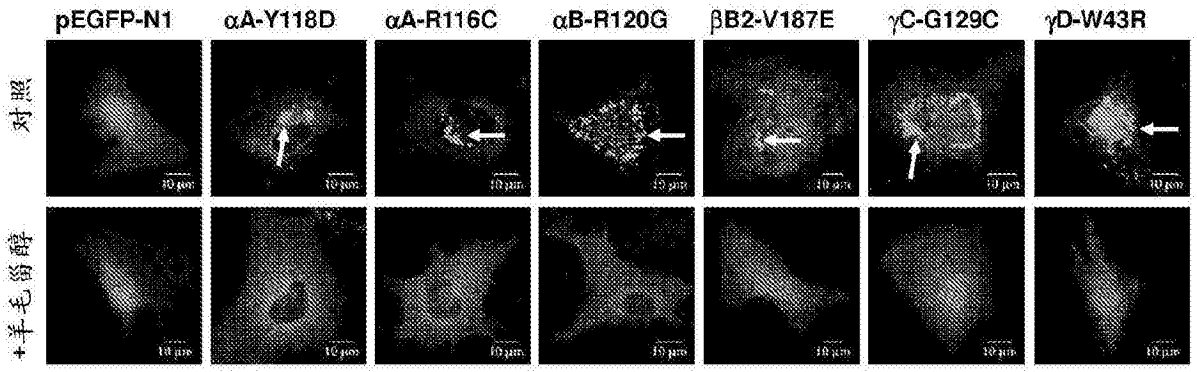


图10A

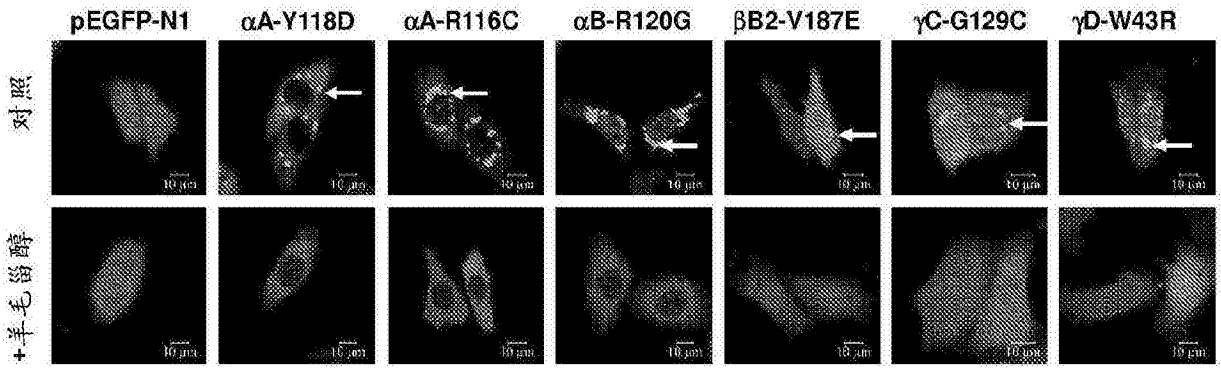


图10B

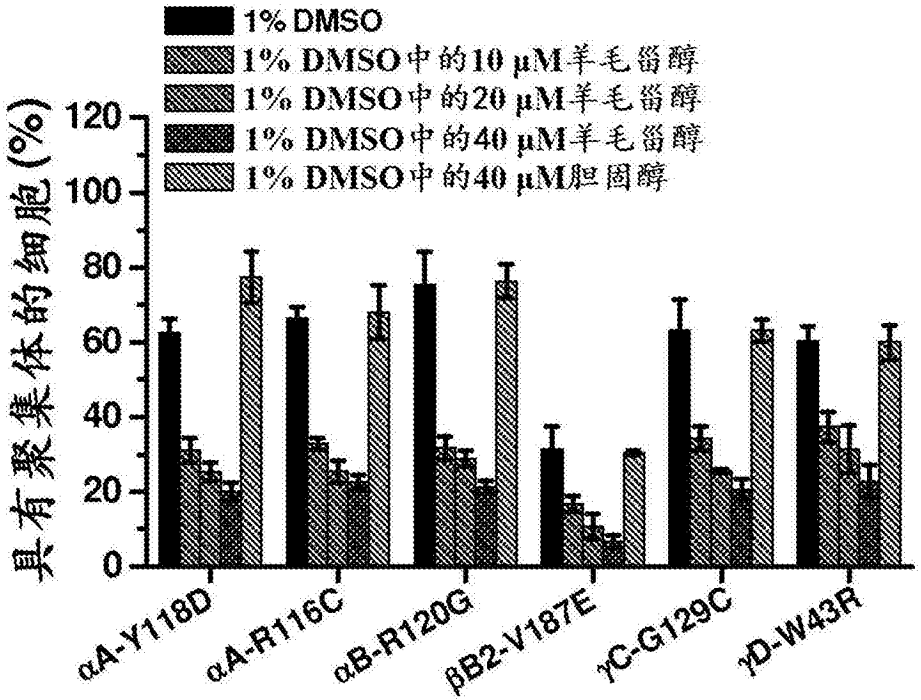

1% DMSO
1% DMSO中的10 μM羊毛甾醇
1% DMSO中的20 μM羊毛甾醇
1% DMSO中的40 μM羊毛甾醇
1% DMSO中的40 μM胆固醇
具有聚集体的细胞(%)
120
100
80
60
40
20
0
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图10C

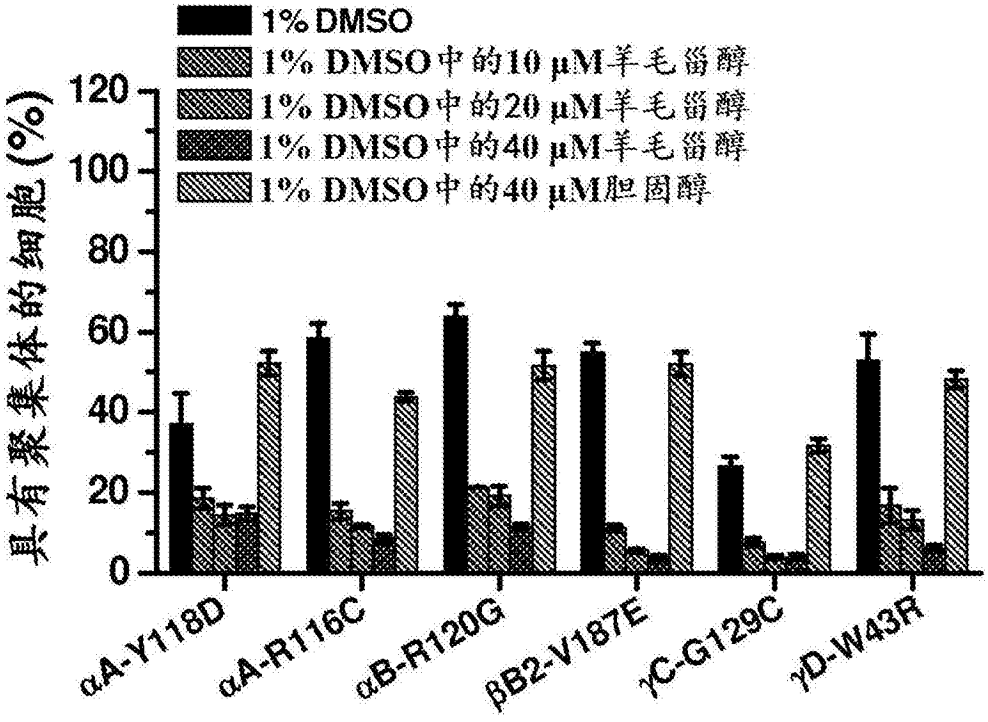

1% DMSO
1% DMSO中的10 μM羊毛甾醇
1% DMSO中的20 μM羊毛甾醇
1% DMSO中的40 μM羊毛甾醇
1% DMSO中的40 μM胆固醇
具有聚集体的细胞(%)
120
100
80
60
40
20
0
αA-Y118D
αA-R116C
αB-R120G
βB2-V187E
γC-G129C
γD-W43R

图10D

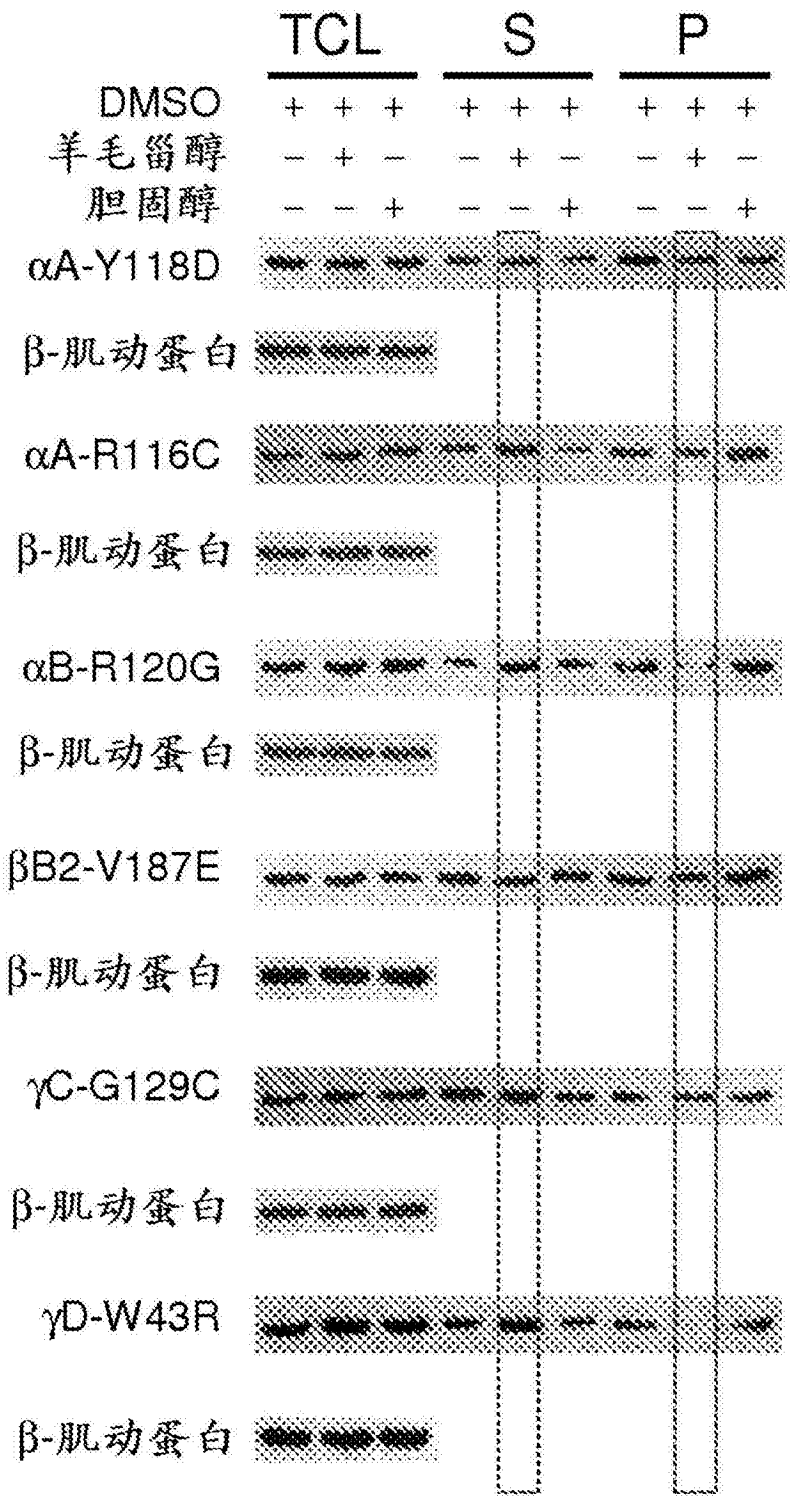

TCL
S
P
DMSO + + + + + + + + +
羊毛甾醇 − + − − + − − + −
胆固醇 − − + − − + − − +
αA-Y118D
β-肌动蛋白
αA-R116C
β-肌动蛋白
αB-R120G
β-肌动蛋白
βB2-V187E
β-肌动蛋白
γC-G129C
β-肌动蛋白
γD-W43R
β-肌动蛋白

图11A

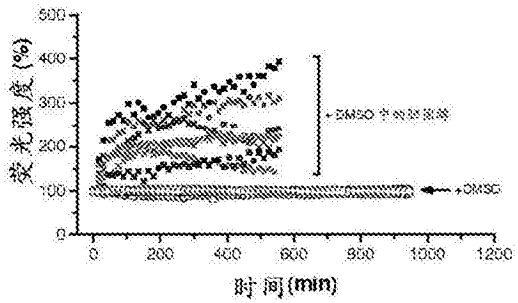


图11B

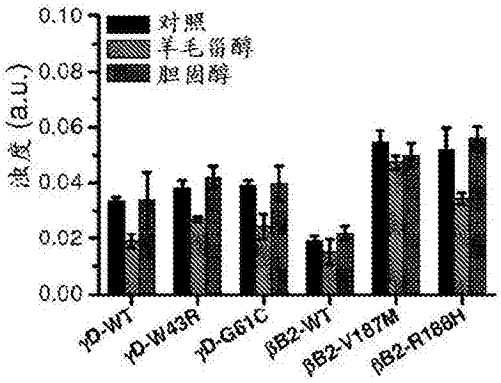


图11C(i)

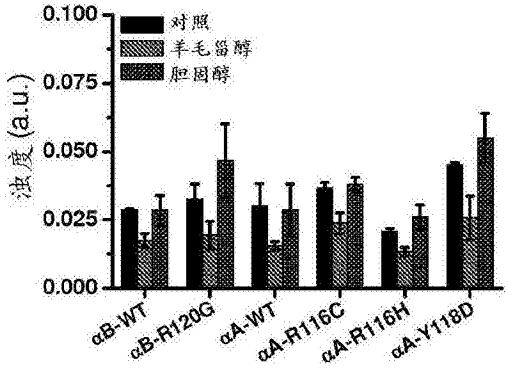


图11C(ii)

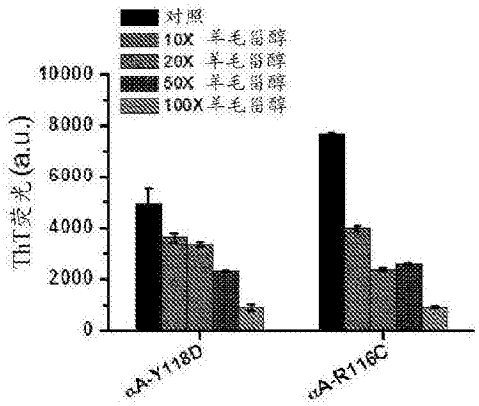


图11D

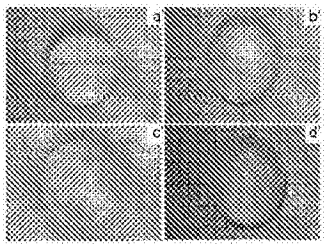


图12A

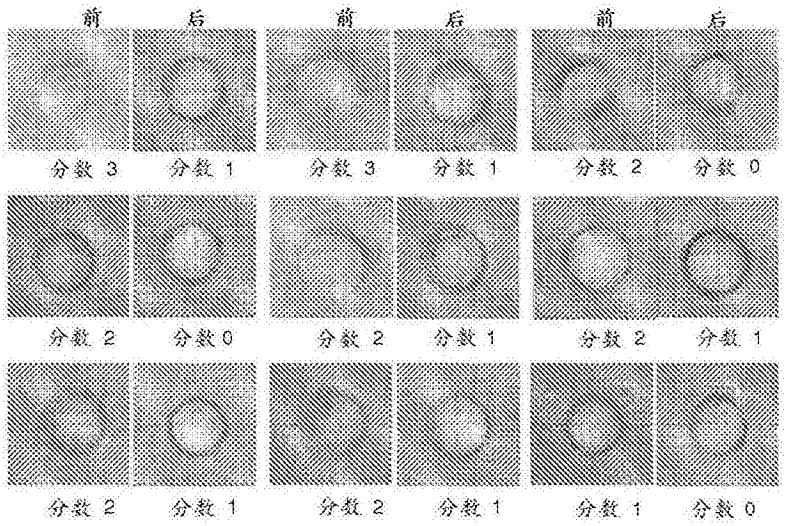


图12B

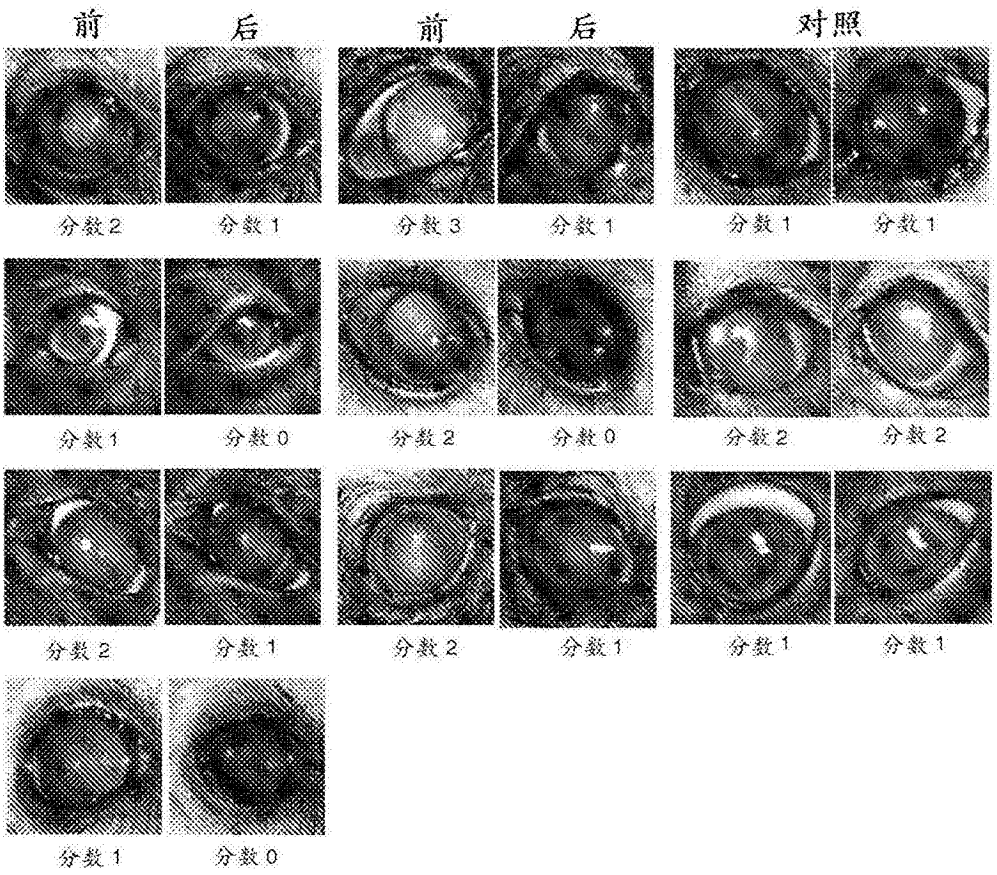

前
后
前
后
对照
分数2
分数1
分数3
分数1
分数1
分数1
分数1
分数0
分数2
分数0
分数2
分数2
分数2
分数1
分数2
分数1
分数1
分数1
分数1
分数0

图12C