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(56) **Prior Art :**
WO 2008/146018 A2 (ROYAL VETERINARY COLLEGE [GB]; SMITH ROGER KENNETH WHEALANDS [GB]; DUD) 4 December 2008
WU ET AL., "Identification of potent and selective MMP-13 inhibitors", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, vol. 15, 2005, pages 4105 - 4109.
US 2005/0245497 A1 (PENFOLD ET AL.) 03 November 2005
US 2010/0278896 A1 (KHAW ET AL.) 04 November 2010

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(54) **Title :** Methods for Drug Screen Using Zebrafish Model and The Compounds Screened Therefrom

(57) **Abstract :**

The invention relates to a platform of using zebra fish in screening candidates for treating and/or preventing myopia and keratoconus disease. The invention is mainly based on that Lumican, one of several SLRPs, plays an important role in the regulation of fibrillogenesis or the genes affecting the size of eyeballs in zebrafish, in addition to playing an important role in clinical myopia. Therefore, the invention uses the established zebra fish model to further identify the drugs affecting the expression of lumican and collagen fibrillogenesis, and/or the re&rulation of eyeball size. These drugs are potential candidates for treating myopia and/or keratoconus disease.

METHODS FOR DRUG SCREEN USING ZEBRAFISH MODEL AND THE COMPOUNDS SCREENED THEREFROM

Field of the Invention

The invention relates to a method for drug screening using zebrafish as a model. The invention particularly relates to a method for identifying candidate compounds for affecting the expression of lumican and collagen fibrillogenesis and for treating a disease medicated by expression of lumican and/or collagen fibrillogenesis and the candidate compounds identified therefrom. Specifically, the method of the invention identifies drugs for treating and/or preventing myopia and/or keratoconus disease.

Background of the Invention

Myopia is the most common eye disorder in the world. In Western countries, the prevalence of myopia is about 16%~27%, whereas in Asian countries it may be even higher, such as an 82% prevalence for the Chinese population in Singapore. A refractive error equal to or below -6 diopters (D), defined as high myopia, is also termed "pathological myopia" since its potential complications, including cataracts, glaucoma, macular degeneration and retinal detachment, might lead to blindness. Genetic and environmental factors may cause myopia. It has been estimated that about half or more of all persons suffering from myopia have axial myopia caused by elongation of the eye along the visual axis. At birth, the human eye is about two-thirds the size of an adult eye and is relatively short in the axial direction. As a consequence, young children tend to be hyperopic. As the eye grows during childhood, compensatory fine tuning of the optical properties of the cornea and lens occurs, increasing the ocular length. Often the entire process takes place virtually flawlessly and the eye becomes emmetropic. When this fine tuning process fails, however, it usually brings about a lengthened eye. As a result, distant images get focused in front of the plane of the retina and axial myopia results. In clinical trials, only anti-cholinergic drugs (such as atropine) have been used to control the progress of myopia. However, the long-term use of atropine needed to control the progress of myopia can cause side effects such as blurred vision, constipation, decreased sweating, difficulty sleeping, dizziness, drowsiness, dry mouth, nose, or skin, headache, loss of appetite, loss of taste, nausea, or nervousness. Therefore, there is a need to develop replacement drugs for controlling, preventing and/or treating myopia.

Thinning of the sclera, particularly at the posterior pole, is a crucial feature of the development of high myopia in humans. In primates, the sclera is a fibrous extracellular matrix (ECM) composed of collagens (mainly Type I collagen), elastin, proteoglycans and other components that are arranged in lamellae produced by scleral fibroblasts (*Alex Gentle et al., The Journal of Biological Chemistry, 2003, Vol. 278, No. 19, pp. 16587-16594*). Scleral remodeling involves the regulation of numerous gene products such as collagens, proteoglycans, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs), including smaller diameter collagen fibrils, reduced glycosaminoglycan (GAG) content, reduced proteoglycan (Decorin) synthesis, and increased MMP-2. Selective changes in mRNA levels also have been found for some proteins, including collagen I, MMP-2, MT1-MMP, TIMP-3, and TGF-β, suggesting that retina-derived signals modulate scleral gene expression to remodel the scleral tissue and modulate scleral creep rate (*John T. Siegwart Jr and Thomas T. Norton, Invest Ophthalmol Vis Sci. 2002 July; 43(7): 2067-2075*). Scleral remodeling is intrinsic to myopia progression, and these biochemical changes are actually a precursor to changes in the biomechanical properties of the sclera and, ultimately, to the development of myopia. The adult human sclera contains three major proteoglycans: aggrecan, biglycan, and decorin, which contribute to the structural properties of the sclera. The ratios of these proteoglycans will change with the condition of the sclera. Decorin and biglycan belong to a class of small leucine-rich proteoglycans (SLRPs), which also includes lumican, DSPG-3 (dermatan sulfate proteoglycan 3, PG-L epiphycan), fibromodulin, PRELP (proline-arginine-rich and leucin-rich repeat protein), keratocan, chondroadherin, and osteoglycin. Decorin, biglycan, lumican, and fibromodulin bind to type I collagen and influence matrix assembly and organization. Animal studies reveal that the proteoglycan synthesis rate significantly influences eyeball growth and myopia development. The synthesis rate of *decorin* in the sclera of marmosets is inversely correlated with vitreous chamber elongation rates. Biglycan and lumican mRNA levels were lowered in the sclera during experimentally induced myopia and increased during recovery. Lumican, a member of small leucine-rich proteoglycan (SLRP) family, is one of the major extracellular components in interstitial collagenous matrices of the corneal stroma, aorta, skin skeletal muscle, lung, kidney, bone, cartilage, and intervertebral discs, etc.

In corneal tissue, lumican contains keratin sulfate chains present as a proteoglycan, whereas in non-corneal tissues, lumican is present as a low or non-sulfated glycoprotein (50-57kDa). Its wide distribution implies that lumican has multiple functions regarding tissue morphogenesis and maintenance of tissue homeostasis. This was best illustrated by the multiple clinical manifestations observed in lumican knockout mice, which exhibited corneal opacity, skin and tendon fragility, delayed wound healing and low fertility. Indeed, lumican has been shown to play essential roles in corneal transparency by regulating collagen fibrillogenesis in wound healing by modulating epithelial cell migration, and in the epithelium-mesnchyme transition of the injured lens. Lumican deficient mice and Lum(-/-)Fmod(-/-)

mice showed collagen fibril diameter alteration and features of high myopia, suggesting that these proteoglycans play an important role in the biomechanical properties of sclera. In addition, linkage studies of high myopia identified potential loci MYP1 (Xq28) and MYP3 (12q21-23), located near or containing several SLRP genes, including biglycan (Xq27ter), decorin (12q21-22), lumican (12q21.3-22), and DSPG3 (12q21). MYP3 may be also responsible for 25% of autosomal dominant high myopia in families in the U.K. Therefore, candidate genes relevant to myopia development that map to MYP3, including decorin, lumican and DSPG3, are of great interest. More recently, novel 14 mutations in SLRP genes have been associated with high myopia; for example, c.893-105G>A in the LUM gene might have a protective role or be in linkage disequilibrium with a protective allele.

The zebrafish is a popular vertebrate model to study biology and the molecular genetics of development. Zebrafish can be easily managed (3–4 cm length as an adult) in large numbers in the laboratory. The ability to combine embryological and genetic methodology has established the zebrafish as a powerful research tool. Transparent embryos allow fundamental vertebrate developmental processes from gastrulation to organogenesis. In addition, the eye, heart beats and blood circulation of the embryo are easily observed. Touch, sight and behavioral responses can also be monitored in live embryos under the dissecting microscope. Several features, such as a short generation time of 3–4 months, make zebrafish particularly suitable for genetic studies. Most previous studies that included a number of eye mutations produced with the chemical mutagen ENU reported a reduced eye size, disorganized retina, cyclopia, reduced ganglion cell layer and loss of photoreceptors. Lung-Kun Yeh *et al.* has isolated and characterized the zebrafish keratocan and lumican gene and found an increased eyeball size after knockdown of zebrafish lumican during their development, which is compatible with and relevant to the clinical findings in children myopia. In children myopia, there were similar findings of axial elongation of eyeballs in children who had an alteration of SNP at the human lumican gene promoter. Decreased zebrafish lumican promoter activity was suspected to have been related to this SNP. In this project, we propose that lumican, one of a number of small leucine rich polypeptides, plays an important role in the regulation of fibrillogenesis and eye development which possibly affect the size of eyeball (Lung-Kun Yeh *et al., Journal of Biological Chemistry, 2010, Vol. 285, No. 36, pp. 28141-28155*). This prior reference also indicates that down-regulation of *zlum* expression by antisense *zlum* morpholinos manifested ocular enlargement resembling axial myopia due to disruption of the collagen fibril arrangement in the sclera and resulted in scleral thinning, and that administration of muscarinic receptor antagonists, e.g. atropine and pirenzepine, effectively subdued the ocular enlargement caused by morpholinos in *in vivo* zebrafish larvae assays. Therefore, this prior reference suggests that zebrafish can be used as an *in vivo* model for screening myopia-treating compounds.

However, there is still a need to further explore applications of the zebrafish model in achieving practical myopia drug screening and finding effective drugs for controlling, preventing and/or treating myopia.

Summary of the Invention

The invention provides a method of using zebrafish with big eye to identify a candidate compound that can be used to affect the expression of lumican and/or collagen fibrillogenesis and/or treat myopia and/or keratoconus disease. The method comprises contacting a test compound with the zebrafish with big eye and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases.

The invention also provides a method of using lumican gene and/or collagen fibrillogenesis-relating gene knockdown zebrafish to identify a candidate compound that can be used to affect the expression of lumican and/or collagen fibrillogenesis and/or treat myopia and/or keratoconus disease. The method comprises contacting a test compound with lumican gene and/or collagen fibrillogenesis-relating gene knockdown zebrafish, determining the number of the big eye in the zebrafish and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases.

Brief Description of the Drawings

Figure 1 shows a series of morphological changes of zLum knockdown fish in 3-7 dpf. Coupled with eye development, zLum KD fish causes the sclera enlargement in progress. On Day 5, retinal detachment can be clearly observed through the microscope.

Figure 2 shows the Effect of lumican gene knowckdown on eye size. 5a. Morphometric measurements of sclera width in red lines and RPE width in white lines. 5b. Results of comparing Lumican morphant with wild type in RPE width / sclera width ratio show knockdown of lumican due to ectasia of the sclera leading to axial elongation.

Figure 3 shows zLum-MO knockdown induces ultrastructural changes in the corneal stroma (CS), anterior sclera (AS) and posterior sclera (PS). (A) WT fish at 12 dpf stage in toludine blue staining. The figure indicates corneal stroma (CS), anterior sclera (AS), and posterior sclera (PS). (B) The diameters of collagen fibril are analyzed in the corneal stroma, anterior and posterior sclera of the 12 dpf-old wild type and zLum-MO-injected groups. Significant increases in the collagen fibril diameter of corneal stroma and anterior sclera are noted in the zLum-MO group, whereas the diameter of collagen fibril in the posterior sclera in both groups is not significantly different. (C-H) Morphological comparison of collagen fibril architecture in the corneal stroma (C-D), anterior scleral tissue (E-F) and posterior scleral tissue (G-H) between the control group (C, E, G) and zLum-MO-injected group (D, F, H) at the 12 dpf stage. (C) TEM micrograph showing regular and smaller fibril architecture of collagen

localized in the corneal stroma of the wild type group. (D) Irregular arrangement and increased collagen fibril diameter is found in the corneal stroma of the zLum-MO-injected group. (E) TEM micrograph showing relatively regular fibril architecture of collagen localized in the anterior sclera of the wild type group. (F) Irregular collagen fibrils with increased fibril diameter are noted in the anterior sclera of the zLum-MO-injected group. (G) Top is adjacent to the retina. TEM micrograph shows fibril architecture of collagen localized in the posterior sclera of the wild type group. (H) Top is adjacent to the retina. TEM micrograph shows irregular collagen fibril architecture in the posterior sclera of the zLum-MO-injected group. (C-H, scale bar: 100 nm).

Figure 4 shows ultrastructural changes in scleral thinning in the zLum-MO group. (A) Top is adjacent to the retina. Two to three layers of scleral fibroblastic cells with collagen fibril formation between the layers found at the posterior sclera of the WT fish at the 7 dpf stage. (B) Top is adjacent to the retina. Only one to two layers of fibroblastic cells at the posterior sclera of the zLum-MO-injected fish at the 7 dpf stage. (C) Scleral thinning is observed obviously in the zLum-MO-injected fish at the 7 dpf stage. The phenomenon is much more prominent in the zLum-MO-injected fish at 12 dpf stage. In particular, significant scleral thinning is observed in the posterior sclera of the zLum-MO-injected fish at 7 and 12 dpf stages as compared with the wild type group (A, B: scale bar: 1.5 um).

Figure 5 shows zLum expression in the zebrafish 44 embryo 2~4 days post-fertilization. zLum mRNA is expressed specifically in the sclera of zebrafish since 3 dpf by whole mount in situ hybridization.

Figure 6 shows western blot and mRNA rescue analyses. (a). In the western blot analysis, the lumican, collgaen 1a1, TGF-beta, and TIMP2 decreased in the lumican morphant. In contrast, the MMP2 expression increased. (b). The abnormally large eyes could be rescued with lumican and collagen 1a1 mRNAs. However, they could also be rescued with ppih, hsp 47 and rx1 mRNAs, which are related to collagen fibrillar formation and eye development, respectively.

Figure 7 shows a zebrafish drug screen assay. (A-B) These two figures illustrate and define the outer margin of the retinal pigmented epithelium layer (RPE(red color)) and the diameter of the scleral coat (D(green color)) in zebrafish. (C) Significant decreases in excessive axial elongation in the zLum-MO-injected fish at the 7 dpf stage after being treated with 0.5% atropine(A) and 0.25% pirenzepine(P), whereas there were no obvious changes in excessive axial elongation after being treated with 0.01% methoctramine(M) (lane1: WT; lane2: MO+ 0.5%A; lane3: MO+ 0.25%P; lane4: MO+ 0.01%M; lane5: MO). Significant decreases in the diameter of the scleral coat of the zLum-MO-injected fish at the 7 dpf stage after being treated with 0.5% atropine and 0.25% pirenzepine, whereas there were no obvious changes in the zLum-MO-injected group treated with 0.01% methoctramine (lane 6: WT; lane 7: MO+ 0.5%A; lane 8: MO+ 0.25%P; lane 9: MO+ 0.01%M; lane 10: MO). (D) Significant decreases in the ratio of RPE/scleral coat (%) were noted during ocular enlargement developed due to the reduction of

zLum protein. Some muscarinic receptor antagonists (atropine and pirenzepine) attenuate the decreasing ratio of the RPE/scleral coat due to the reduction of zLum protein, whereas there are no obvious changes in the decreased ratio of the RPE/scleral coat in the methoctramine-treated group (lane1: WT; lane 2: MO+ 0.5%A; lane 3: MO+ 0.25%P; lane 4: MO+ 0.01%M; lane 5: MO).

Figure 8 shows (A) Normal phenotype of WT fish at the 7 dpf stage. (B) Normal phenotype of RS-MO-injected embryos at the 7 dpf stage. (C) Significantly enlarged eyeball of zlum-MO-injected fish at the 7 dpf stage. (D) Significant decreases in ocular enlargement were noted in the zlum-MO-injected larvae at the 7 dpf stage after being treated with 0.5% atropine for 2 days. (E) Decreases in ocular enlargement were also found in the zLum-MO-injected larvae at the 7 dpf stage after being treated with 0.25% pirenzepine for 2 days. (F) No obvious changes in the phenotypes of zlum-MO-injected fish at the 7 dpf stage after being treated with 0.01% methoctramine.

Figure 9 shows atropine rescues zLum knockdown morphant. It can reverse the expressions of lumican, collagen 1a1, TGF-beta, MMP2 and TIMP2 which decreased in lumican morphant with atropine.

Figure 10 shows the big eye ratios of the zebrafish treated with marimastat, doxycycline, captopril, minocycline hydrochloride, atropine, aspirin, propofol and N-acetylcysteine.

Figure 11 shows the big eye ratios of the zebrafish treated with tetracycline (Fig. 11 (a)), minocycline (Fig. 11 (b)), doxycycline (Fig. 11 (c)), marimastat (Fig. 11 (d)) and batimastat (Fig. 11 (e)).

Detailed Description of the Invention

The invention uses a platform for using zebrafish to screen candidates for treating and/or preventing myopia and keratoconus disease. The invention found that lumican, one of a number of SLRPs, plays an important role in the regulation of fibrillogenesis and genes that influence the size of eyeballs in zebrafish, in addition to playing an important role in clinical myopia. Therefore, the invention uses an established zebrafish model to further identify the drugs affecting the expression of lumican and collagen fibrillogenesis, and the regulation of eyeball size. These drugs are potential candidates of treating myopia and keratoconus disease, including, but not limited to, metalloprotease (MMP) inhibitors, TGF-beta inhibitors, anticholinergic or muscarinic compounds and COX inhibitors.

As used in the specification and claims, the singular form "a", "an" and "the" include their plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, "expression" refers to the process by which a polynucleotide is transcribed into mRNA and/or the process by which the transcribed mRNA (also referred to as "transcript") is subsequently translated into peptides, polypeptides, or proteins.

A "control" is an alternative subject or sample used in an experiment for comparison purposes.

The terms "test compound" and "candidate compound" refer to any chemical entity, pharmaceutical, drug, or the like that is a candidate for being used to achieve the utility mentioned herein, such as increasing the expression of lumican and collagen fibrillogenesis and/or treating or preventing myopia and/or keratoconus disease. Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic through the screening methods of the present invention.

The term "big eye" denotes that an eye with a value of the axial length of the retinal pigmented epithelium layer divided by the axial length of the scleral coat is less than 0.7.

The term "treat" and "treatment" mean cause, or the act of causing, a postponement of development of a disorder and/or a reduction in the severity of symptoms that will or are expected to develop. The terms further include ameliorating existing symptoms or preventing symptoms.

The term "therapeutically effective amount" means that amount of a drug or pharmaceutical agent that will elicit the biological or medical response of a tissue system, animal or human that is being sought by a researcher or clinician, resulting in a beneficial effect for at least a statistically significant fraction of patients, such as a improvement of symptoms, a cure or a reduction in disease load.

The term "subject" is intended to include living organisms susceptible to conditions or diseases, disease states or conditions as generally disclosed, but not limited to, throughout this specification. Examples of subjects include humans, dogs, cats, cows, goats, and mice. The term "subject" is further intended to include transgenic species.

The term "alkyl" as used herein means a saturated straight chain or branched non-cyclic hydrocarbon having an indicated number of carbon atoms (e.g., C_1-C_{20}, C_1-C_{10}, C_1-C_8, C_1-C_6, C_1-C_4, etc.). Representative saturated straight chain alkyls include -methyl, -ethyl, -n-propyl, -n-butyl, -n-pentyl, -n-hexyl, -n-heptyl, -n-octyl, -n-nonyl and -n-decyl; while representative saturated branched alkyls include -isopropyl, -sec-butyl, -isobutyl, -tert-butyl, -isopentyl, 2-methylbutyl, 3-methylbutyl, 2-methylpentyl, 3-methylpentyl, 4-methylpentyl, 2-methylhexyl, 3-methylhexyl, 4-methylhexyl, 5-methylhexyl, 2,3-dimethylbutyl, 2,3-dimethylpentyl, 2,4-dimethylpentyl, 2,3-dimethylhexyl, 2,4-dimethylhexyl, 2,5-dimethylhexyl, 2,2-dimethylpentyl, 2,2-dimethylhexyl, 3,3-dimtheylpentyl, 3,3-dimethylhexyl, 4,4-dimethylhexyl, 2-ethylpentyl, 3-ethylpentyl, 2-ethylhexyl, 3-ethylhexyl, 4-ethylhexyl, 2-methyl-2-ethylpentyl, 2-methyl-3-ethylpentyl, 2-methyl-4-ethylpentyl, 2-methyl-2-ethylhexyl, 2-methyl-3-ethylhexyl, 2-methyl-4-ethylhexyl, 2,2-diethylpentyl, 3,3-diethylhexyl, 2,2-diethylhexyl, 3,3-diethylhexyl and the like.

The term "alkenyl" by itself or as part of another substituent, as used herein, refers to an unsaturated branched, straight-chain or cyclic alkyl having at least one carbon-carbon double bond

derived by the removal of one hydrogen atom from a single carbon atom of a parent alkene. The group may be in either the cis or trans conformation about the double bond(s). Typical alkenyl groups include, but are not limited to, ethenyl; propenyls such as prop-1-en-1-yl, prop-1-en-2-yl, prop-2-en-1-yl, prop-2-en-2-yl, cycloprop-1-en-1-yl; cycloprop-2-en-1-yl; butenyls such as but-1-en-1-yl, but-1-en-2-yl, 2-methyl-prop-1-en-1-yl, but-2-en-1-yl, but-2-en-2-yl, buta-1,3-dien-1-yl, buta-1,3-dien-2-yl, cyclobut-1-en-1-yl, cyclobut-1-en-3-yl, cyclobuta-1,3-dien-1-yl, etc.; and the like. In preferred embodiments, the alkenyl group is (C2-C6) alkenyl.

The term "alkynyl" by itself or as part of another substituent, as used herein, refers to an unsaturated branched, straight-chain or cyclic alkyl having at least one carbon-carbon triple bond derived by the removal of one hydrogen atom from a single carbon atom of a parent alkyne. Typical alkynyl groups include, but are not limited to, ethynyl; propynyls such as prop-1-yn-1-yl, prop-2-yn-1-yl, etc.; butynyls such as but-1-yn-1-yl, but-1-yn-3-yl, but-3-yn-1-yl, etc.; and the like. In preferred embodiments, the alkynyl group is (C2-C6) alkynyl.

The term "aryl" by itself or as part of another substituent, as used herein, refers to a monovalent aromatic hydrocarbon group having the stated number of carbon atoms (i.e., C5-C15 means from 5 to 15 carbon atoms) derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Typical aryl groups include, but are not limited to, groups derived from aceanthrylene, acenaphthylene, acephenanthrylene, anthracene, azulene, benzene, chrysene, coronene, fluoranthene, fluorene, hexacene, hexaphene, hexylene, as-indacene, s-indacene, indane, indene, naphthalene, octacene, octaphene, octalene, ovalene, penta-2,4-diene, pentacene, pentalene, pentaphene, perylene, phenalene, phenanthrene, picene, pleiadene, pyrene, pyranthrene, rubicene, triphenylene, trinaphthalene, and the like, as well as the various hydro isomers thereof. In preferred embodiments, the aryl group is (C5-C15) aryl, with (C5-C10) being even more preferred.

The term "heteroaryl" by itself or as part of another substituent, as used herein, refers to a monovalent heteroaromatic group having the stated number of ring atoms (e.g., "5-14 membered" means from 5 to 14 ring atoms) derived by the removal of one hydrogen atom from a single atom of a parent heteroaromatic ring system. Typical heteroaryl groups include, but are not limited to, groups derived from acridine, benzimidazole, benzisoxazole, benzodioxan, benzodiaxole, benzofuran, benzopyrone, benzothiadiazole, benzothiazole, benzotriazole, benzoxazine, benzoxazole, benzoxazoline, carbazole, .beta.-carboline, chromane, chromene, cinnoline, furan, imidazole, indazole, indole, indoline, indolizine, isobenzofuran, isochromene, isoindole, isoindoline, isoquinoline, isothiazole, isoxazole, naphthyridine, oxadiazole, oxazole, perimidine, phenanthridine, phenanthroline, phenazine, phthalazine, pteridine, purine, pyran, pyrazine, pyrazole, pyridazine, pyridine, pyrimidine, pyrrole, pyrrolizine, quinazoline, quinoline, quinolizine, quinoxaline, tetrazole, thiadiazole, thiazole, thiophene, triazole, xanthene, and the

like, as well as the various hydro isomers thereof. In preferred embodiments, the heteroaryl group is a 5-14 membered heteroaryl, with 5-10 membered heteroaryl being particularly preferred.

The term "pharmaceutically acceptable salts and prodrugs," as used herein, refers to those carboxylate salts, acid addition salts or base addition salts, and prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use of the compounds of the invention. The term "salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared in situ during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium and the like, as well as non-toxic ammonium, quaternary ammonium, and amine cations including, but not limited to, ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. (See, for example, Berge S. M., et al., "Pharmaceutical Salts," J. Pharm. Sci., 1977; 66:1-19 which is incorporated herein by reference.)

In one aspect, the invention provides a method of using zebrafish with big eye to identify a candidate compound that can be used to affect the expression of lumican and/or collagen fibrillogenesis, and/or treat myopia and/or keratoconus disease. The method comprises contacting a test compound with the zebrafish with big eye and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases. In one embodiment, the test compound is identified as a candidate compound if the ratio of the big eye in the zebrafish decreases relative to the total number of the big eyes in the control zebrafish that is not treated with the test compound.

In another aspect, the invention provides a method of using lumican gene and/or collagen fibrillogenesis-relating gene knockdown zebrafish to identify a candidate compound that can be used to affect the expression of lumican and/or collagen fibrillogenesis and/or treat myopia and/or keratoconus disease. The method comprises contacting a test compound with lumican gene and/or collagen fibrillogenesis-relating gene knockdown zebrafish, determining the number of the big eye in the zebrafish and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases. In one embodiment, the test compound is identified as a candidate compound if the ratio of the big eye in the zebrafish decreases, relative to the total number of the eyes in the zebrafishes or that of the big eyes in the control zebrafish that is not treated with the test compound.

In one embodiment, the invention provides a method of identifying a candidate compound affecting the expression of lumican and collagen fibrillogenesis and/or the regulation of eyeball size, comprising:

(a) introducing an antisense mRNA of lumican gene and/or collagen fibrillogenesis-relating gene or an analog of the antisense mRNA into plural fertilized embryos of zebrafish;

(b) exposing the zebrafish obtained from (a) to a test compound for a sufficient length of time and then collecting the zebrafish; and

(c) determining the number of the big eye in the zebrafish and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases.

Preferably, the antisense mRNA in (a) are lumican or keratocan antisense mRNA. Preferably, the knockdown zebrafish in (b) are exposed to the test compound at their optic cup formation. Preferably, the resulting zebrafish in (b) are collected at their cornea establishment stage. Preferably, the test compound in (c) is identified as a candidate compound if the ratio of the big eye in the zebrafish decreases, relative to the total number of the eyes of the zebrafish or the total number of the big eyes in the control zebrafish. In view of the above, the method comprises the following steps:

(a) introducing an antisense mRNA of lumican gene and/or collagen fibrillogenesis-relating gene or an analog of the antisense mRNA into plural fertilized embryos of zebrafish;

(b) exposing the zebrafish obtained from (a) to a test compound for a sufficient length of time and then collecting the zebrafish; and

(c) determining the number of the big eye in the zebrafish and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases relative to the total number of the eyes in the zebrafishes or that of the big eyes in the control zebrafish that is not treated with the test compound.

In another embodiment, the invention provides a method of identifying a candidate compound treating and/or preventing myopia and/or keratoconus disease, comprising:

(a) introducing an antisense mRNA of lumican gene and/or collagen fibrillogenesis-relating gene or an analog of the antisense mRNA into plural fertilized embryos of zebrafish;

(b) exposing the zebrafish obtained from (a) to a test compound for a sufficient length of time and then collecting the zebrafish; and

(c) determining the number of the big eye in the zebrafish and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases.

Preferably, the antisense mRNA in (a) are lumican or keratocan antisense mRNA. Preferably, the knockdown zebrafish in (b) are exposed to the test compound at their optic cup formation. Preferably, the resulting zebrafish in (b) are collected at their cornea establishment stage. Preferably, the test compound in (c) is identified as a candidate compound if the ratio of the big eye in the zebrafish decreases, relative to the total number of the eyes of the zebrafish or the total number of the big eyes in the control zebrafish. In view of the above, the method comprises the following steps:

(a) introducing an antisense mRNA of lumican gene and/or collagen fibrillogenesis-relating gene or an analog of the antisense mRNA into plural fertilized embryos of zebrafish;

(b) exposing the zebrafish obtained from (a) to a test compound for a sufficient length of time and then collecting the zebrafish; and

(c) determining the number of the big eye in the zebrafish and identifying the test compound as a candidate compound if a ratio of the big eye in the zebrafish decreases relative to the total number of the eyes in the zebrafishes or that of the big eyes in the control zebrafish that is not treated with the test compound.

The screening assays described herein provide methods for identifying compounds that affect the expression of lumican and collagen fibrillogenesis and the regulation of eyeball size, and treat and/or prevent myopia and keratoconus disease using the decrease of the ratio of enlarged eyeballs in lumincan knockdown zebrafish as an indicator of compounds that affect the expression of lumican and collagen fibrillogenesis and treat and/or prevent myopia and keratoconus disease.

Compounds identified in the assays described herein are candidate compounds that can be used (i) to affect the expression of lumican and collagen fibrillogenesis and the regulation of eyeball size and/or (ii) as lead compounds to develop related compounds that can be used to treat and/or prevent myopia and keratoconus disease.

Keratocan and Lumican Genes in Zebrafish

Lumican, one of several SLRPs, plays an important role in the regulation of fibrillogenesis or the genes affecting the size of eyeballs in zebrafish, in addition to playing an important role in clinical myopia. Similar to keratocan and lumican genes of human and mice, zebrafish keratocan and lumican genes have all the structural features of SLRPs, i.e. a central domain of leucine- rich repeats flanked by N- and C-terminal domains with conserved cysteines. The size and structure of the zebrafish keratocan and lumican genes are similar to the mammalian keratocan and lumican genes. Interestingly, both the zebrafish lumican and keratocan genes have been mapped to the same genome. In addition, similar to the mammalian keratocan and lumican genes, the zebrafish keratocan and lumican genes are TATA-less genes. Also, the most striking difference between keratocan and lumican expression in the corneas of zebrafish and mammal is that they are expressed mainly in the corneal epithelial layer in the case of the former instead of the stromal layer (keratocytes). It is also a very promising field to explore in developmental biology.

Knockdown Zebrafish

Surprisingly, an increased size of eyeball (i.e., big eye) was associated with the knockdown of zebrafish lumican, keratocan and/or collagen fibrillogenesis-relating gene(s) during the development of zebrafish, a clinical manifestation similar to clinical findings in children myopia. In children myopia, axial elongation of eyeballs in children was correlated with an alteration of SNP at the human lumican

gene promoter in patients. Decreased expression level of zebrafish lumican, keratocan and/or collagen fibrillogenesis-relating gene(s) by knockdown using an antisense or its analog may mimic the molecular mechanism causing the axial elongation observed in patients with this SNP. According to the invention, an antisense mRNA of lumican is introduced into a fertilized embryo of zebrafish to obtain a lumican knockdown zebrafish. In one embodiment, the lumican antisense is morpholino. Preferably, the morpholino has the sequence: 5'-GATCCCAGAGCAAACATGGCTGCAC-3'.

Exposure of Knockdown Zebrafish to a Test Compound and Collection of the Resulting Zebrafish

External development and optical clarity during embryogenesis allow for visual analyses of early developmental processes, and high fecundity and short generation times facilitate genetic analyses. The adult zebrafish eye is emmetropic, and it is able to transmit both visible and ultraviolet wavelengths, evidenced by adult responsiveness to ultraviolet wavelengths. The development of the zebrafish eye is similar to eye development in other species of fish and mammals. It begins with the optic primordial at about 12 h postfertilization (hpf). By 24 hpf, the eyecups are well developed, and by about 30 hpf, ganglion cells are found in a small area of the ventronasal retina. At 50 hpf, the retinal layers become apparent across parts of the retina. Young zebrafish are hyperopic and become emmetropic by 72 hpf, which is the same time the extraocular muscles appear to be adult-like, and the optokinetic response is evident.

According to the invention, the knockdown zebrafish are exposed to a test compound at the optic cup formation stage thereof. Generally, at around 24 hours after fertilization, the optic cup of zebrafish is formed. The knockdown zebrafish can be exposed to the test compound at the optic cup formation stage. After contacting the knockdown zebrafish with the test compound, the test compound, if it is a potential candidate, may start to activate the expression of lumican and collagen fibrillogenesis, thereby decreasing the enlargement of eyeball and treating and/or preventing myopia and/or keratoconus disease. Retina lenses are established around 48 hours after fertilization, and the sclera and cornea are established around 72 hours after fertilization. At the scleral and corneal establishment stage, the zebrafish are collected.

Determination of Big Eye Zebrafish and Identification of Candidate Compounds

Big eye of a zebrafish is an indicator of myopia. As used herein, "big eye" refers to an eye with an enlarged axial length of eyeball and denotes the value of the axial length of the retinal pigmented epithelium layer divided by the axial length of the scleral coat less than 0.7. The axial length of a retinal pigmented epithelium layer and the axial length of a scleral coat can be measured by any method known in the art; for example, dissecting microscopy.

The test compound can be identified as a candidate compound affecting the expression of lumican and/or collagen fibrillogenesis and/or regulation of eyeball size, and/or treating and/or preventing myopia and/or keratoconus disease if the ratio of the number of big eyes decreases relative to the total

number of eyes in zebrafish or that of big eyes in control zebrafish that is not contacted with the test compound. Preferably, the test compound can be identified as a candidate compound if the ratio of the number of big eyes is less than 30% relative to the total number of eyes in zebrafish or that of big eyes in control zebrafish that is not contacted with the test compound. Preferably, the ratio is less than 15%. More preferably, the ratio decreases to about 0% to about 30%, about 0% to about 25%, about 0% to about 20%, about 0% to about 15%, about 0% to about 10%, about 1% to about 30%, about 1% to about 25%, about 1% to about 20% or about 1% to about 15%.

In a further aspect, screening of test compounds is accomplished by identifying those in a group of test compounds that decrease the ratio of the big eye of zebrafish to less than 30%. Test compounds that decrease the big eye ratio are also referred to herein as "candidate compounds."

The new screening methods of the invention can be used to identify compounds, e.g., small organic or inorganic molecules (molecular weight less than 1,000 Da), oligopeptides, oligonucleotides, or carbohydrates that decrease the big eye ratio of the lumican knockdown zebrafish. As used herein, a "test compound" can be any chemical compound, for example, a macromolecule (e.g., a polypeptide, a protein complex, glycoprotein, or a nucleic acid) or a small molecule (e.g., an amino acid, a nucleotide, an organic or inorganic compound). A test compound can have a formula weight of less than about 10,000 grams per mole, less than 5,000 grams per mole, less than 1,000 grams per mole, or less than about 500 grams per mole. The test compound can be naturally occurring (e.g., an herb or a natural product), synthetic, or can include both natural and synthetic components. Examples of test compounds include metalloprotease inhibitors, collagenase inhibitors, TGF-β pathway activators, TGF-β inhibitors and Cox inhibitors.

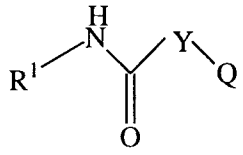
Metalloprotease Inhibitors for Use in A Method for Affecting the Expression of Lumican and/or Collagen Fibrillogenesis, and/or Treating Myopia and/or Keratoconus Disease

In one embodiment, the invention provides a method for treating a disease medicated by expression of lumican and/or collagen fibrillogenesis and/or treating myopia and/or keratoconus disease, comprising administering to the subject a therapeutically effective amount of a MMP inhibitor.

Metalloproteases (MMPs) are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense. Inhibitors of metalloproteases are known. Examples include natural biochemicals such as tissue inhibitors of metalloproteinases (TIMPs), α2-macroglobulin and their analogs or derivatives. A number of smaller peptide-like compounds that inhibit metalloproteases have been described. Thiol group-containing amide or peptidyl amide-based metalloprotease (MMP) inhibitors are known as is shown in, for example, WO95/12389, WO96/11209 and U.S. Pat. No. 4,595,700. Hydroxamate group-containing MMP inhibitors are disclosed in a number of published patent applications such as WO 95/29892, WO 97/24117, WO 97/49679 and EP 0 780 386 that disclose carbon back-boned compounds, and WO

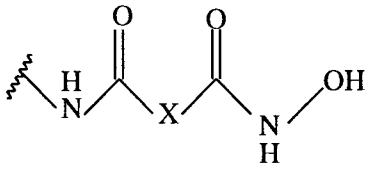
90/05719, WO 93/20047, WO 95/09841 and WO 96/06074 that disclose hydroxamates that have a peptidyl back-bones or peptidomimetic back-bones. In addition, other pyrimidine-based MMP inhibitors, hydroxypyrone-based MMP inhibitors, phosphorous-based MMP inhibitors and tetracycline-based MMP inhibitors have also been reported (Cancer Metastasis Rev., 2006, 25:115-136.)

According to one embodiment of the invention, the MMP inhibitor is a peptidomimetic hydroxamate MMP inhibitor having the following Formula (I) or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof,



(I)

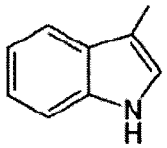
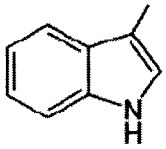
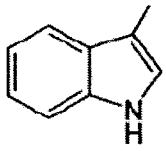
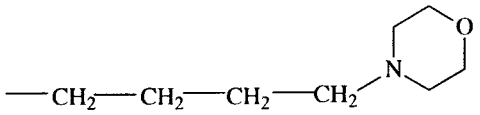
wherein

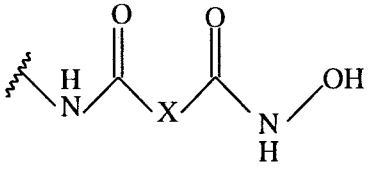
Q is absent or  ;

X is C_{1-10}alkylene, C_{2-10} alkenylene or C_{2-10} alkynylene, unsubstituted or substituted by one or more OH, C_{1-10} straight or branched alkyl, C_{2-10} straight or branched alkenyl, C_{1-10}alkylC_{5-15}aryl, C_{1-10}alkenylC_{5-15}aryl, C_{1-10}alkynylC_{5-15}aryl, C_{1-10}alkylsulfanylC_{5-15}aryl, C_{1-10}alkylsulfonylC_{5-15}aryl, C_{1-10}alkylsulfinylC_{5-15}aryl, C_{1-10}alkyloxy or C_{5-15}aryl;

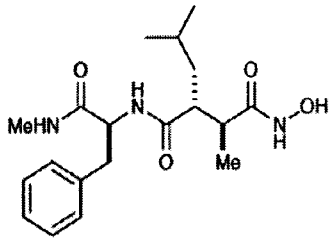
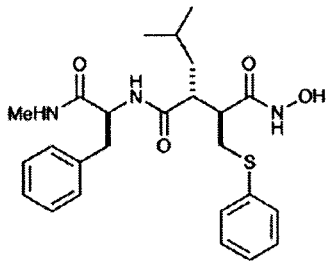
Y is C_{1-10}alkylene, C_{2-10} alkenylene or C_{2-10} alkynylene, unsubstituted or substituted by one or more OH, C_{1-10} straight or branched alkyl, C_{2-10} straight or branched alkenyl, C_{1-10}alkylC_{5-15}aryl, C_{1-10}alkenylC_{5-15}aryl, C_{1-10}alkynylC_{5-15}aryl, C_{1-10}alkylsulfanylC_{5-15}aryl, C_{1-10}alkylsulfonylC_{5-15}aryl, C_{1-10}alkylsulfinylC_{5-15}aryl, C_{1-10}alkyloxy, C_{5-15}aryl, C_{1-10}alkylC_{5-15}aryl, C_{5-14}heteroaryl, C_{1-10}alkylC_{5-14}heteroaryl, or C_{1-10}alkylsulfanylC_{5-14}heteroaryl, provided that when Q is absent, Y is C_{5-14}heteroaryl; wherein the heteroaryl is optionally substituted and has 1 to 3 heteroatoms independently selected from N, O and S; and

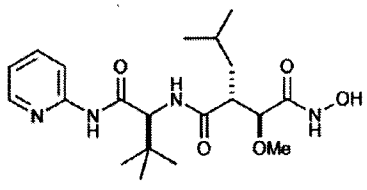
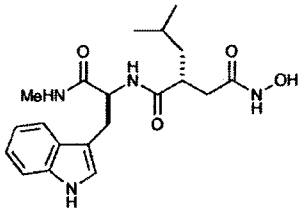
R^1 is H, OH, C_{1-10}alkyl, C_{2-10}alkenyl, C_{2-10}alkynyl, C_{5-15}aryl, C_{1-10}alkylC_{5-15}aryl, C_{5-14}heteroaryl, or C_{1-10}alkylC_{5-14}heteroaryl.

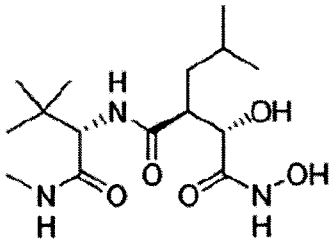
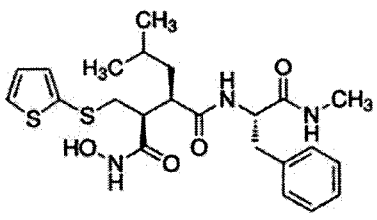
Preferably, when Q is absent, Y is  ; more preferably, when Q is absent, Y is  and R_1 is C_{5-15}heteroaryl; most preferably, when Q is absent, Y is  and R_1 is  .

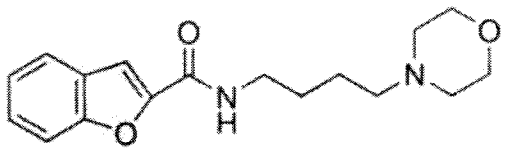
Preferably, when Q is  , X is $-CH_2-$, $-CH(CH_2CH_2(CH_3)_2)-$, or $-CH_2CH_2-$, $-CH(CH_2CH_2(CH_3)_2)CH(CH_3)-$, $-CH(CH_2CH_2(CH_3)_2)CH(CH_2\text{-S-phenyl})-$, $-CH(CH_2CH_2(CH_3)_2)CH(OCH_3)-$, $-CH(CH_2CH_2(CH_3)_2)-$, or $-CH_2CH_2-$, $-CH(CH_2CH_2(CH_3)_2)CH(CH_3)-$, $-CH(CH_2CH_2(CH_3)_2)CH_2-$, $-CH(CH_2CH_2(CH_3)_2)CH(OH)-$, or $-CH(CH_2CH_2(CH_3)_2)CH(CH_2\text{-S-thienyl})-$; Y is $-CH(CH_2\text{-phenyl})-$, $-CH(C(CH_3)_3)-$ or $-CH(CH_2\text{-indolyl})-$; and R_1 is CH_3 or phenyl.

More preferably, the compound of Formula (I) is selected from the group consisting of:

 KB-R-7785,  GI-129471,

 Salimostat,  Ilomastat,

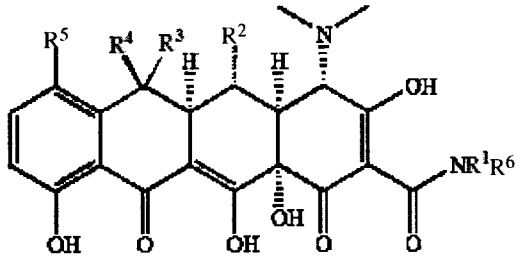
 Marimastat,  Batimastat, and



CL-82198,

or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof.

According to another embodiment of the invention, the MMP inhibitor is a tetracyclic-based MMP inhibitor having the following Formula (II) or a tautomer or pharmaceutically acceptable salt, prodrug or solvate thereof,



(II)

wherein

R^1 and R^6 are each independently H, C_{1-10}alkylC_{5-14}heteroaryl, or $C_{1-10}NR^7R^8$;

R^2 is hydrogen or OH;

R^3 and R^4 are each independently H, OH, NH_2, NO, CN, C_{1-10}alkyl, C_{1-10}alkenyl or C_{1-10}alkynyl;

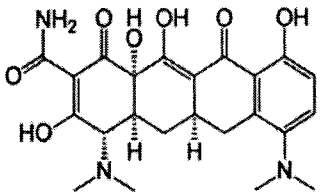
R^5 is hydrogen, halogen, NH_2, OH, NO, CN, C1-10 alkyl, NHC_{1-10}alkyl, $N(C_{1-10}alkyl)_2$, C_{5-15}aryl or C_{5-14}heteroaryl; and

R^7 and R^8 are each independently H, C_{1-10}alkyl C_{1-10}alkylNH_2COOH or taken together with the nitrogen atom to which each is attached form a 3 to 8 membered heteroaryl;

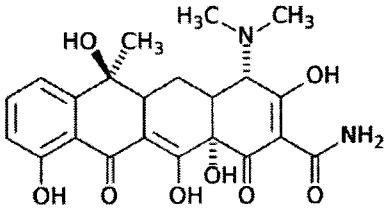
wherein heteroaryl has 1 to 3 heteroatoms independently selected from N, O and S.

Preferably, R^1 is H; R^6 is H, -CH_2-pyrrolyl, -CH_2-NH-CH_2-CH_2-CH_2-CH_2-CH(NH2)-COOH; R^2 is H or oxo; R3 is H or OH; R4 is H or OH and R^5 is NH_2, $N(CH_3)_2$or halogen.

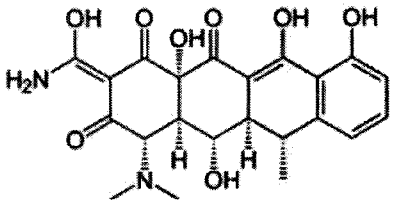
More preferably, the compound of Formula (II) is selected from the group consisting of:



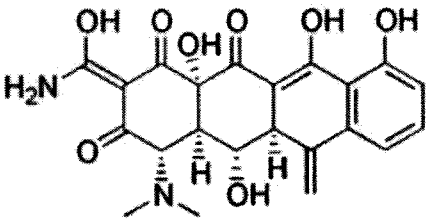
Minocycline hydrochloride,



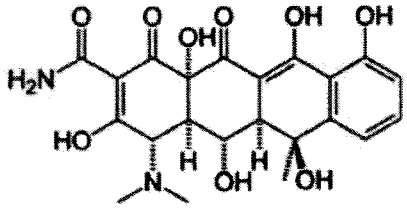
Tetracycline,



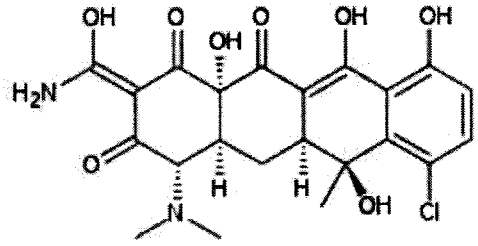
Doxycycline,



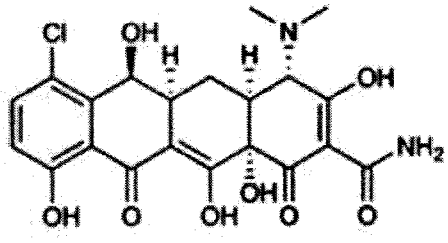
Metacycline,



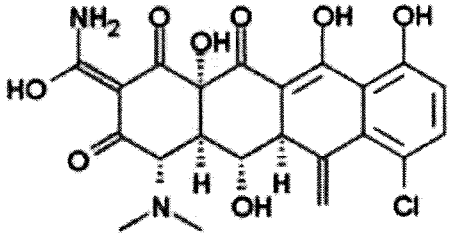
Oxytetracycline,



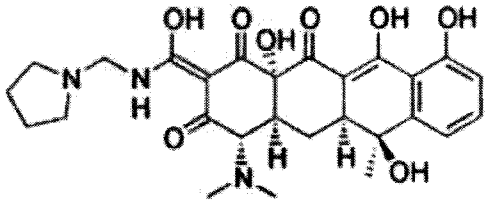
Chlortetracycline,



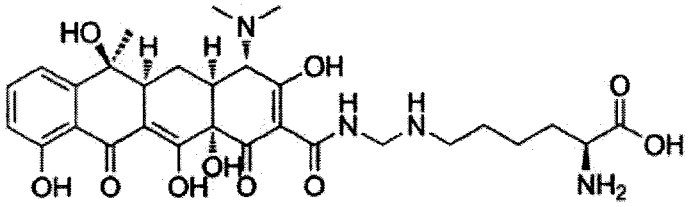
Democlocycline,



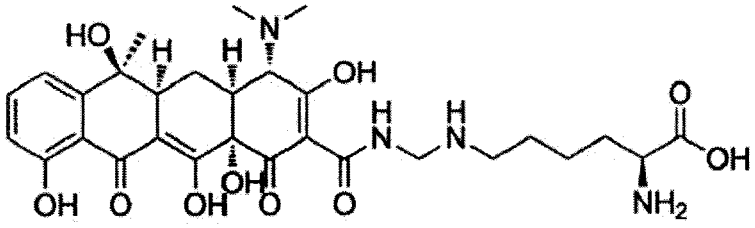
Meclocycline,



Rolitetracycline,



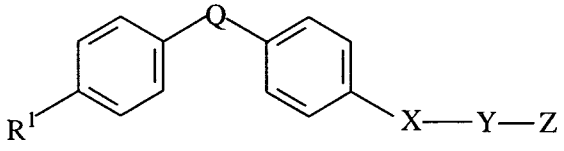
Lymccycline, and



Tigecycline,

or a tautomer or pharmaceutically acceptable salt, prodrug or solvate thereof.

According to another embodiment of the invention, the MMP inhibitor is a diaryl ether hydroxamate having the following Formula (III) or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof,



(III)

wherein

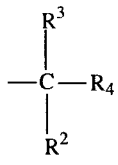
R^1 is halogen, OH, NH_2, OC_{1-10}alkyl unsubstituted or substituted by 1-3 halogen, or NH_2;

Q is absent or O;

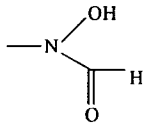
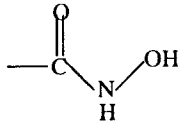
X is O or $S(O)_2$;

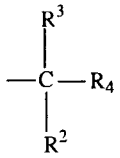
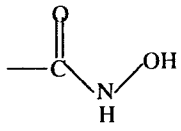
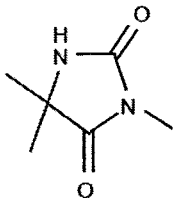
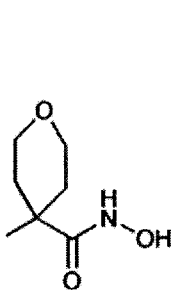
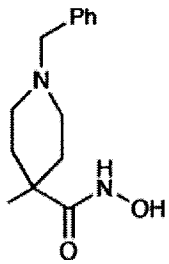
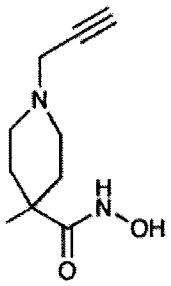
Y is CH_2 or NH;

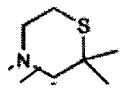
Z is C_{5-14}heteroaryl having 1 to 3 heteroatoms independently selected from N, O and S or

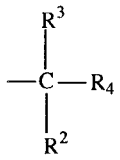


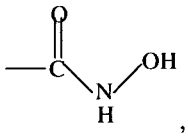
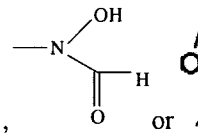
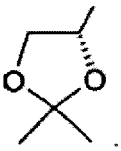
, and

R^2, R^3 and R^4 are each independently H, C_{1-10}alkyl,  ,  ,or unsubstituted or substituted C_{5-14}heteroaryl having 1 to 3 heteroatoms independently selected from N, O and S; or R^2 and R^4 are taken together with the carbon atom to which each is attached form a 5 membered saturated heterocyclyl ring which is unsubstituted or substituted by CN or C_{1-10}alkyl, C_{1-10}alkylC_{5-15}aryl.

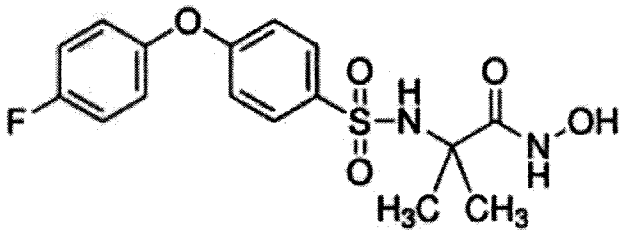
Preferably, when Q is absent, R^1 is $OC(halogen)_3$, X is O, Y is CH_2, Z is  , and R^2, R^3 and R^4 are each independently H,  , or  ,or R^2 and R^4 are taken together with the carbon or nitrogen atom to form  ,  or  .

Preferably, when Q is O; R^1 is halogen or $OC(halogen)_3$, X is $S(O)_2$, and Z is  .

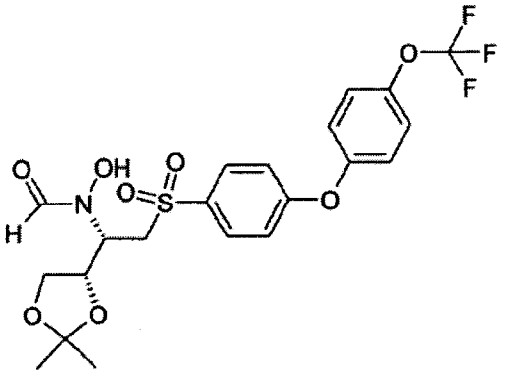
Preferably, when Q is O; R^1 is halogen or $OC(halogen)_3$, X is $S(O)_2$, Y is NH; Z is  ;

and R^2, R^3 and R^4 are each independently H, $C_{1\text{-}10}$alkyl,  ,  or  .

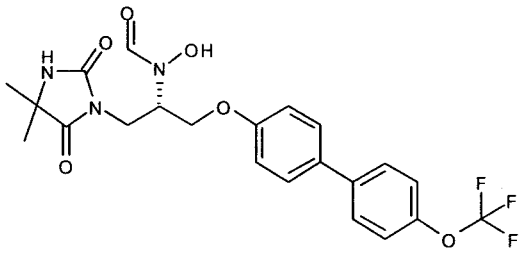
More preferably, the compound of Formula (III) is selected from the group consisting of:



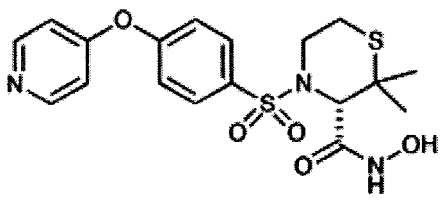
CP471474,



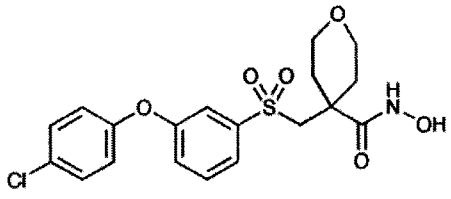
ABT-518,



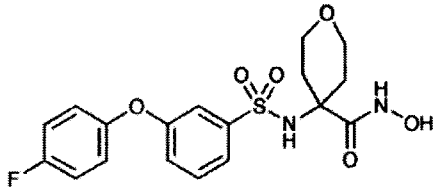
ABT-770,



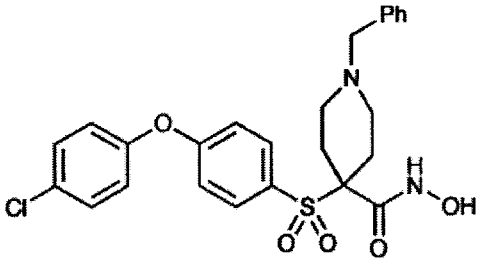
Prinomastat,



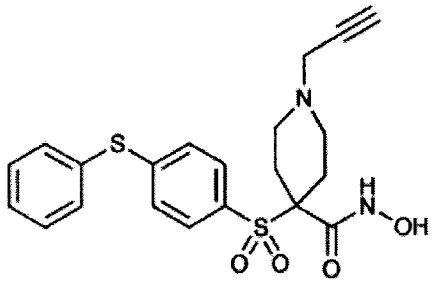
RS-130830,



CAS No. 230954-09-3,



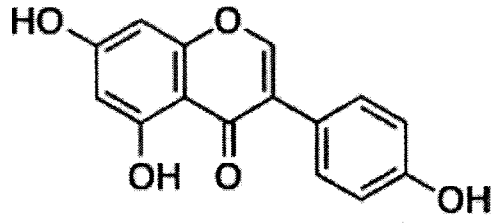
CAS NO. 239796-97-5, and



SC-276,

or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof.

According to a further another embodiment of the invention, the MMP inhibitor is a compound having the following formula:



Genistein,

or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof.

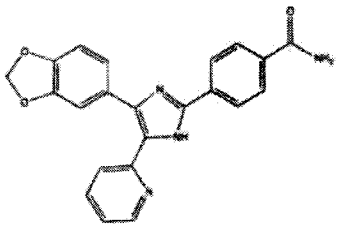
More preferably, the MMP inhibitor is Marimastat, Batimastat, CL-82198, Minocycline, Tetracycline or Doxycycline.

TGF-beta Inhibitors for Use in A Method for Affecting the Expression of Lumican and/or Collagen Fibrillogenesis, and/or Treating Myopia and/or Keratoconus Disease

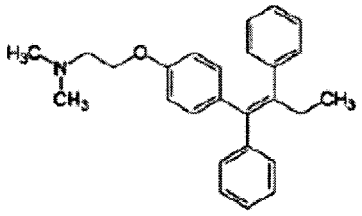
In an another embodiment, the invention provides a method for treating a disease medicated by expression of lumican and/or collagen fibrillogenesis, and/or treating myopia and/or keratoconus disease, comprising administering to the subject a therapeutically effective amount of a TGF-beta inhibitor.

Transforming growth factor-beta (TGF-beta) belongs to a large super-family of multifunctional polypeptide factors. TGF-beta is a potent inducer of growth arrest in many cell types, including epithelial cells. This activity is the basis of the tumor suppressor role of the TGF-beta signaling system in carcinomas. Other activities, including TGF-beta-induced epithelial-to-mesenchymal differentiation, contribute to cancer progression. PCT patent application WO 02/0948332 describes a genus of dihydropyrrolopyrazole compounds useful for treating disorders associated with enhanced TGF-beta signaling activity or overproduction. US 7,638,537 and US 7,635,702 provide pyrazole compounds and imidazole compounds as potent inhibitors of TGF-signaling pathway.

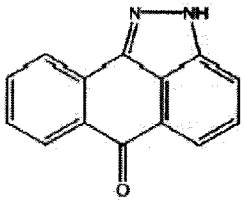
According to one embodiment of the invention, the TGF-beta inhibitor is selected from the group consisting of:



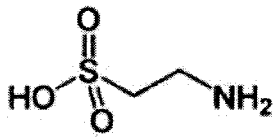
SB-431542,



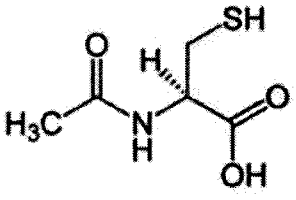
Tamoxifen,



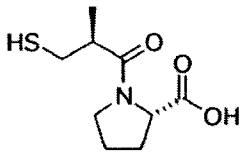
SP600125,



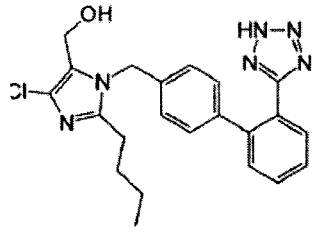
Taurine,



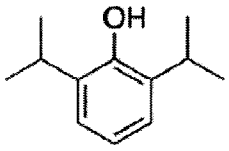
N-acetylcysteine,



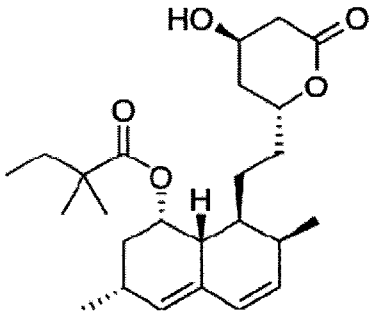
Captopril,



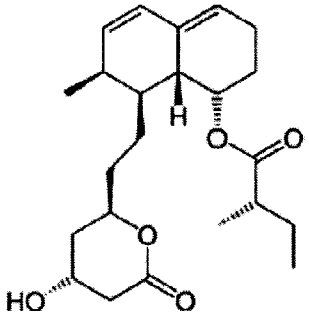
Losartan,



Propfol,



Simvastatin, and



Mevastatin.

or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof.

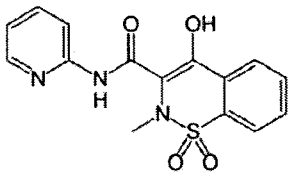
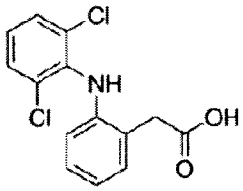
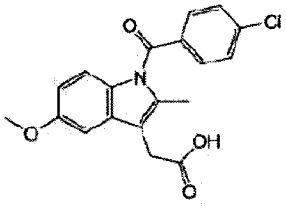
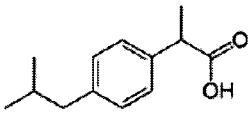
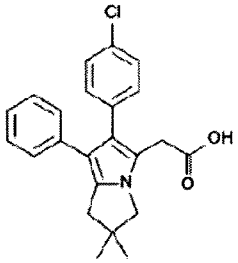
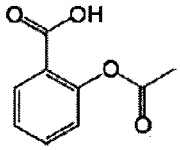
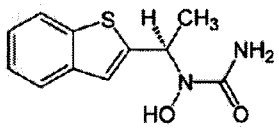
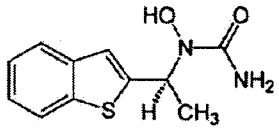
Preferably, the TGF-beta inhibitor is Losartan, N-acetylcysteine, Propofol and Captopril.

COX/LOX Inhibitors for Use in A Method for Affecting the Expression of Lumican and/or Collagen Fibrillogenesis, and/or Treating Myopia and/or Keratoconus Disease

In a further embodiment, the invention provides a method for treating a disease medicated by expression of lumican and/or collagen fibrillogenesis and/or treating myopia and/or keratoconus disease, comprising administering to the subject a therapeutically effective amount of a COX/LOX inhibitor.

COX enzymes convert arachidonic acid to the prostaglandin endoperoxide PGH2, from which other prostaglandins are formed. A number of drugs inhibit the action of either the COX or the LOX enzymes.

According to the invention, the COX/LOX inhibitor is selected from the group consisting of:


Piroxicam,

Voltaren (Diclofenac),

Indomethacin,

Ibuprofen,

Licofelone,

Aspirin, and


and Zileuton;

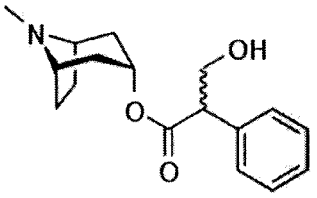
or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof.

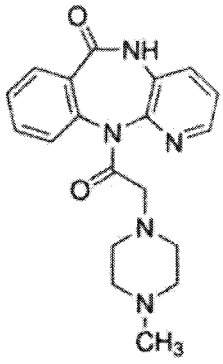
Preferably, the COX/LOX inhibitor is Aspirin.

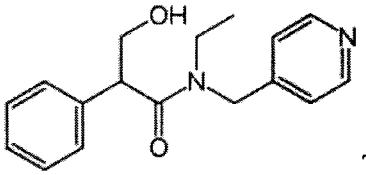
Anticholinergic & Muscarinic Serier Compounds for Use in A Method for Affecting the Expression of Lumican and/or Collagen Fibrillogenesis, and/or Treating Myopia and/or Keratoconus Disease

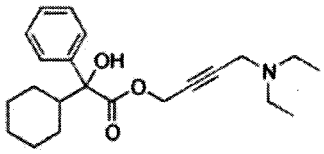
Further, in a another embodiment, the invention provides a method for treating a disease medicated by expression of lumican and/or collagen fibrillogenesis and/or treating myopia and/or keratoconus disease, comprising administering to the subject a therapeutically effective amount of an anticholinergic or muscarinic compound.

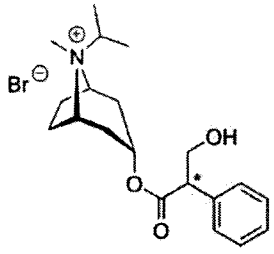
According to the invention, the anticholinergic or muscarinic compound is selected from the group consisting of:

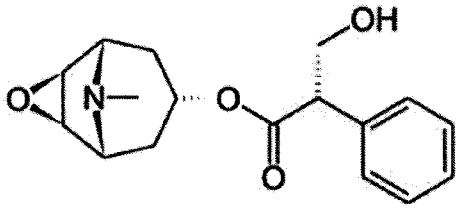

Atropine,


Pirenzepine,


Tropicamide,


Oxybutynin,


(Tavor), Ipratropium bromide (Atrovent), and


Scopolamine,

or a pharmaceutically acceptable salt, prodrug, solvate, stereoisomer or enantiomer thereof.

Preferably, the anticholinergic or muscarinic compound is atropine.

Illustrating examples of the candidate compounds are listed in the table below:

Name	Type
Propofol	TGF beta activation
Simvastatin	TGF beta activation
Mevastatin	TGF beta activation
SB-431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide)	TGF-beta receptor inhibitor
Tamoxifen	TGF-Beta1 inhibitor

SB-505124 (2-[4-(1,3-Benzodioxol-5-yl)-2-(1,1-dimethylethyl)-1*H*-imidazol-5-yl]-6-methyl-pyridine)	TGF-beta receptor inhibitor
RepSox (SB-4696)	TGF-beta receptor inhibitor
Captopril	TGFB inhibitor
SP600125[Anthrapyrazolone]	TGFB inhibitor
N-Acetylcysteine	Collagenase inhibitor
Vita min E succinate	TGF beta activation
Concanavalin A (Con A)	TGF beta activation
Statin	TGF beta activation
SB525334 (6-[2-tert-Butyl-5-(6-methyl-pyridin-2-yl)-1H-imidazol-4-yl]-quinoxaline)	TGF-beta receptor inhibitor
Doxorubicin	TGFB inhibitor
Norrin	TGFB inhibitor
AP 12009 (Trabedersen)	TGFB inhibitor

Doxycycline	MMP inhibitor
Genistein	MMP inhibitor
Marimastat	MMP inhibitor
Minocycline hydrochloride	MMP inhibitor
CL-82198 (N-[4-(4-Morpholinyl)butyl]-2-benzofurancarboxamide hydrochloride)	MMP inhibitor
Ilomastat (GM6001)	MMP inhibitor
Batimastat	MMP inhibitor
CP471474 (2-[[[4-(4-Fluorophenoxy)phenyl]sulfonyl]amino]-N-hydroxy-2-methylpropanamide)	MMP inhibitor
Tetracycline	MMP inhibitor

Aspirin	COX inhibitor
naproxen	COX inhibitor
Indomethacin	COX inhibitor

Piroxicam	COX inhibitor
Zileuton	COX inhibitor
Voltaren	COX inhibitor
Iduprofen	COX inhibitor

Atropine	Anticholinergic
Tropicamide	Anticholinergic
Ipratropium bromide	Anticholinergic
Oxybutynin	Antimuscarinic
Scopolamine hydrobromide	Antimuscarinic
Pirenzepine dihydrochloride	Antimuscarinic
Taurine	Glycine receptors inhibitor

Losartan	Angiotensin-II receptor inhibitor

PD-166866 (6-aryl-pyrido[2,3-d]pyrimidines)	FGF inhibitor
PD-161570 (N-[6-(2,6-Dichlorophenyl)-2-[[4-(diethylamino)butyl]amino]pyrido[2,3-d]pyrimidin-7-yl]-N'-(1,1-dimethylethyl)urea)	FGF inhibitor
PD 173074 (N-[2-[[4-(Diethylamino)butyl]amino]-6-(3,5-dimethoxyphenyl)pyrido[2,3-d]pyrimidin-7-yl]-N'-(1,1-dimethylethyl)urea)	FGF inhibitor
(E/Z)-BCI hydrochloride	FGF inhibitor
1-Methyl-2-piperidinemethanol	FGF inhibitor
2-Thiohydantoin	FGF inhibitor
SU5402 (2-[(1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-4-methyl-1H-pyrrole-3-	FGF inhibitor

propanoic acid)	
PD166285 (the 6-aryl-pyrido[2,3-d]pyrimidines)	FGF inhibitor
N-Methyl-4-piperidinol	FGF inhibitor
PD-089828 (6-aryl-pyrido-[2,3-d]pyrimidines)	FGF inhibitor
NP603 ((Z)-3-(5-(6-(3,5-Dimethoxyphenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrol-3-yl)-propionic acid)	FGF inhibitor
SU4984 ([3-[4-(1-formylpiperazin-4-yl)-benzylidenyl]-2-indolinone]	FGF inhibitor
TSU-68 (SU 6668) (Orantinib)	FGF inhibitor
Brivanib	FGF inhibitor
BIBF1120 (Vargatef)	FGF inhibitor
Ponatinib (AP24534)	FGF inhibitor
Danusertib (PHA-739358)	FGF inhibitor
Masitinib	FGF inhibitor
Brivanib (BMS-540215)	FGF inhibitor
Brivanib alaninate (BMS-582664)	FGF inhibitor

Preferred examples include, but are not limited to, those listed in the table below.

Name
Atropine
Tropicamide
Ipratropium bromide (Atrovent)
Oxybutynin (Tavor)
Scopolamine hydrobromide
Pirenzepine dihydrochloride
SB 431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide)
Tamoxifen
SB-505124 (2-[4-(1,3-Benzodioxol-5-yl)-2-(1,1-dimethylethyl)-1*H*-imidazol-5-yl]-6-methyl-pyridine)

RepSox (SB-4696)
Doxycycline hyclate (Dermostat,Periostat)
Genistein
Marimastat
Taurine
Minocycline hydrochloride
n-acetylcysteine
Aspirin
Propofol
SP600125 (Anthrapyrazolone)
Zileuton
Mevastatin
Indomethacin
Piroxicam
Captopril
Simvastatin

Any of the above-mentioned compounds can be combined with a pharmaceutically acceptable carrier to form a formulation, composition, combination or preparation (each term can be used interchangeably). The phrase "pharmaceutically acceptable carrier" used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a compound(s) of the present invention within or to the subject such that it can perform its intended function. Typically, such compounds are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline;

Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for intravenous, oral, nasal, topical, transdermal, buccal, sublingual, rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 1 percent to about ninety-nine percent of active ingredient, preferably from about 5 percent to about 70 percent, most preferably from about 10 percent to about 30 percent.

The pharmaceutical formulations of the invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions/formulations, at their art-established usage levels. Thus, for example, the compositions/formulations may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the therapeutic compounds of the invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the therapeutic compounds of the formulation. Additionally, it will be appreciated that other pharmaceutical formulations, which can conveniently be presented in unit dosage form, can be prepared according to conventional techniques well known in the pharmaceutical industry. In general, such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). The formulations are

typically prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; humectants, such as glycerol; disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; solution retarding agents, such as paraffin; absorption accelerators, such as quaternary ammonium compounds; wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; absorbents, such as kaolin and bentonite clay; lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluents.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the compound in the proper medium. Absorption enhancers can also be used to increase the

flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the active compound in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention. Such solutions are useful for the treatment of any ophthalmic disease. 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 or ointments. Aqueous solutions and diluents for suspensions that are used in preparation of eye drops can include distilled water, physiological saline, and the like. Non-aqueous solutions and diluents for suspensions can include vegetable oil, liquid paraffin, mineral oil, propylene glycol, p-octyldodecanol as well as similar solvents. Various additives may be contained in eye drops, ophthalmic gels and/or ophthalmic ointments as needed. These include, but not limited to, buffering agents, isotonizers, preservatives, thickeners, stabilizers, antizoxidants, pH-adjusting agents, chelating agents. 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, and acetate buffer. Buffering agents are included in an amount that provides sufficient buffer capacity for the expected physiological conditions. In addition to a buffer, isotonizers can be added to eye drops to make the preparation isotonic with the tear. Isotinizers include, but are not limited to, sugars such as 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 and sodium succinate. Isotonizers are added in an amount that makes the osmotic pressure of the eye drop equal to that of the tear. 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, benzalkonium chloride, parabens, chlorobutanol and benzylic alcohol. 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 and carboxyvinyl polymers. 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 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. 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. Sterilization methods can include, but are not limited to, heat sterilization, irradiation and filtration. 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.

The therapeutic compounds described herein can also be administered in a time release formulation, for example in a composition which includes a slow release polymer. These compounds can be prepared with carriers that will protect these compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are generally known to those skilled in the art.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be desirable. In some embodiments, topical formulations include those in which the therapeutic compounds described herein are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Exemplary lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). The therapeutic compounds described herein may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, these compounds can be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C.sub.1-10 alkyl ester (for example, isopropylmyristate, IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

In some embodiments of the invention, pharmaceutical compositions may be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 um in diameter. (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety.

When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants can also be present in emulsions as needed. Pharmaceutical emulsions can also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion. Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion can be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that can be incorporated into either phase of the emulsion. Emulsifiers can broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants can be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285). Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar

inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate. A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Intravenous injection administration is preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes,

without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, epidural, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of ordinary skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

The invention proposes that Lumican, one of several SLRPs, plays an important role in the regulation of fibrillogenesis or the genes affecting the size of eyeballs in zebrafish, in addition to playing an important role in clinical myopia The invention is to use our established zebrafish model to further identify the drugs affecting the expression of lumican and collagen fibrillogenesis and the regulation of eyeball size. Test compounds are tested based on the regulation of lumican and collagen synthesis through TGF-β pathway and subsequent MMP2 and TIMP regulation, test compounds are tested. In the invention, about 30 clinically-available and FDA-approval drugs which are currently used in clinical and relevant regulation of the TGF-β pathway or MMP and TIMP activity were tested. The results revealed that MMP inhibitors (marinastat, doxycycline and minocycline), collagenase inhibitors (n-acetylcysteine),

TGF-β pathway activators (propofol), TGF-β inhibitor (Captopril) and Cox inhibitor (Aspirin) are effective candidate compounds.

Example

Materials and Methods

Aquaculture

Zebrafish are raised and maintained according to previously established protocols (Soules KA, Link BA. Morphogenesis of the anterior segment in the zebrafish eye. BMC Dev Biol 2005;5:12). All experiments are performed on Tuebingen AB zebrafish raised at 28°C on a 14-h light and 10-h dark cycle and maintained using standard methods. Embryos are staged according to morphological criteria (somite number) (Kimmel CB, Ballard WW, Kimmel SR, et al. Stages of embryonic development of the zebrafish. Dev Dyn 1995;203(3):253-310) and timed in hours after fertilization. Embryos are generated by natural pair-wise mating, as described in the zebrafish handbook (Westerfield M. The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio). University of Oregon Press, Eugene,2nd edition 300P. , 1993) . For each mating, 4±5 pairs are set up and, on an average; 100± 150 embryos per pair are generated. The zebrafish embryo is optically transparent, making it possible to detect functional and morphological changes in internal organs without having to kill or dissect the organism. Chorions are removed manually with Dumont Watchmaker's Forceps No. 5.

Zebrafish Lumican clone

The zebrafish genome has now been sequenced by the Sanger Center, and there have been substantial annotations on the genome conducted by the trans-National Institutes of Health Zebrafish Genome Initiative. To identify the zebrafish expressed sequence tag (EST) clone encoding a putative protein that shares a high sequence similarity with the human and mouse SLRP family proteins, we applied a Basic Local Alignment Search Tool (BLAST) analysis of the GenBank database using the full-length human lumican cDNA sequence. An approximately 4.6 kb Not I/MluI zebrafish genomic DNA fragment containing the 5' portion of the zebrafish Lumican gene is amplified by polymerase chain reaction (PCR) and subcloned into the pBluescript SK vector (Stratagene, La Jolla, CA). The insert is sequenced, using T3, T7 and walk-in primers, by the DNA core of the Department of Molecular Genetics at the National Taiwan University. The 5'- and 3'-ends of the zLum mRNA are amplified using the 5'-Rapid Amplification of cDNA END (5'-RACE) and 3'-RACE Systems, respectively (Invitrogen, Carlsband, CA). For the 5'-RACE experiment, 1 μg of total RNA from zebrafish eyes is reverse transcribed with a first lumican-specific primer corresponding to a sequence in exon 2 of the zLum gene. The RNA templates are degraded by treatment with an RNase mix. A poly-dCTP tail is added to the 3'-end of the cDNAs with terminal deoxynucleotidyl transferase. The cDNA is amplified with a second gene-specific primer corresponding to a sequence from the junction between exon 1 and exon 2 in

conjunction with the abridged anchor primer. The resulting PCR products are diluted 100-fold and used as templates to be reamplified with a third gene-specific primer in conjunction with the universal amplification primer. For 3'-RACE, PCRs are performed using a forth gene-specific primer corresponding to a sequence in exon 3 of the zLum gene. The cycling conditions are: 34 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min followed by a 10-min extension at 72°C at the end of the cycles. Finally, the 5'-RACE and 3'- RACE PCR products are gel purified, and the sequences are determined with a dideoxy sequencing protocol. The transcription initiation and termination sites of the zLum gene are determined by a sequence comparison between genomic DNA, the 5'-RACE product, and the 3'-RACE product, respectively (Yeh LK, Liu CY, Kao WW, et al. Knockdown of zebrafish lumican gene (zlum) causes scleral thinning and increased size of scleral coats. J Biol Chem 2010;285(36):28141-55).

First lumican-specific primer: 5'-AGTAGAGGTATTTGATTCCGGTC-3';

Second lumican-specific primer: 5'-GCACAAGAAGGTGATGAAACG-3';

Third lumican-specific primer: 5'-CAGACTTAGAAGTCCAGCCAAC-3';

Forth gene-specific primer: 5'-GCCTCAGAGATCATCTTTGAATAG-3';

Abridged anchor primer: 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3';

Universal amplification primer: 5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3'.

Morpholino Knockdown

Morpholinos are chemically modified antisense oligonucleotides that can be designed to hybridize to the translation-initiation or splicing acceptor/donor sites of specific mRNAs (Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. Nat Genet 2000;26(2):216-20). A morpholino-antisense oligonucleotide (Gene Tools, Philomath, OR) are designed and synthesized to target the 5'-untranslated and/or flanking regions, including the translation start codon of the respective genes. The MO sequence is designed as follows: zLum-MO, 5'-GATCCCAGAGCAAACATGGCTGCAC-3'. This oligonucleotide complemented the sequence from -8 through +17 with respect to the translation initiation codon. A random sequence MO (RS-MO) serves as a control for zLum- MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'. This RS-MO is obtained from Gene Tools as a standard control oligonucleotide with no target specificity (Yeh LK, Liu CY, Kao WW, et al. Knockdown of zebrafish lumican gene (zlum) causes scleral thinning and increased size of scleral coats. J Biol Chem 2010;285(36):28141-55).

Morpholino is resuspended in sterile water to a concentration of 1 mmol/L and diluted to 680ng/nL with sterile water. The morpholinos are injected at the single-cell stage in a volume of 0.0023 nL. Here, we identified the effects of morpholinos on protein levels are assayed with western blotting from injected embryos with GAPDH as a control.

Whole-embryo in situ hybridization

The main advantage of whole-mount ISH is that it is a quick and efficient method to establish spatial and temporal gene expression patterns in embryos and early larvae. Embryos are obtained at different stages and fixed in 4% paraformaldehyde in 1x PBS overnight at 4°C. After rinsed with PBS 3 times, we transferred these embryos into 100% methanol, and stored at -20°C until use. All embryos are treated with 0.003% phenylthiourea (PTU) to prevent melanogenesis. Whole mount RNA in situ hybridization is carried out according to the nature protocol (Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 2008;3(1):59-69). The hybridization signals are visualized with anti- digoxigenin (DIG) antibody-alkaline phosphatase conjugates using procedures recommended by Roche (Roche Applied Science, Indianapolis, IN).

Antibody

Zebrafish Lumican Antibody- An affinity-purified anti-zLum antibody against a synthetic peptide N-terminal peptide (N'-CNERNLKFIPIVPTGIKY-C') corresponding to the 18 N-terminal amino acid residues deduced from the zLum cDNA is generated to detect zebrafish lumican. The peptides are conjugated to keyhole limpet hemocyanin for antibody production in rabbits. The antibodies are purified through an immune absorbent column of the above zebrafish Lumican oligopeptide conjugated to Sulfolink gel (Pierce, Rockford, IL) according to the manufacturer's instructions. Fractions containing purified anti-zebrafish lumican antibody are pooled and concentrated, and the protein concentration is measured by spectrophotometer at 280 nm (Yeh LK, Liu CY, Kao WW, et al. Knockdown of zebrafish lumican gene (zlum) causes scleral thinning and increased size of scleral coats. J Biol Chem 2010;285(36):28141-55).

We used several antibodies to evaluate the effects of lumican knockdown and all can be obtained from different commercial supplies. Rabbit anti-TGFβ2, Rabbit anti-MMP-2, Goat anti-TIMP-2, Goat anti-Col1a1 (L-19) and Goat anti-PDI are obtained from Santa Cruz. Mouse anti-GAPDH is obtained from Abnova.

Western Blotting

Proteins are extracted from zebrafish embryos in RIPA buffer (50 mM Tris-HC1, pH 7.4,150 mM NaCl, 1 mM EDTA, 1% Na-deoxycholate, 1% NP-40) containing protease inhibitor (0.5mM AEBSF, 0.3uM Aprotinin, 10uM Bestatin, 10uM E-64, 10uM Leupeptin). For the preparation of Western Blot, 100 embryos from 3 day-post-fertilization are sonicated in 100ul of RIPA buffer and microfuged for 20 min. Prior to SDS/PAGE, aliquots of some scleral protein extracts is digested with 0.1 unit/ml endo-β-galactosidase (Sigma, St. Louis,MO) at 37°C overnight and the clear supernatant proteins are resolved by SDS-PAGE (30ug per lane) in an 10% polyacrylamide gel and transferred to 0.2 μm pore size nitrocellulose membrane (Invitrogen) for immunoblotting. Lumican is detected using the anti-zebrafish lumican Nterminal peptide antibody (0.1 μg/ml, detail as described above). GAPDH is immunostained with a monoclonal antibody (Abnova) as a control for equivalent loading. Anti-rabbit (1:1,000) and anti-

goat (1:2,500) HRP conjugated secondary antibodies are used with the appropriate primary antibodies. Antibody reactivity is detected through chemiluminescence (Immobilion® Western HRP and AP Chemiluminescent Substrates, Millipore).

Human sclera cells were seeded in 6-well culture plates at 4×10^5cells/well and incubated with different concentrations of MMP inhibitors at 37°C for 24 hours. The culture without MMP inhibitors act as controls. After 24 hours incubation, cells were harvested for protein extraction. Proteins were extracted and homogenized in RIPA buffer containing protease inhibitor. Protein content is quantified by spectrophotometry. Samples with equal protein content were electrophoresed on 10% polyacrylamide gels and electrophoretically transferred to PVDF membranes. The blot membranes are incubated with PBS solution containing 5% skim milk overnight at 4°C to block nonspecific antigens and then incubated with primary antibody diluted for1- 2 hours. Primary antibody used in this experiment are as follows: anti–TGFβ1, anti–TGFβ2, anti–TGFβ3, anti-MMP2, anti-MMP9, anti-TIMP2 and GAPDH. After the primary antibody reaction, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti–rabbit IgG as the secondary antibody at room temperature for 1 h, detected by Chemiluminescence Reagent Plus, and exposed to film. The protein expression pattern between cells treated with/without different MMP inhibitors were compared.

zLumican Promoter Transgenic Fish

Gene function can be rapidly and robustly studied in zebrafish using antisense morpholino oligonucleotides (Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. Nat Genet 2000;26(2):216-20; Heasman J. Morpholino oligos: making sense of antisense? Dev Biol 2002;243(2):209-14). Furthermore, techniques for generating transgenic lines70 (Davidson AE, Balciunas D, Mohn D, et al. Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon. Dev Biol 2003;263(2):191-202; Kurita K, Burgess SM, Sakai N. Transgenic zebrafish produced by retroviral infection of in vitro-cultured sperm. Proc Natl Acad Sci U S A 2004;101(5):1263-7), targeted mutations (reverse genetics) (Wienholds E, Schulte-Merker S, Walderich B, Plasterk RH. Target-selected inactivation of the zebrafish rag1 gene. Science 2002;297(5578):99-102) and cloning by nuclear transfer (Lee KY, Huang H, Ju B, et al. Cloned zebrafish by nuclear transfer from long-term-cultured cells. Nat Biotechnol 2002;20(8):795-9) have been developed. Here, we established a transgenic line expressed under the control of the zebrafish lumican promoter. Genomic DNA, both 1.7 kb and 0.48kb from the 5'-untranslated region of the zLum gene, are amplified with specific PCR primers and inserted into the multiple cloning sites of pBluescript II SK vectors (Stratagene, La Jolla,CA) containing an EGFP sequence59. The recombinant plasmids are prepared in Escherichia coli DH5α and purified with a QIAGEN Plasmid Purification Maxi kit. Purified plasmid DNA is adjusted to 50 ng/μl in distilled water and microinjected into one-cell-stage zebrafish embryos under a dissecting microscope. The following day, embryos with GFP expression are imaged and selected by using a Leica dissection scope

equipped with epifluorescence (MZFLII). Only embryos displaying fluorescence are grown to adulthood. Pairs of sibling adults grown from injected embryos with fluorescence are intercrossed to identify germ line founders. Subsequently, individual adults from positive pairs are outcrossed to identify the individual founder fish. These functional and morphological changes may be further highlighted by this lumican promoter transgenic fish.

PCR primers:

Forward primer I: 5'-ATAAGAATGCGGCCGCTCCATTAATTCGACAGACCAG-3' ;

Forward primer II: 5'-ATAAGAATGCGGCCGCAGGTAGACAACACGGTTATGT-3' ;

Reverse primer: 5'-CGACGCGTGGCTGCACAACTTAAATTAAACCT-3' ;

Chemicals Used for the Primary Drug Screening

The chemicals and drugs for drug screening will include TGF-receptor inhibitors (Atropine、tropicamide、ipratropium bromide (Atrovent)、oxybutynin (Tavor)、scopolamine hydrobromide、Pirenzepine dihydrochloride、SB 431542、Tamoxifen、SB-505124、RepSox (SB-4696)、Doxycycline hyclate (Dermostat, Periostat)、Genistein、Marimastat、Taurine、Minocycline hydrochloride、n-acetylcysteine、Losartan、aspirin、zileuton、SP600125、Propofol、Statin、indomethacin、Ibuprofen、naproxen、piroxicam、nabumetone、Licofelone、Captopril、Procyanidin、Heterotaxin、Simvastatin、Lovastatin、Rosuvastatin). All of these compounds have been well investigated before for their pharmacological activities against the pathways involved the lumican-regulated collagen fibrillogenesis.

Results

We have successfully established a zebrafish model to study ocular development and diseases and characterized lumican gene (*zLum*) expression in the cornea and the sclera of zebrafish. Knockdown of zLum causes scleral thinning and increased size of scleral coats during the ocular development of zebrafish, compatible with the clinical findings in child myopia. As shown in Figures 4 and 5 of The Journal of Biological Chemistry, 2010, Vol. 285, No. 36, pp. 28141-28155, it is clearly demonstrated the expression of zebrafish lumican (zLum) in the zebrafish, especially in the scleral coat, cornea and periocular matrices. Interestingly, the lumican in the scleral coat is non-sulfated in contrast with that of sulfated lumican in the corneal stroma.

After lumican underwent knockdown, in Figure 1 to 5, the scleral coat became enlarged and was similar to the scleral changes of human myopia, i.e. axial elongation. As mentioned above, we also showed the alteration of a SNP in the lumican promoter and its haplotype were strongly associated with development of high myopia in Taiwanese population. Our animal study recaptured these findings in the

human myopia in which the importance of lumican gene in the development of axial elongation was emphasized.

In Figure 6, we showed that the phenotype of lumican knockdown in zebrafish could be rescued with the TGF-β. The lumican knockdown fish also demonstrated by the increased expression of MMP2 and decreased expression of TIMP which further confirmed the role of lumican in the regulation of scleral remodeling as shown in experimental myopia of other species. Importantly, the scleral coat enlargement could be inhibited with the administration of atropine in lumican knockdown fish (Figures 7 to 9). The expression of MMP-2 and TIMP also returned to the normal level with this treatment.

We have tested about 30 clinically-available drugs relevant to TGF-β pathway. The first drug, marinastat (BB 2516), a proposed anti-neoplastic drug, acting as a broad-spectrum matrix metalloproteinase inhibitor, is also considered a good candidate. Our preliminary results revealed that marinastat could prevent the scleral coat enlargement very efficiently in *zLum*MO knockdown fish (2% of scleral enlargement in experimental group vs. 30% of scleral enlargement in control group). The results of marinastat indeed showed the MMPs are the effectors and targets for scleral coat enlargement after lumican knockdown. Marinastat can be a potential target for myopia prevention and clinical drug testing.

Tetracyclines have been used both systemically and locally in the treatment of various infections caused by gram-negative bacteria. During recent years it has been established that tetracyclines exert biological functions entirely independent of their antimicrobial property. Furthermore, several investigations involving both *in vitro* and *in vivo* animal studies have shown that tetracycline antibiotics and their chemically modified analogues with no antimicrobial activity can inhibit mammalian collagenase activity and collagen breakdown. Doxycycline and minocycline were second-generation tetracyclines. Doxycycline and chemically modified tetracyclines CMT-1 and CMT-6 had direct inhibitory effects on both 92-kDa (MMP-9) and 72-kDa (MMP-2) gelatinaes. Minocycline also inhibit various MMPs, including MMP-9 and MMP-2. These drugs were considered as a good candidate. Our results revealed that doxycycline and minocycline could prevent the scleral coat enlargement very efficiently in zLum - morpholinos (MO) knockdown model (6.8% and 4.7% of scleral enlargement in experimental group vs. 30% of scleral enlargement in control group). The another antibiotic, minocycline, also presented the effectiveness on the prevention of sclera enlargement. Minocycline belongs to the second generation class of cyclines. Minocycline has an anti-infectious property with a spectrum similar to that of other cyclines, notably against Chlamydias, Treonema and Proprionibacterium acenes. The anti-inflammatory and anti-collagenase activity associated with this anti-infectious action is greater than that of first generation cyclines specifically with a modulator effect on epidermal cytokines. Therefore, it is reasonable to expect tetracycline to demonstrate efficacy in the collagen synthesis of the scleral coat. Aspirin causes several different effects in the body, mainly the reduction of inflammation, analgesia (relief of pain), the prevention of clotting, and the reduction of fever. Aspirin's ability to

suppress the production of prostaglandins and thromboxanes is due to its irreversible inactivation of the cyclooxygenase (COX) enzyme. Cyclooxygenase is required for prostaglandin and thromboxane synthesis. Aspirin acts as an acetylating agent where an acetyl group is covalently attached to a serine residue in the active site of the COX enzyme. This makes aspirin different from other NSAIDs (such as diclofenac and ibuprofen), which are reversible inhibitors. Our results revealed that aspirin could prevent the scleral coat enlargement very efficiently in *zLum-* MO knockdown model (9.6% of scleral enlargement in experimental group vs. 30% of scleral enlargement in control group).

N-acetylcysteine, an effective antioxidant which inhibit the formation of extracellular reactive oxygen intermediates128, also was a collagenase inhibitor. Several papers reported that N-acetylcysteine shows inhibition of matrix MMP-2 expression and activity. Our results revealed that n-acetylcysteine could prevent the scleral coat enlargement very efficiently in zLum - morpholinos (MO) knockdown model (11.7% of scleral enlargement in experimental group vs. 30% of scleral enlargement in control group).

Propofol (2,6-diisopropylphenol) is one of the most popular agents used to induce anesthesia in surgical procedures for long-term sedation and to treat postoperative nausea in critically-ill patients. Propofol could induce endothelial cells to express latent TGF-β, which was converted into active TGF-β by PBMCs *in vivo*. Our results revealed that propofol also could prevent the scleral coat enlargement very efficiently in *zLum*-MO knockdown model (12% of scleral enlargement in experimental group vs. 30% of scleral enlargement in control group).

In summary, the results of marinastat, doxycycline, minocycline, n-acetylcysteine, aspirin, Propofol showed the TGF-β and MMPs are the effectors and targets for scleral coat enlargement after lumican knocking down. These drugs could be a potential target for myopia prevention and clinical drug testing. Accordingly, we have proven zebrafish was an excellent *in vivo* animal model to observe the development of axial myopia and for screening compounds in treating myopia. The big eye ratios of the zebrafish treated with marimastat, doxycycline, captopril, minocycline hydrochloride, atropine, aspirin, propofol and N-acetylcysteine are shown in Figure 10. Fig. 11 (a)-(e) shows Figure 11 shows the big eye ratios of the zebrafish treated with tetracycline, minocycline, doxycycline, marimastat and batimastat at various concentrations. Other test compounds and their big eye ratios are shown in the table below.

	Name	Conc. I choice	Big eye rate(%)
1	Atropine	0.50%	14.5%
2	Tropicamide	1mM	22.3%
3	Ipratropium bromide (Atrovent)	50mM	26.0%
4	Oxybutynin (Tavor)	2uM	22.7%

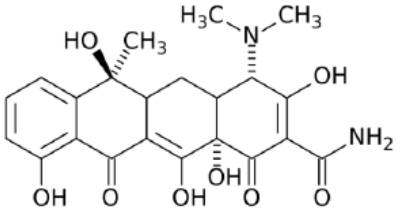
5	Scopolamine hydrobromide	100uM	27.0%
6	Pirenzepine dihydrochloride	0.25%	19.1%
7	SB 431542	25uM	23.4%
8	Tamoxifen	20uM	17.7%
9	Doxycycline hyclate (Dermostat,Periostat)	100ppm	6.8%
10	Genistein	25uM	36.2%
11	Marimastat	50uM	2%
12	Taurine	0.2M	21.8%
13	Minocycline hydrochloride	50uM	4.7%
14	n-acetylcysteine	10uM	11.7%
15	Aspirin	50mg/L	9.6%
16	Propofol	0.5mM	12.0%
17	SP600125	2.5uM	29.5%
18	Zileuton	100uM	21.3%
19	Mevastatin	0.01nM	27.0%
20	Indomethacin	10uM	18%
21	Piroxicam	10uM	22%
22	Captopril	1mM	7.6%
23	Simvastatin	0.1uM	26.0%

Furthermore, human scleral fibroblast cell was isolated and cultured for testing. The primary human scleral fibroblasts was cultured from explants of human donor eyes after death as previous described (Barathi, V.A., S.R. Weon, and R.W. Beuerman, Expression of muscarinic receptors in human and mouse sclera and their role in the regulation of scleral fibroblasts proliferation. Mol Vis, 2009. 15: p. 1277-93; Wang, Q., et al., Role of bone morphogenetic proteins in form-deprivation myopia sclera. Mol Vis, 2011. 17: p. 647-57). The scleral was dissected from the donner eye and washed immediately with cold phosphate buffered solution three times. Then human sclera was be trimmed into pieces approximately 1 mm × 1 mm and cultured in 60 mm × 15 mm cell culture dishes (Corning Ltd) in Dulbecco's modified Eagle's medium (DMEM)/F12 with high glucose supplemented (Invitrogen), 10% fetal bovine serum (FBS; Gibco). And then the cells were incubated at 37°C in a humidified incubator containing 5% CO2. The growth medium was changed every 3 or 4 days. When achieve a heavy primary monolayer, the cells will be dispersed by incubating with 0.25% trypsin/0.5 mM EDTA (Sigma) for 5 min at 37°C, and subcultured into a 25 mm2 flask (Corning Ltd). All cells used in experiments are between passages 1 and 3. The purity of fibroblast cultures were confirmed by immunofluorescence

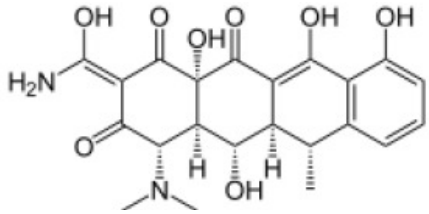
staining of anti-vimentin. After the western blotting assay and chemiluminescence assay as mentioned above, the above-mentioned screened MMP inhibitors affect the expression of expression of lumican and/or collagen fibrillogenesis.

Claim

1. A pharmaceutical composition for use in the treatment of myopia, comprising a therapeutically effective amount of an MMP inhibitor, wherein the MMP inhibitor is selected from the group consisting of



Tetracycline and



Doxycycline, or a tautomer or a pharmaceutically acceptable salt or solvate thereof, and wherein Doxycycline has a concentration from 2 mM to 2 μM, and Tetracycline has a concentration from 2.5 mM to 250 μM.

METHODS FOR DRUG SCREEN USING ZEBRAFISH MODEL AND THE COMPOUNDS SCREENED THEREFROM

Abstract of the Disclosure

The invention relates to a platform of using zebrafish in screening candidates for treating and/or preventing myopia and keratoconus disease. The invention is mainly based on that Lumican, one of several SLRPs, plays an important role in the regulation of fibrillogenesis or the genes affecting the size of eyeballs in zebrafish, in addition to playing an important role in clinical myopia. Therefore, the invention uses the established zebrafish model to further identify the drugs affecting the expression of lumican and collagen fibrillogenesis, and/or the regulation of eyeball size. These drugs are potential candidates for treating myopia and/or keratoconus disease.

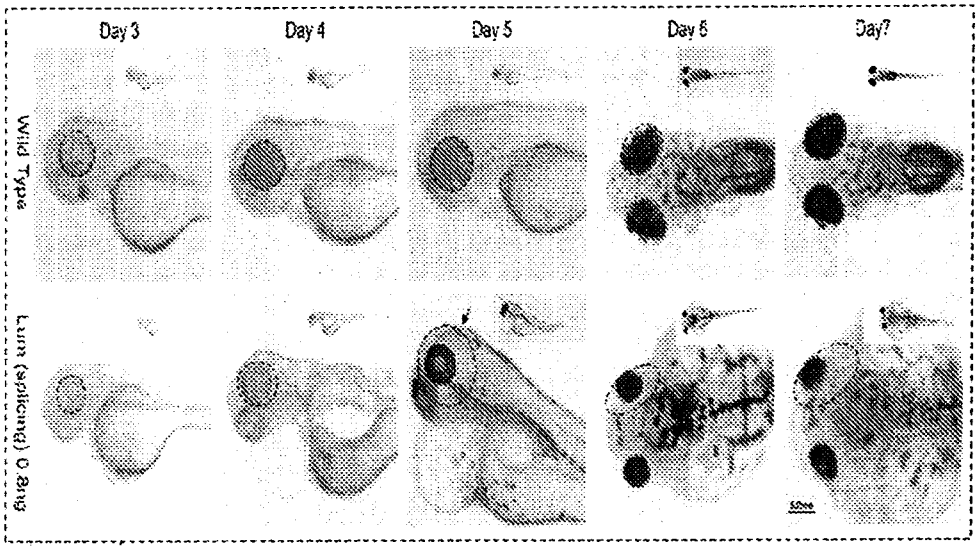


Figure 1

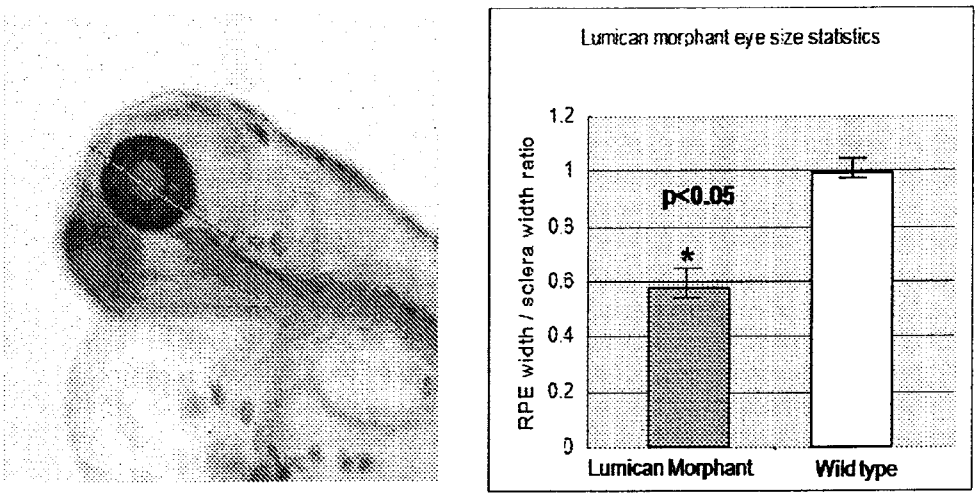

Lumican morphant eye size statistics
RPE width / sclera width ratio
1.2
1
0.8
0.6
0.4
0.2
0
p<0.05
*
Lumican Morphant
Wild type

Figure 2

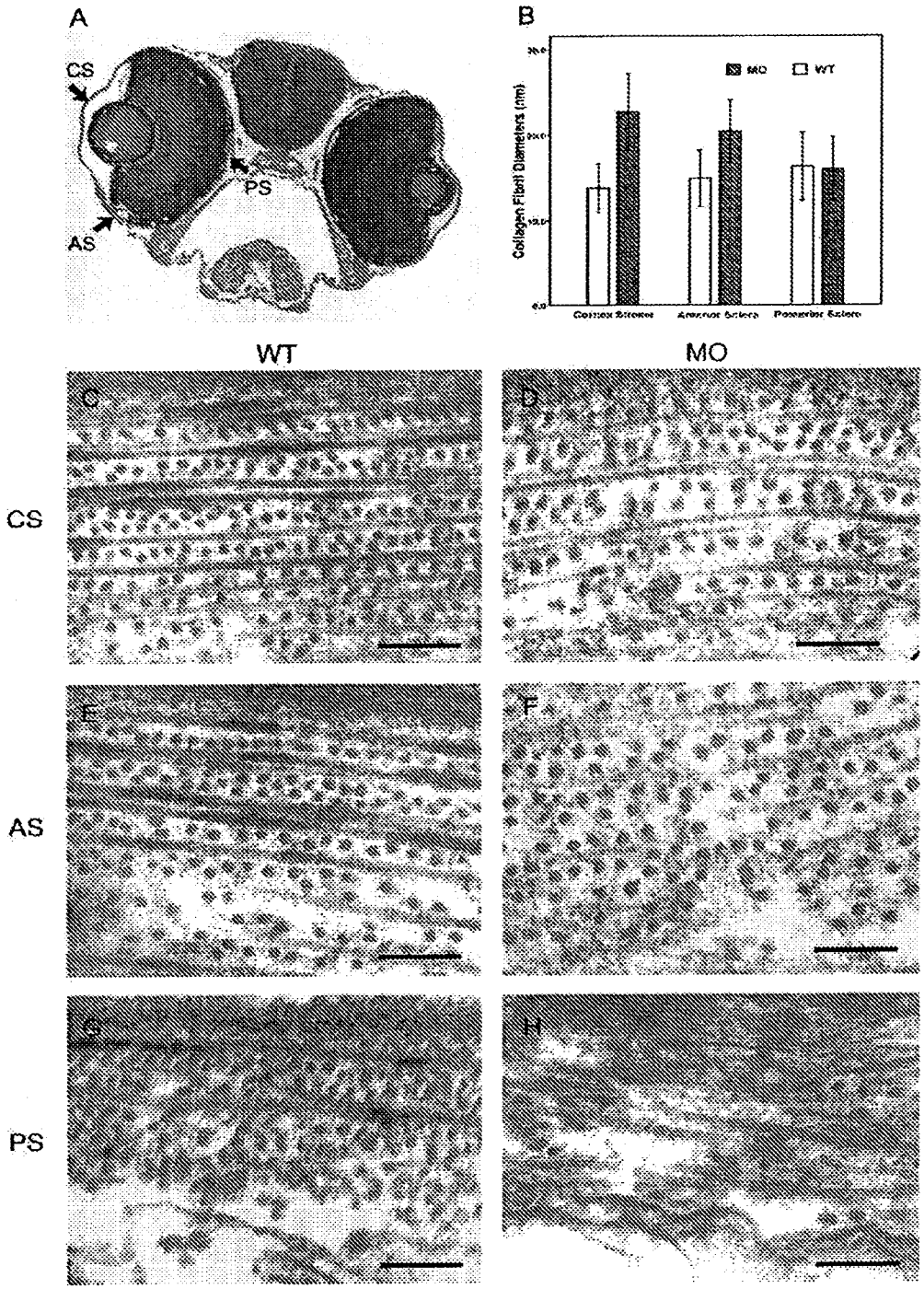

A
CS
PS
AS
B
MO
WT
Collagen Fibril Diameters (nm)
WT
MO
CS
AS
PS
C
D
E
F
G
H

Figure 3

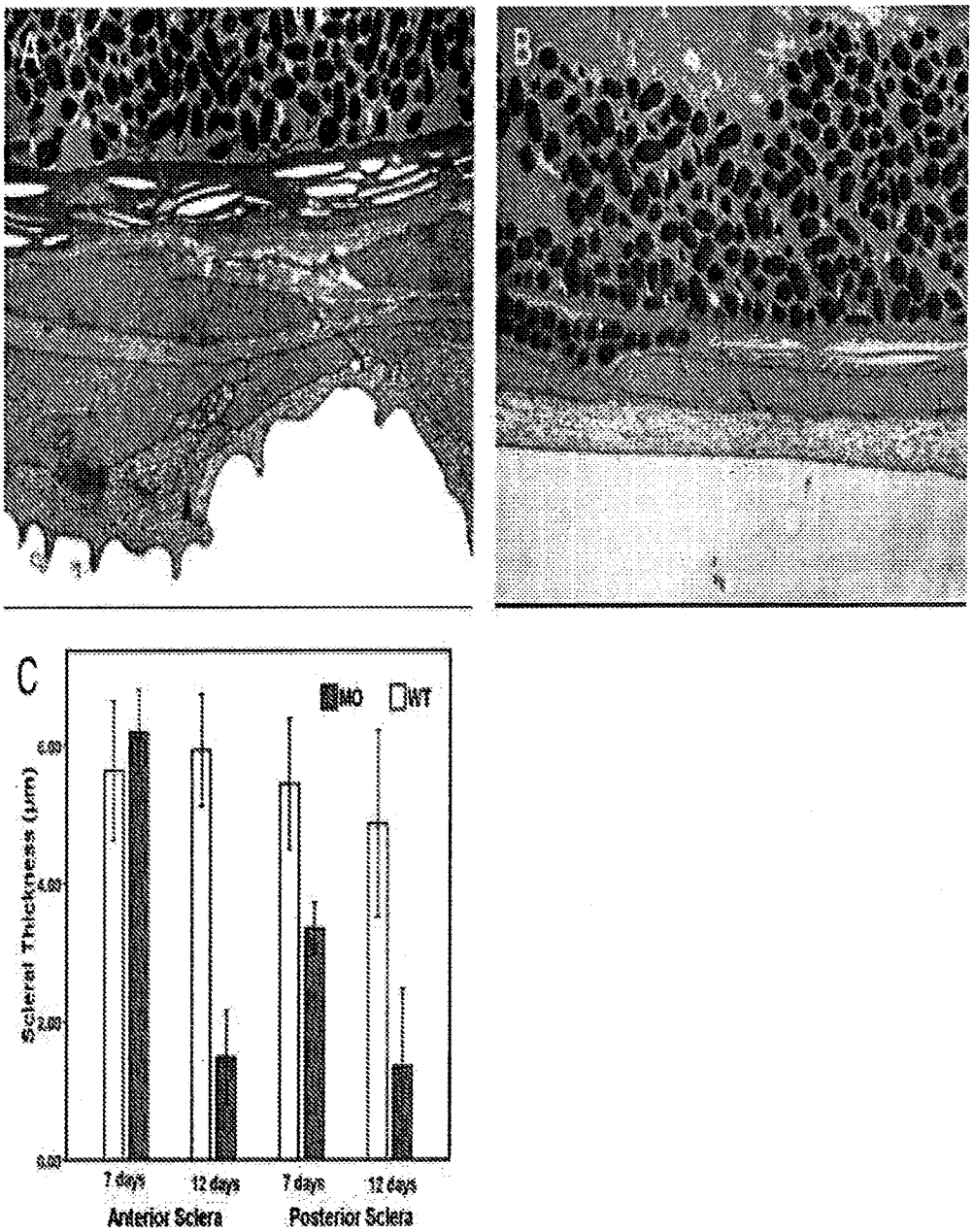

A
B
C
MO
WT
Scleral Thickness (μm)
7 days
12 days
7 days
12 days
Anterior Sclera
Posterior Sclera

Figure 4

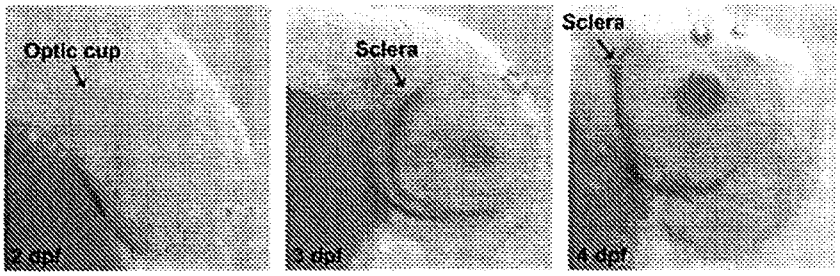


Figure 5

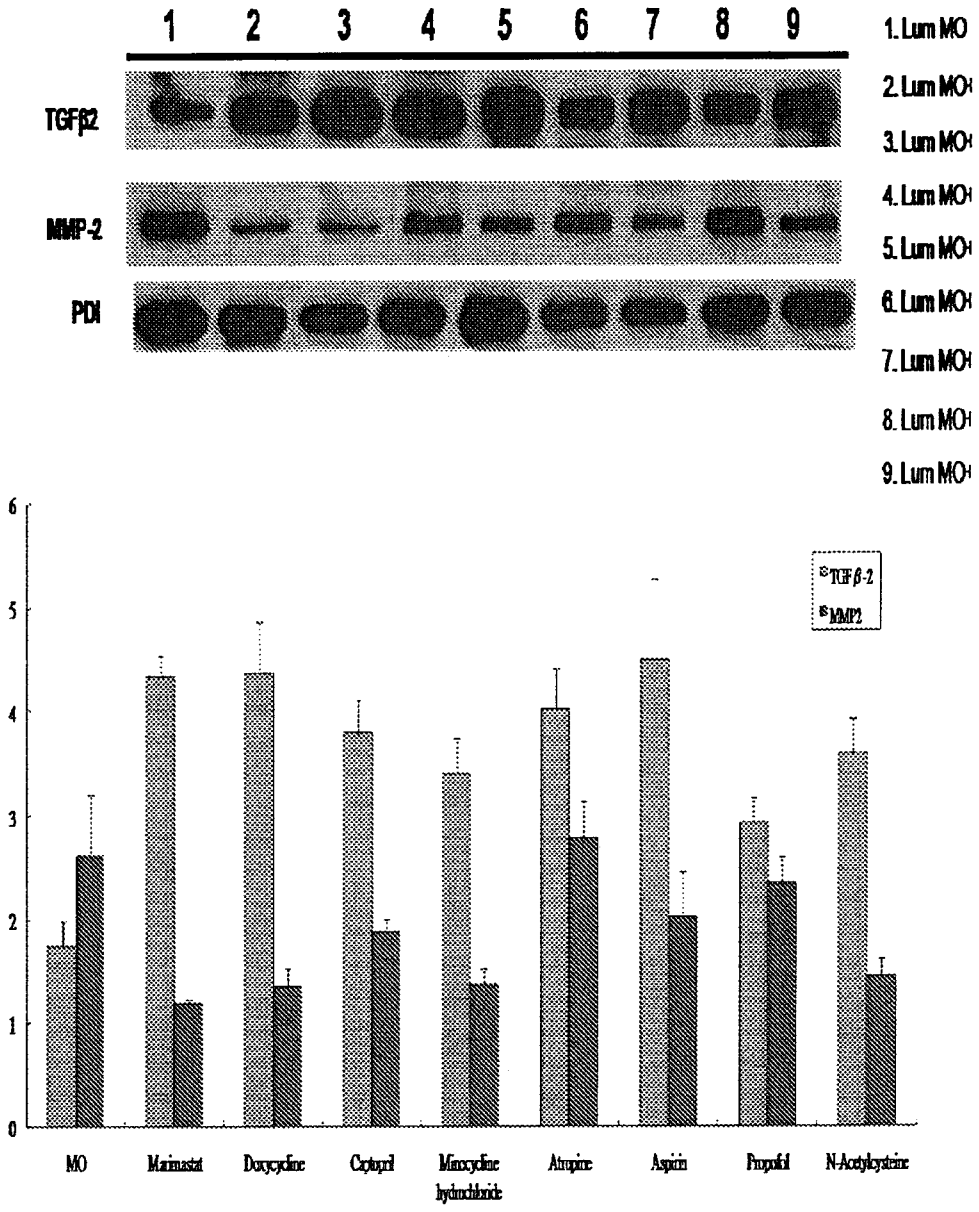

1 2 3 4 5 6 7 8 9
TGFβ2
MMP-2
PDI
1. Lum MO
2. Lum MO
3. Lum MO
4. Lum MO
5. Lum MO
6. Lum MO
7. Lum MO
8. Lum MO
9. Lum MO
TGF β-2
MMP2
0
1
2
3
4
5
6
MO
Marimastat
Doxycycline
Captopril
Minocycline hydrochloride
Atropine
Aspirin
Propofol
N-Acetylcysteine

Figure 6

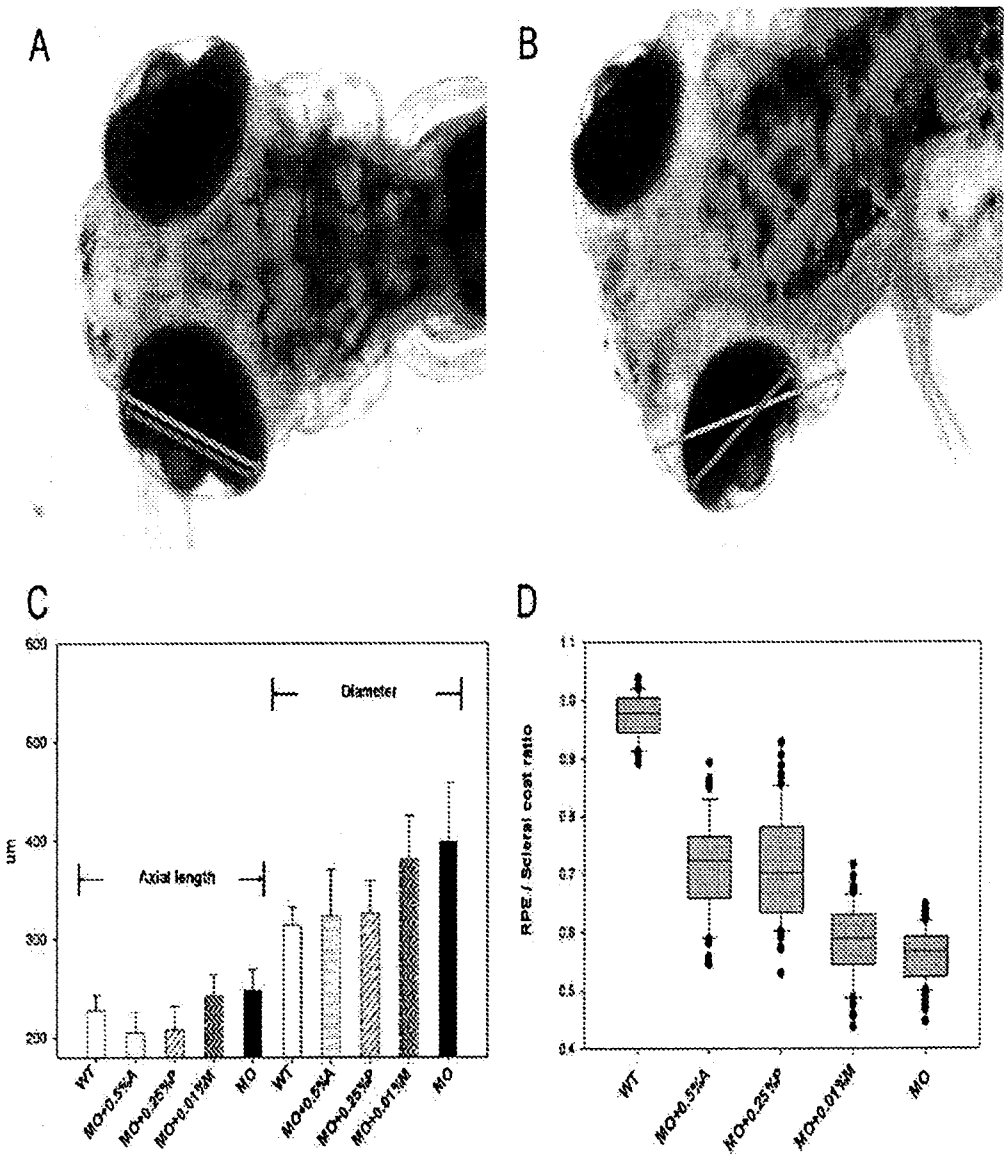

A
B
C
D
um
Diameter
Axial length
WT
MO+0.5%A
MO+0.25%P
MO+0.01%M
MO
RPE / Scleral cost ratio

Figure 7

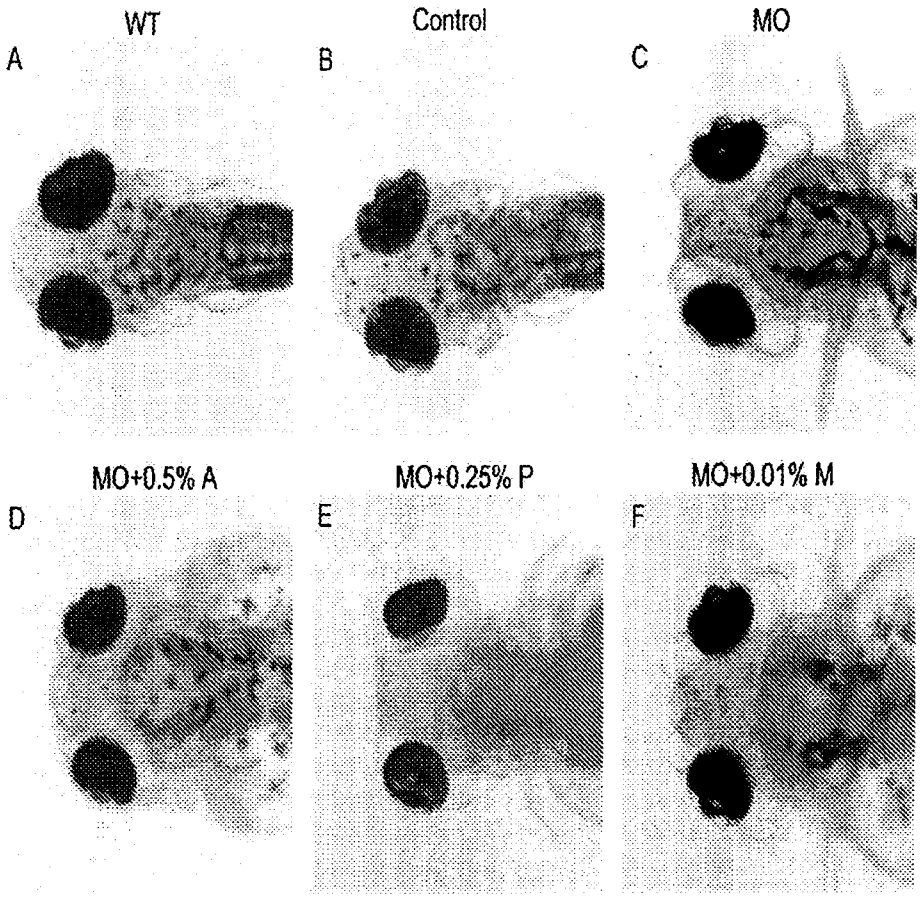


Figure 8

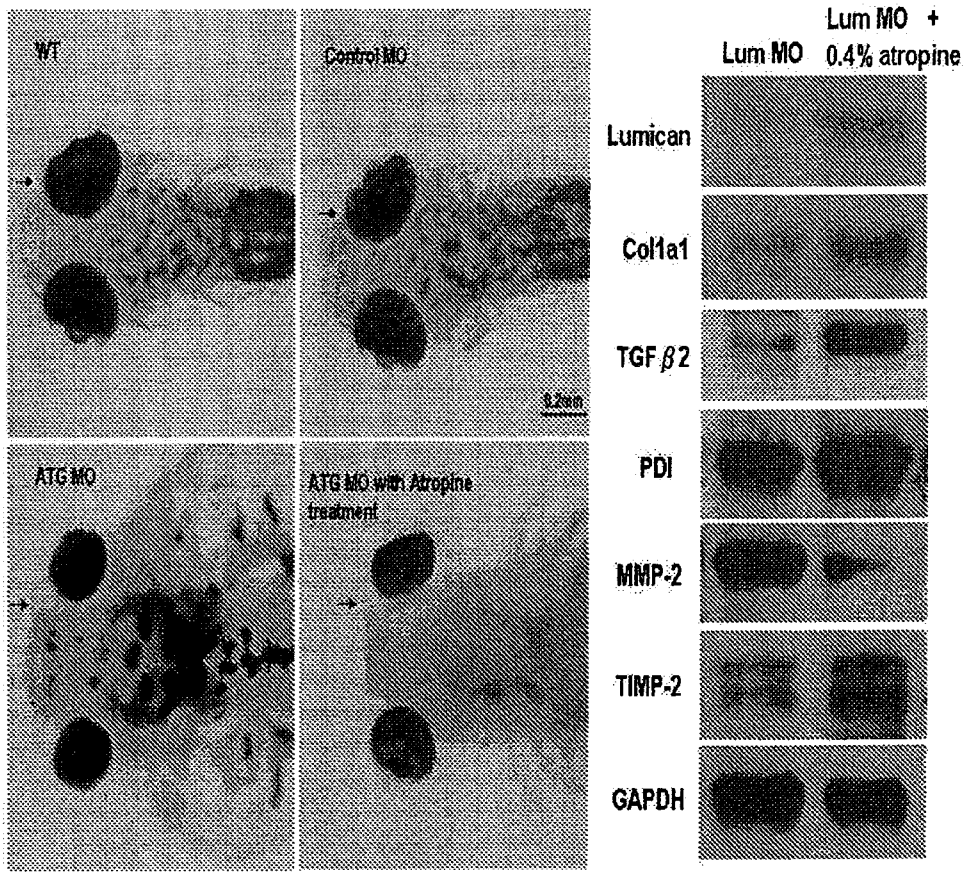

WT
Control MO
ATG MO
ATG MO with Atropine treatment
Lum MO
Lum MO + 0.4% atropine
Lumican
Col1a1
TGFβ2
PDI
MMP-2
TIMP-2
GAPDH

Figure 9

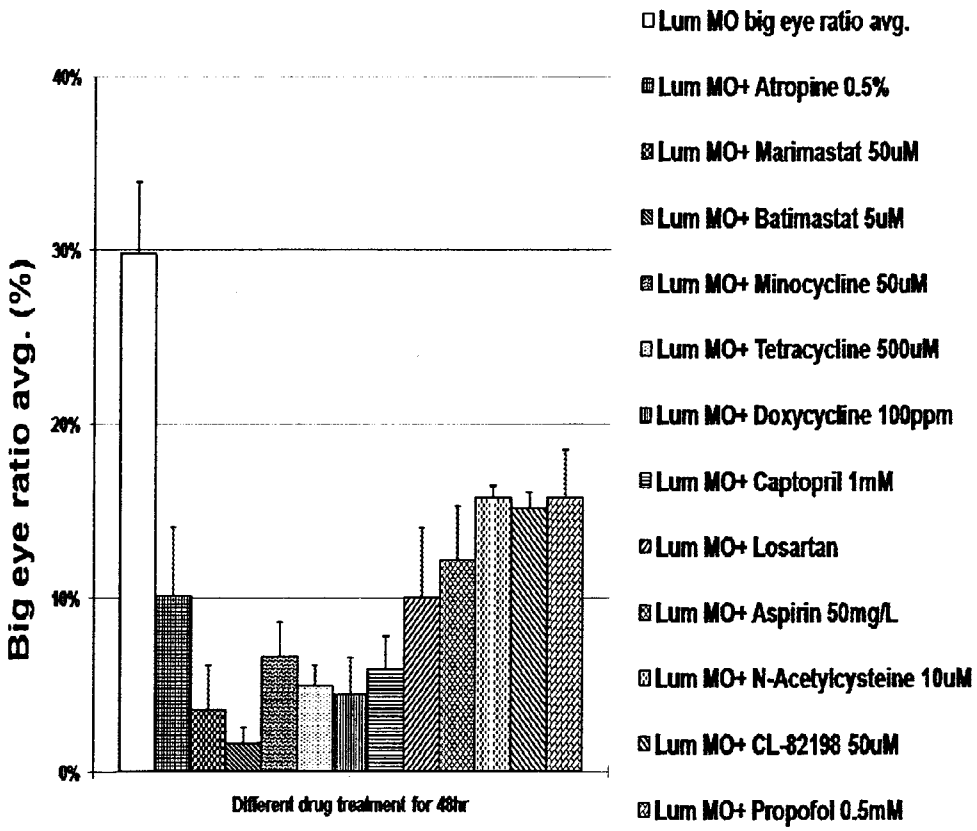

Big eye ratio avg. (%)
40%
30%
20%
10%
0%
Different drug treatment for 48hr
Lum MO big eye ratio avg.
Lum MO+ Atropine 0.5%
Lum MO+ Marimastat 50uM
Lum MO+ Batimastat 5uM
Lum MO+ Minocycline 50uM
Lum MO+ Tetracycline 500uM
Lum MO+ Doxycycline 100ppm
Lum MO+ Captopril 1mM
Lum MO+ Losartan
Lum MO+ Aspirin 50mg/L
Lum MO+ N-Acetylcysteine 10uM
Lum MO+ CL-82198 50uM
Lum MO+ Propofol 0.5mM

Figure 10

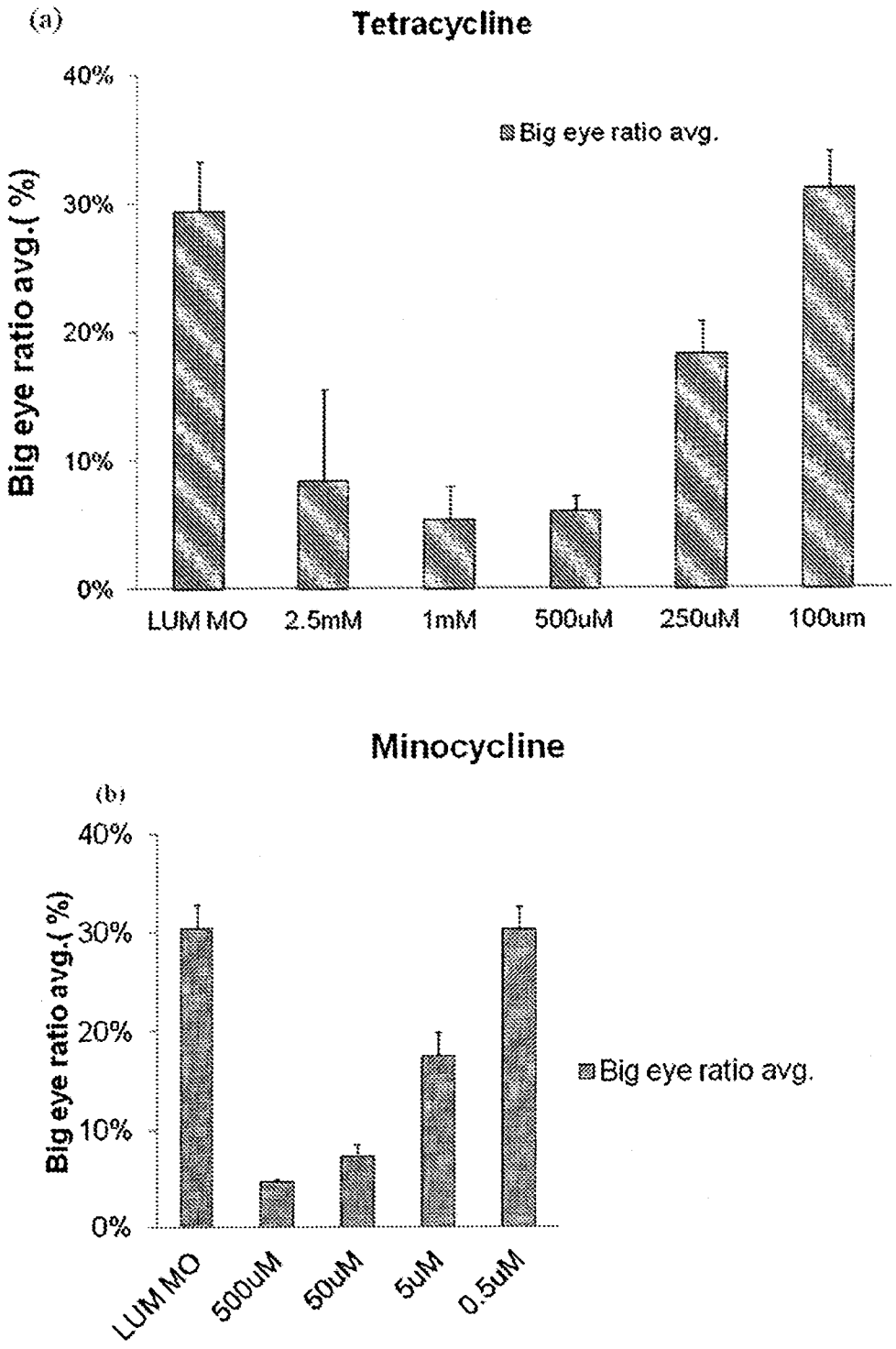
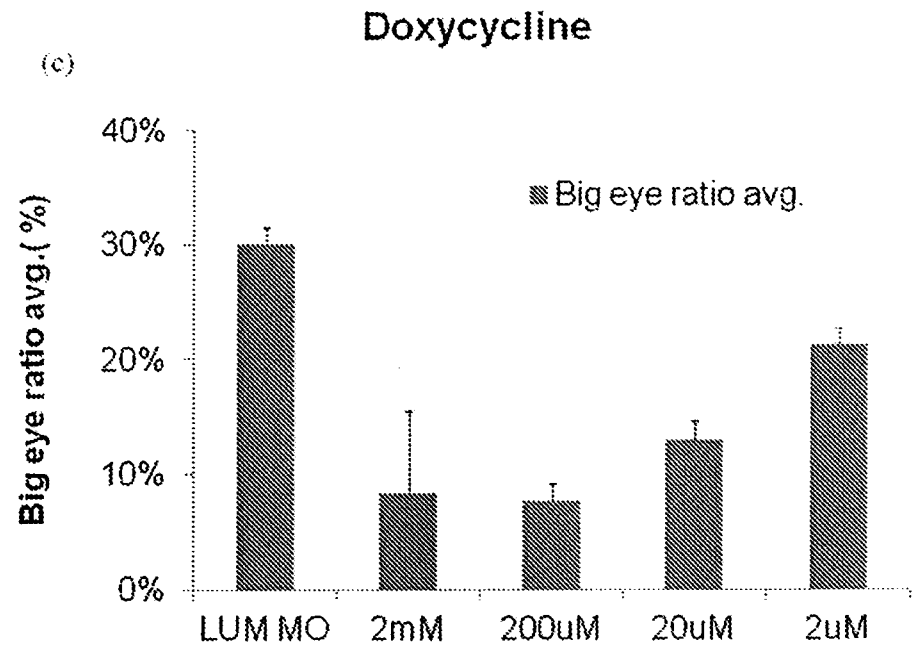

(a)
Tetracycline
40%
30%
20%
10%
0%
Big eye ratio avg.(%)
Big eye ratio avg.
LUM MO
2.5mM
1mM
500uM
250uM
100um
Minocycline
(b)
40%
30%
20%
10%
0%
Big eye ratio avg.(%)
Big eye ratio avg.
LUM MO
500uM
50uM
5uM
0.5uM

Figure 11



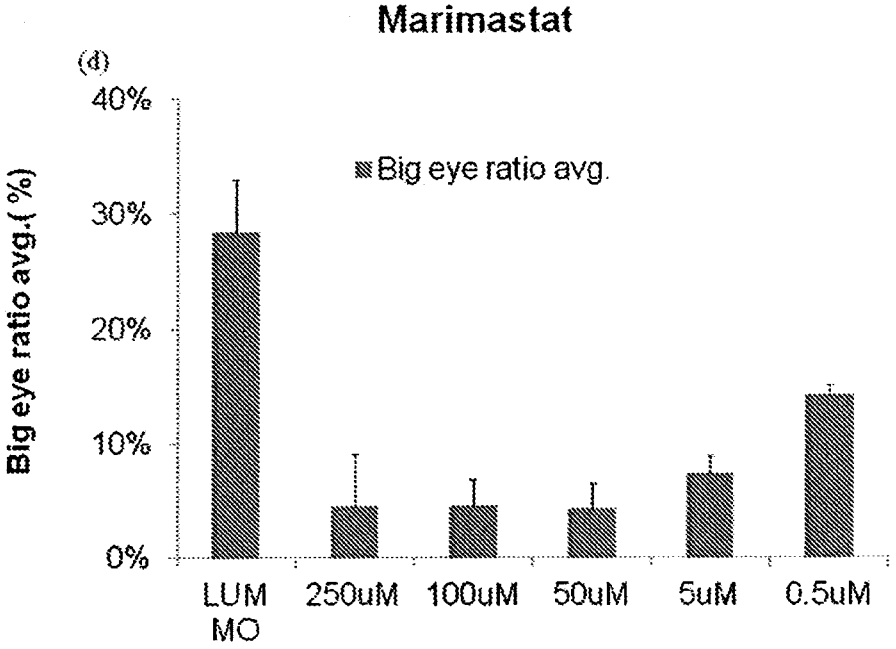


Figure 11 (Continued)

(e)

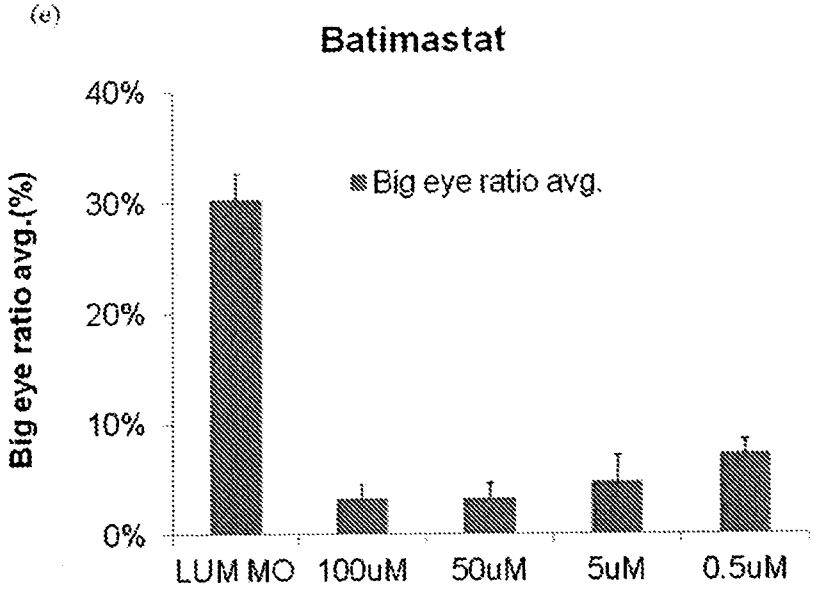


Figure 11 (Continued)