Title: NUTLIN-3A FOR TREATMENT OF PROLIFERATIVE VITREORETINOPATHY

FIG. 2A

Abstract: The invention provides compositions and methods for treatment of proliferative vitreoretinopathy.
RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/683,887 filed August 16, 2012, the contents of which are hereby incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

The contents of the text file named "36770-527001WO_ST25.txt", which was created on August 16, 2013 and is 8.7 KB, are hereby incorporated by reference in its entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was funded in part by the U.S. Government under grant number EY012509, awarded by the National Eye Institute. The Government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates generally to the field of ophthalmology.

BACKGROUND OF THE INVENTION

Proliferative vitreoretinopathy (PVR) is a blinding disease that afflicts 5-11% of patients that undergo surgery to correct a rhegmatogenous retinal detachment. There are between 1,700 and 3,700 cases of PVR annually in the US. The current treatment for PVR is repeat surgery, which is anatomically successful in only 60-80% of cases, and the procedure carries the risk of recurrence. Prior to the invention described herein, efforts to identify non-surgical, i.e., pharmacological, approaches to treat PVR have not been successful. Thus, there is a pressing need for new therapy options for individuals who are afflicted by this blinding disease.

SUMMARY OF THE INVENTION

The invention is based on the surprising discovery that preventing the reduction of intra-ocular p53 by administering the small molecule, Nutlin-3, prevents retinal detachment, the most sight-threatening component of proliferative vitreoretinopathy. The invention provides compositions and methods for inhibiting or reducing the severity of proliferative vitreoretinopathy (PVR) in a subject.
Accordingly, a method for inhibiting or reducing the severity of PVR is carried out by identifying a subject suffering from or at risk of developing PVR, and administering a composition comprising an agent that inhibits or reduces an intra-ocular reduction of the level of p53 associated with PVR. The subject is preferably a mammal in need of such treatment, e.g., a subject that has been diagnosed with PVR or a predisposition thereto. For example, the level of p53 associated with PVR is reduced by 10%, 25%, 50%, or reduced by 2-fold, 10-fold, or more, and administration of compositions of the present invention prevents, inhibits, or reduces the reduction of p53 levels associated with PVR. Alternatively, the administration of the compositions of the present invention causes a 10%, 20%, 30%, 40%, or 50% increase in p53 levels compared to the p53 levels associated with PVR or the p53 levels prior to treatment, or a 2-fold, 3-fold, 4-fold, or 5-fold increase in p53 levels compared to the levels associated with PVR or the p53 levels prior to treatment. The mammal can be, e.g., any mammal, e.g., a human, a primate, a mouse, a rat, a dog, a cat, a horse, as well as livestock or animals grown for food consumption, e.g., cattle, sheep, pigs, chickens, and goats. For example, the mammal is a performance mammal, such as a racehorse or racedog (e.g., greyhound). Preferably, the mammal is a human.

In some cases, the subject suffering from or at risk of developing PVR has undergone rhegmatogenous retinal detachment surgery. A subject that is suffering from PVR is identified by presenting with any PVR indication. PVR indications include the appearance of vitreous haze and retinal pigment epithelial (RPE) cells in the vitreous humor, a wrinkling of the edges of a retinal tear or the inner retinal surface, or by the presence of retinal membranes. A subject that is at risk of developing PVR is identified by presenting with any PVR risk factor. Risk factors for PVR include age, aphakia/pseudophakia, high levels of vitreous proteins, duration of retinal detachment before corrective surgery, the size of the retinal hole or tear, intra-ocular inflammation, vitreous hemorrhage, intraocular pressure, extended retinal detachments, reinterventions, scleral surgery, and trauma or injury to the eye. Other subjects at risk for developing PVR are individuals that engage in activities with increased risk for trauma or injury to or in the proximity of the eye. Examples of such subjects include, but are not limited to, boxers, wrestlers, military personnel, young males. Preferably, the subject has not been diagnosed with cancer, such as an ocular cancer. Preferably, the subject has not been diagnosed with a retinoblastoma. The subject is greater than 3 months old, 6 months old, 9 months old, 12 months old, 18 months old, 24 months old, 30 months old, or 36 months old. Preferably, the subject is an adolescent or an adult.
Preferably, the agent that inhibits or reduces an intra-ocular reduction of the level of p53 associated with PVR is an agent that prevents, inhibits, or reduces p53 from interacting with human double min 2 (Hdm2). Thus, the agent increases the level of p53. In some cases, the reduction of the level of p53 is a platelet-derived growth factor receptor a (PDGFRa)-mediated reduction. For example, the agent that inhibits the intra-ocular reduction of the level of p53 is a polynucleotide, a polypeptide, an antibody, or a small molecule. The upper molecular weight limit for a small molecule is approximately 800 Daltons which allows for the possibility to rapidly diffuse across cell membranes so that the molecule can reach intracellular sites of action. Nutlins, a family of cis-imidazoline analogues, are small-molecule double min 2 antagonists that inhibit the interaction or association between p53 and Hdm2. Preferably, the small molecule comprises Nutlin-3a (RG7112/RO5045337). Preferably, the agent is a functional analog of Nutlin-3a, in which the analog prevents, inhibits, or reduces p53 from interacting or associating (e.g., binding) with Hdm2. Methods for identifying such functional analogs are also described herein.

The structure of Nutlin-3a is reproduced below:

![Structure of Nutlin-3a](image)

Nutlin-3a is administered at a concentration ranging from 0.1 µM to 500 µM, e.g., between 0.5 µM and 400 µM; between 1.0 µM and 300 µM; between 2.0 µM and 200 µM; between 5 µM and 175 µM; between 10 µM and 150 µM; between 20 µM and 125 µM; between 30 µM and 100 µM; or between 50 µM and 75 µM. Preferably, Nutlin-3a is administered at a concentration ranging from 2 to 50 µM. Preferably, Nutlin-3a is administered at a concentration of 200 µM at a dose of 0.1 ml/day, or scaled-up to an amount appropriate for human therapy.

Nutlin-3a is present in the compositions of the present invention at a concentration range of 0.1-10%, with preferred ranges between 1-5% and 2-2.5% (mg/ml). Exemplary liquid formulations for eye drops contain 2-2.5% (mg/ml) of the composition. Preferred formulations are in the form of a solid, a paste, an ointment, a gel, a liquid, an aerosol, a mist,
a polymer, a film, an emulsion, or a suspension. The formulations are administered intravitreally or subconjunctivally.

The composition is administered every 96 hours, every 72 hours, every 48 hours, every 24 hours, every 12 hours, every 6 hours, every 3 hours, or every 1 hour. The composition is administered for a duration of 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 14 days, 20 days, 30 days, 60 days, 90 days, 120 days, 180 days or 365 days. For example, Nutlin-3a is administered intravitreally or subconjunctivally once per day for 7 days. Preferably, the administration is a local administration.

In a preferred method, the administration is intravitreal injection. Preferably, multiple intravitreal injections are administered to the subject over a period of at least 7 days, at least 14 days, at least 28 days. The multiple injections can be every day, every other day, every three days, every four days, every five days, every six days, or weekly for the duration of the treatment. Preferably, for each intravitreal injection, Nutlin-3a is administered at a concentration ranging from 2 to 50 µM.

In another preferred method, the administration is subconjunctival. For subconjunctival administration, a single administration is preferred, wherein the Nutlin-3a is at a concentration ranging from 2 to 50 µM, or preferably at a higher concentration than given over multiple injections, for example, ranging from 10 to 50 µM, 20 to 50 µM, 30 to 50 µM, or 40 to 50 µM. Preferably, the Nutlin-3a administered in a formulation suitable for sustained-release or slow-release of the active ingredient, such that Nutlin-3a is disseminated to or throughout the retina and/or proximal ocular tissues over time, for example, over at least one week, two weeks, three weeks, one month, or two months. Suitable formulations for a single administration include, but are not limited to, membranes, gels, creams, wafers, sponges, or degradable pellets.

The invention provides a composition comprising an agent that inhibits or reduces an intra-ocular reduction of the level of p53 associated with PVR, and/or prevents p53 from interacting or associating with human double min 2 (Hdm2). The composition is used for inhibiting or reducing the severity of proliferative vitreoretinopathy (PVR) in a subject suffering from or at risk of developing PVR. The intra-ocular reduction of the p53 level is mediated by platelet-derived growth factor receptor a (PDGFRa). The agent is a polynucleotide, a polypeptide, an antibody, or a small molecule, e.g., Nutlin-3a (RG7112/RO5045337) or an analog thereof. Nutlin-3a, or analogs thereof, is administered at a concentration of 0.1 µM, 0.5 µM, 1.0 µM, 2.0 µM, 5 µM, 10 µM, 20 µM, 30 µM, or 50 µM. Nutlin-3a, or analog thereof, is present in the composition at a concentration of
0.1-10% (mg/ml). The composition further comprises a pharmaceutically acceptable carrier. In some preferred embodiments, the composition is a solid, a paste, an ointment, a gel, a liquid, an aerosol, a mist, a polymer, a film, an emulsion, or a suspension.

Also within the invention is a pharmaceutical composition comprising a Nutlin-3a compound, or an analog thereof, and a pharmaceutically acceptable carrier and/or ophthalmic excipient. The pharmaceutical composition comprising a Nutlin-3a compound, or analog thereof, and a pharmaceutically acceptable carrier and/or an ophthalmic excipient is for use for inhibiting or reducing the severity of proliferative vitreoretinopathy (PVR).

Exemplary pharmaceutically acceptable carrier include a compound selected from the group consisting of a physiological acceptable salt, poloxamer analogs with carbopol, carbopol/hydroxypropyl methyl cellulose (HPMC), carbopol-methyl cellulose, carboxymethylcellulose (CMC), hyaluronic acid, cyclodextrin, and petroleum.

All compounds of the invention are purified and/or isolated. Specifically, as used herein, an "isolated" or "purified" small molecule (e.g., Nutlin-3a or a functional analog or variant thereof), nucleic acid molecule, polynucleotide, polypeptide, or protein, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized. Purified compounds are at least 60% by weight (dry weight) the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. For example, a purified compound is one that is at least 90%, 91%, 92%, 93%, 94%, 95%, 98%, 99%, or 100% (w/w) of the desired compound by weight.

Purity is measured by any appropriate standard method, for example, by column chromatography, thin layer chromatography, or high-performance liquid chromatography (HPLC) analysis. A purified or isolated polynucleotide (ribonucleic acid (RNA) or deoxyribonucleic acid (DNA)) is free of the genes or sequences that flank it in its naturally occurring state. Purified also defines a degree of sterility that is safe for administration to a human subject, e.g., lacking infectious or toxic agents.

Similarly, by "substantially pure" is meant a nucleotide or polypeptide that has been separated from the components that naturally accompany it. Typically, the nucleotides and polypeptides are substantially pure when they are at least 60%, 70%, 80%, 90%, 95%, or even 99%, by weight, free from the proteins and naturally-occurring organic molecules with they are naturally associated.

An "isolated nucleic acid" is a nucleic acid, the structure of which is not identical to that of any naturally occurring nucleic acid, or to that of any fragment of a naturally
occurring genomic nucleic acid spanning more than three separate genes. The term covers, for example: (a) a DNA which is part of a naturally occurring genomic DNA molecule, but is not flanked by both of the nucleic acid sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner, such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybridgene, i.e., a gene encoding a fusion protein. Isolated nucleic acid molecules according to the present invention further include molecules produced synthetically, as well as any nucleic acids that have been altered chemically and/or that have modified backbones. Isolated nucleic acid molecules also include messenger ribonucleic acid (mRNA) molecules.

Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid and the phrase "nucleic acid sequence" refers to the linear list of nucleotides of the nucleic acid molecule, the two phrases can be used interchangeably.

By the terms "effective amount" and "therapeutically effective amount" of a formulation or formulation component is meant a sufficient amount of the formulation or component, alone or in a combination, to provide the desired effect. For example, by "an effective amount" is meant an amount of a compound, alone or in a combination, required to prevent PVR in a mammal. Ultimately, the attending physician or veterinarian decides the appropriate amount and dosage regimen.

The terms "treating" and "treatment" as used herein refer to the administration of an agent or formulation to a clinically symptomatic individual afflicted with an adverse condition, disorder, or disease, so as to effect a reduction in severity and/or frequency of symptoms, eliminate the symptoms and/or their underlying cause, and/or facilitate improvement or remediation of damage. The terms "preventing" and "prevention" refer to the administration of an agent or composition to a clinically asymptomatic individual who is susceptible or predisposed to a particular adverse condition, disorder, or disease, and thus relates to the prevention of the occurrence of symptoms and/or their underlying cause.

The transitional term "comprising," which is synonymous with "including," "containing," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. By contrast, the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. The
transitional phrase "consisting essentially of limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All published foreign patents and patent applications cited herein are incorporated herein by reference. Genbank and NCBI submissions indicated by accession number cited herein are incorporated herein by reference. All other published references, documents, manuscripts and scientific literature cited herein are incorporated herein by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photomicrograph of a western blot and a bar chart demonstrating that Nutlin-3a prevented both the rabbit vitreous humor (RV)-induced decline in the level of p53 and contraction. Figure 1A is a photograph of a western blot showing the impact of Nutlin-3a on RV-mediated signaling events. Primary rabbit conjunctival fibroblasts (RCFs) were pre-treated with either Nutlin-3a (10 µM), or vehicle for 30 min and then exposed to either RV (diluted 1:3 in Dulbecco's Modified Eagle Medium (DMEM)), or DMEM for 2 hr. The resulting cell lysates were subjected to western blot analysis using the indicated antibodies.

The numbers are a ratio of p-PDGF/ PDGF, p-Akt/Akt or p53/RasGAP (Ras GTPase) activating protein. This data presented is representative of three independent experiments. Figure 1B is a bar chart showing the impact of Nutlin-3a on RV-mediated contraction. RCFs were subjected to the collagen contraction assay as described above. Nutlin-3a (10 µM) or vehicle was included as indicated. The data were subjected to a paired t test; "*" denoted a statistically significant difference.

Figure 2 is a dot plot and a series of photomicrographs showing that Nutlin-3a prevented retinal detachment and elevated p53 in epiretinal membranes. Figure 2A is a dot plot illustrating the impact of Nutlin-3a on experimental PVR. RCFs were used to induce PVR as described below. The indicated rabbits received no additional injections (none) or
0.1 cc injections of either vehicle, or Nutlin-3a (20 µM) on day 0, 2 and 4. The data were subjected to an unpaired t test or one way Analysis of Variance (ANOVA) test; "#" denoted a statistically significant difference. Figure 2B is a series of photographs showing that Nutlin-3a enhanced expression of p53 in epiretinal membranes. At the end of the experiment (day 28), the eyes were enucleated, and paraffin sections of whole eyes were subjected to p53 immunohistochemistry. The arrows point to the epiretinal membrane (the arrowheads denote the retina and the brown color indicates p53). The left and right panels are from a Nutlin-3a injected animal, whereas the middle panel is from a rabbit treated with vehicle. The left and middle panels were stained with the p53 antibody, whereas the right panel was stained with non-immune IgG. The scale bar is 50 µm.

Figure 3 is a series of western blots and photographs showing that molecularly suppressing expression of p53 rescued the ability of PDGFRa-deficient cells to contract. Figure 3A is a photograph of a western blot showing the knockdown RCFs. Lentiviruses were used to stably express short hairpin ribonucleic acids (shRNAs) directed against green fluorescent protein (GFP), PDGFRa, or p53 in primary RCFs. The resulting cell lysates were subjected to western blot analysis using the indicated antibodies. The signal intensity was quantified and expressed as a ratio of the loading control (RasGAP). The data presented are representative of two independent experiments. Figure 3B is a photograph of a western blot showing the expression of PDGFRa potentiated RV-mediated suppression of p53. The cells described in panel A were left resting or exposed to vitreous (diluted 1:3 in DMEM) from normal rabbits (RV) for 2 hr, lysed and total cell lysates were subjected to western blot analysis with the indicated antibodies. The signal intensity was quantified and the data presented are representative of three independent experiments. The data were subjected to a paired t test; "#" denoted a statistically significant difference. Figure 3C is a bar chart showing RV induced contraction of RCF-loaded collagen gels, which required PDGFRa-mediated suppression of p53. The cells described in panel A were subjected to a collagen contraction assay. RV (diluted 1:3 in DMEM) was added on top of the gels on day 0 and the replenished every 24 hr; the experiments was terminated on day 3. The panel below the bar graph shows photographs of representative gels. The data in the bar graph are triplicates within a single experiment. The data were subjected to a paired t test; "*" denoted a statistically significant difference. The data presented are representative of 3 independent experiments.

Figure 4 is a dot plot showing that molecularly suppressing expression of p53 restored the ability of PDGFRa-deficient cells to induce retinal detachment. The cells described in
Figure 3A were compared for their PVR potential in a rabbit model of this disease. The insert indicates the type of lenti-short hairpin RNA (shRNA) used to modify the cells. PVR was induced as described below. Each symbol represents the response of an individual rabbit on the indicated day. PVR was graded according to the Fastenberg scale of classification: stage 0, no disease; stage 1, epiretinal membrane; stage 2, vitreoretinal traction without retinal detachment; stage 3, localized retinal detachment (1-2 quadrants); stage 4, extensive retinal detachment (2-4 quadrants without complete detachment); stage 5, complete retinal detachment. The data were subjected to an unpaired t test or one way ANOVA test; "*" denotes a statistically significant difference.

Figure 5 is a series of bar charts demonstrating the importance of PDGFRA and suppression of p53 for RV-dependent cell proliferation and protection from apoptosis and senescence. Figure 5A is a bar chart showing the results of RCFs (sh GFP, sh PDGFRA, sh PDGFRA/sh p53, and sh p53) seeded into a 24-well plate at a density of 5 x 10⁴ cells/well in DMEM + 10% fetal bovine serum (FBS). After 6 hours the cells had attached, the medium was changed to either 0.5 ml DMEM, or rabbit vitreous (diluted 1:3 in DMEM). The media were replaced every day. The cells were counted with a hemocytometer on day 3. The mean +/- standard deviation of three independent experiments is shown; * denotes P<0.05 using a paired t-test. Figure 5B is a bar chart showing the results of RCFs described in A seeded into 60 mm dishes at a density of 100,000 cells/dish in DMEM + 10% FBS. After 6 hours the cells had attached, the medium was changed to either 3 ml DMEM, or rabbit vitreous (diluted 1:3 in DMEM). The media were replaced every day. On day 3, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) in an apoptosis assay kit by following the manufacturer’s instructions. Cells that were stained with annexin V and/or PI were detected and quantified by flow cytometry in Coulter Beckman XL (Coulter C). The mean +/- standard deviation of three independent experiments is shown; * denotes P<0.05 using a paired t-test. Figure 5C is a bar chart showing the results of RCFs described in A seeded into a 12-well plate at a density of 10,000 cells/well in DMEM + 10% FBS. After 6 hours the cells had attached, the medium was changed to either 1 ml DMEM, or rabbit vitreous (diluted 1:3 in DMEM). The media were replaced every day. On Day 3, the β-galactosidase activity was measured as outlined in the manufacturer’s instructions. Both stained and unstained cells were counted and photographed under an inverted microscope. The mean +/- standard deviation of three independent experiments is shown; * denotes P<0.05 using a paired t-test. The scale bar is 50 µm.
Figure 6 is a schematic representation showing that RV engaged PDGFRα, which triggered two signaling pathways that drive cellular response intrinsic to PVR. Signaling pathway #1 leads to a reduction in the level of p53, and this is sufficient for protection from apoptosis and senescence. Contraction and proliferation require an additional set of signaling events that constitute pathway #2.

Figure 7 is a photograph of a western blot and a bar chart showing p53 attenuated human vitreous humor (HV)-driven collagen gel contraction. Figure 7A is a photograph of a western blot showing the impact of Nutlin-3a on HV-mediated signaling events. Methods were the same as Figure 3A, but the RPE cells from a human patient epiretinal membrane were used in place of RCF, and HV (a pool of vitreous from 5 PVR patients diluted 1:3 in DMEM) was used in place of RV. The data presented are representative of three independent experiments. Figure 7B is a bar chart showing the impact of Nutlin-3a on HV-mediated contraction. This experiments were the same as Figure 3B with the modification noted in Figure 7A. The data were subjected to a paired t test. "*" denoted a statistically significant difference. The data shown are representative of three independent experiments.

Figure 8 is a series of photographs showing the minimum effective dose and maximum tolerated dose of Nutlin-3a. Figure 8A is a series of photomicrographs of western blots showing RCFs pretreated with the indicated concentrations of Nutlin-3a for 30 min, and then exposed to RV (diluted 1:3 in DMEM) for 2 hours. Cells were lysed and resulting lysates were subjected to western blot analysis using the indicated antibodies. The data shown are representative of three independent experiments. Figure 8B is a series of photographs showing RCFs grown to near confluence in DMEM + 10% FBS, the medium was replaced with DMEM + 5% FBS + the indicated concentration of Nutlin-3a. The medium was replaced every 24 hours, and the photographs were taken on day 3. The data shown are representative of two independent experiments. The scale bar is 50 µm.

Figure 9 is a series of photographs showing epiretinal membranes from rabbits subjected to the PVR protocol. Rabbits that underwent a gas vitrectomy were intravitreally injected with the indicated concentration of Nutlin-3a on day 0, 2 and 4. The rabbits underwent a fundus examination on day 1, 3, 5, 7 and 14. Following the last fundus exam rabbits were euthanized, eyes were enucleated and fixed in 10% formalin. Sections were prepared, stained with hematoxylin and eosin, and then photographed. Representative photos of a Nutlin-3a (Figure 9A) and vehicle (Figure 9B) injected eye are presented. Arrowheads and arrows point to the retina and epiretinal membrane, respectively; scale bar: 50 µm. The lack of the retinal pigment epithelial cell layer is an artifact related to processing of the tissue.
Figure 10 is a series of bar graphs showing that inhibiting PDGFRa kinase activity blocked RV-induced collagen contraction. The indicated RCFs (50,000 cells/ml) (Figure 10A) or F cells (1 million cells/ml) (Figure 10B) were subjected to a collagen contraction assays as described below. F cells were immortalized mouse embryo fibroblasts derived from PDGFR knock-out mice that do not express either of the two PDGFR genes, Fa and Fβ cells are F cells in which PDGFRa or PDGFRβ has been re-expressed. After the collagen was solidified (90 minutes), DMEM or RV (diluted 1:3 in DMEM) supplemented with Imatinib (10 µM) or its vehicle was added, and replenished every 24 hours; the experiments were terminated on day 3. The panels below the bar graphs show photographs of representative gels; the data presented are representative of 3 independent experiments. The data were subjected to a paired t test; "*" denoted a statistically significant difference.

Figure 11 is a series of photographs of western blots and bar charts showing that PDGFRa did more than suppress p53 to mediate RV-dependent contraction. Figure 11A is a photograph showing western blot analysis of knockdown RCFs. Lentiviruses were used to stably express shRNAs directed against GFP, PDGFRa, or p53 in primary RCFs. The resulting cell lysates were subjected to western blot analysis using the indicated antibodies. The signal intensity was quantified and expressed as a ratio of the loading control (RasGAP). The data presented are representative of two independent experiments. Figure 11B is a photograph of a western blot showing that expression of PDGFRa potentiated RV-mediated suppression of p53. The cells described in panel A were left resting or exposed to vitreous (diluted 1:3 in DMEM) from normal rabbits (RV) for 2 hours, lysed and total cell lysates were subjected to western blot analysis with the indicated antibodies. The signal intensity was quantified and the data presented are representative of three independent experiments. Figure 11C is a bar chart showing RV induced contraction of RCF-loaded collagen gels, which required PDGFRa-mediated suppression of p53. The cells (50,000 cells/ml) described in panel A were subjected to a collagen contraction assay. RV (diluted 1:3 in DMEM) was added on top of the gels on day 0 and the replenished every 24 hours; the experiments were terminated on day 3. The panel below the bar graph shows photographs of representative gels; the data presented are representative of 3 independent experiments. The data were subjected to a paired t test; "*" denoted a statistically significant difference.

Figure 12 is a series of photographs showing p53 was undetectable in human PVR membranes. Epiretinal membranes from patients afflicted with PVR subjected to p53 immunohistochemistry. While the positive control (rat colon cancer) demonstrated that this approach could readily detect p53 protein (panel B), p53 was undetectable in membranes.
from 7 different patients, three of which are shown (panels D-F). The green/brown pigment is routinely observed in such samples, which contain a large number of retinal pigment epithelial cells; they were also present in the section of a PVR membrane that was stained with an non-immune, isotype-matched control antibody (panel C). Panel A is a section of rat colon cancer that serves as a negative control for panel B.

**DETAILED DESCRIPTION**


Nutlin-3a was first identified as a potent and selective small molecule inhibitor of the p53-MDM2 interaction. Subsequent studies showed that Nutlin-3a administration in vitro caused p53 stabilization and activation of the p53-pathway. Researchers have investigated the effects of Nutlin-3a administration to the eye, specifically in the context of a therapeutic strategy for treating retinoblastoma (Brennan et al., 2011 Cancer Res, 71(12): 4205-13).

Retinoblastoma is a malignant tumor of the retina and it is estimated that up to 40% of retinoblastomas are hereditary. Retinoblastoma is a childhood cancer, and usually diagnosed in very young children between 12 months and 24 months of age. Because retinoblastomas retain wild-type p53 (instead, having a mutated RBI gene that drives tumorigenesis),
administration of Nutlin-3a may be useful as a cancer therapeutic by inducing effective p53-mediated apoptosis, senescence, or growth arrest in the tumor cells.

The data described herein demonstrate the surprising results that Nutlin-3a administration inhibited or reduced proliferative vitreoretinopathy and retinal detachment. These results are particularly intriguing, even in light of Brennan et al., because PVR and retinoblastoma are distinct ocular conditions. First, the etiologies of PVR and retinoblastoma are completely different- retinoblastoma is the uncontrolled growth and division of cells driven most often by mutations in the \textit{Rbl} (retinoblastoma) gene, while PVR is caused by a spontaneous event occurring after injury, trauma, or surgical procedure. Second, Nutlin-3a was a known p53 activator, and thus, the anti-tumorigenic effects in a wild-type p53 retaining cancer, such as retinoblastoma, were well known in the cancer field. However, unlike in cancers, the role of p53 and/or MDM2 has never been identified or implicated, prior to the invention, in a non-cancer setting, e.g., the development or mechanisms of PVR or retinal detachment. And finally, the patient populations affected by retinoblastoma and PVR are also entirely distinct. Retinoblastoma can develop in utero, and is usually diagnosed between 12 and 24 months of age. Moreover, many retinoblastoma patients inherited the disease. In contrast, PVR is associated with retinal detachment, which is a spontaneous event occurring after injury, trauma, or surgical procedure. PVR and retinal detachment often occur in the elderly (e.g., greater than 65 years of age), very near-sighted individuals, or individuals with a family history of retinal detachment. Thus, the patients affected by PVR are typically older than 12 or 24 months, and/or have suffered from a previous injury, trauma or surgical procedure to or near the proximity of the eye.

**Proliferative vitreoretinopathy**

PVR is a blinding disease associated with rhegmatogenous retinal detachment, for which there is currently no satisfactory treatment. The term "proliferation" in "PVR" refers to the proliferation of retinal pigment epithelial and glial cells, while the terms "vitreo" and "retinopathy" identify the tissues which are affected, namely the vitreous humor (or simply vitreous) and the retina. Specifically, PVR is a disease that develops as a complication, secondary to rhegmatogenous retinal detachment. PVR occurs in about 8-10% of patients undergoing primary retinal detachment surgery, and can prevent the successful surgical repair of rhegmatogenous retinal detachment. Prior to the invention described herein, there were no prophylactic/preventative options available to patients that were at risk of PVR, e.g., those patients that had undergone retinal surgery.
The full-thickness retinal break (e.g., tears and holes) that is quintessential to rhegmatogenous retinal detachment results in exposure of cells to vitreous, a rich source of growth factors and cytokines (Oh K, Hartnett M, Landers IM: Pathogenic mechanisms of retinal detachment. Edited by Ryan S. Philadelphia, Elsevier Mosby, 2006). The accumulation of fluid in the sub-retinal space, along with the tractional force of the vitreous on the retina results in rhegmatogenous retinal detachment. Specifically, the RPE cells migrate into vitreous, proliferate, and synthesize extracellular matrix proteins (Han D: Proliferative vitreoretinopathy. Edited by Albert D, JW. M, DT. A, BA. B. Philadelphia, Elsevier Saunders, 2008, pp. 2315-2324). The cytokines present in the vitreous humor trigger the ability of the RPE to proliferate and migrate. This series of events culminates in the formation of a retina-associated membrane, which contracts and thereby causes retinal detachment and vision loss (Campochiaro P; The pathogenesis of proliferative vitreoretinopathy. Edited by Ryan S. Philadelphia, Elsevier Mosby, 2006).


Predisposing factors for postoperative PVR are preoperative PVR, aphakia, high levels of vitreous proteins, duration of retinal detachment before corrective surgery, the size of the retinal hole or tear, intra-ocular inflammation, vitreous hemorrhage, vitreous liquidity, and trauma or injury to the eye. As described in Rodriguez de la Rua E et al., 2005 Curr Eye Res, 30: 147-153, incorporated herein by reference, the risk for PVR was higher in patients > 70 years, with intraocular pressure lower than 14 (OR: 3.84; CI 95%: 2.04-7.30), in retinal breaks larger than “1 clock hour” (OR: 2.54; CI: 1.28-5.05), extended retinal detachments (OR: 4.01; CI: 1.98-8.10), and reinterventions (OR: 1.55; CI: 1.14-9.22). Scleral surgery also was a risk factor for PVR (OR: 3.89; CI: 2.12-7.14) and aphakia/pseudophakia when scleral surgery is performed (OR: 3.33; CI: 1.54-7.22). In particular, some subjects that have
undergone ocular surgeries, such as surgery to correct retinal detachments, are at increased risk for developing PVR.

The interaction of P53 and Nutlin-3a

Various forms of cellular stress increase expression and activate p53, a tetrameric transcription factor, and thereby trigger the p53 pathway, which leads to cell cycle arrest, apoptosis and/or senescence (Levine AJ et al., 2009 Nat Rev Cancer, 9:749-758). The finding that p53 and/or the p53 pathway is mutated in approximately 50% of solid tumors (Hainaut P and Hollstein M, 2000 Adv Cancer Res, 77:81-137) has lead to the development of pharmacological agents that stimulate the p53 pathway. For instance, the small molecule Nutlin-3a activates the p53 pathway by preventing p53 from interacting with Mdm2/Hdm2 (murine double min 2, also called Hdm2 in humans) (Vassilev LT et al., 2004 Science, 303:844-848), which reduces the level of p53 by a variety of mechanisms (Prives C, 1998 Cell, 95:5-8; Ofir-Rosenfeld Y et al., 2008 Mol Cell, 32:180-189; Sasaki M et al., 2011 Nat Med, 17:944-951).

The amino acid sequence of human p53 (Genbank Accession No. AAD28535.1) is as follows (SEQ ID NO: 1):

MEEPQDSVEPPLSQETFSDDLWKLLPENNVLSPSLPSQAMDDMLSPDDIEQWFTDGPIDEAPRMPFEAA
PRVAPAPAATPAAAPAPSWPLLSSVPQKTQYQSYGFRGLFHLHSTAKSVTCSPLALKMFCQLA
CPVQILWVDSTPPGTRVRAAI YKQSQHIMTEWRRCPVHBRC3SDLAPQHQL RVEAGNLREVYLDLN
MVRSRQMCNTNMSVDTGVTTSQPASEQETLVRPKPLLKLLKSVMGKQDKTYTMEKVLFLGQYIMFTK
RLYDEKQOH IYCSNDELGLDILGSPSFVSKHEKRI YTMIPRNVLWNVQESSDSGTSSSRENCHLEGSD
QKDLQVLQELQKSSPHLVSRSPTSSRRRAI SETEENSDLSEGRQKRRKSDS 1 SLSFDESALCVIRE
ICERSSSSESTGTFNPDALGVEHSGDQLQDVSQSVFEVLSDEYSLSSEGQELSSEDDE

Orthologs for human p53 can be readily identified and are known in the art, for example, mouse (Genbank Accession No. AAC05704), rat (Genbank Accession No. AAH81788), cat (Genbank Accession No. P41685), dog (Genbank Accession No. AF060514) and horse (Genbank Accession No. X91793). Other isoforms and transcriptional variants of p53 are also known in the art.

The amino acid sequence of human double min 2 (Hdm2) (Genbank Accession No. NP_002383) is as follows (SEQ ID NO: 2):

NP_0002383

Orthologs for Hdm2 can be readily identified and are known in the art, for example, mouse (Genbank Accession No. AAB09030), rat (Genbank Accession No. NP_00110569.1), cat
(Genbank Accession No. NP_001009346.1), dog (Genbank Accession No. AAG42840.0) and horse (Genbank Accession No. AAF28866.1).

In 2004, Vassilev and co-workers (Hoffman-La Roche Inc., Nutley, New Jersey) described a class of antagonists that inhibited the murine double min 2 (MDM2)-p53 complex. These antagonists are a group of cis-imidazoline analogues designated as the Nutlins. Through x-ray crystallography, the MDM2-p53 complex showed a well defined hydrophobic cleft which represented the binding site for p53. In addition, the structure revealed that this cleft was filled by only three side chains of the helical region of p53: Phel9, Leu26 and Trp23. This observation led to the possibility that a small molecular inhibitor could mimic these three amino acids and their orientation. The inhibitor could disrupt the MDM2-p53 interaction by binding specifically in this cleft, liberating functional p53. A class of small molecules, called Nutlins, were found to bind specifically into the p53-binding pocket of Mdm2/Hdm2, thereby preventing Mdm2 interaction with p53 and inhibiting Mdm2-dependent degradation of p53. Nutlin-3a (RG7112/RO5045337) is currently in clinical trials for certain tumors in which the Hdm2/p53 pathway is intact (Secchiero P et al., 2011 Curr Pharm Des, 17:569-577). Furthermore, an ophthalmic formulation of Nutlin-3a has been developed (Brennan RC et al., 2011 Cancer Res, 71:4205-4213). The results presented below demonstrate that the correlation between the PDGFRa-mediated decline in the level of p53 and development of PVR is causally related, and that Nutlin-3a-mediated stabilization of p53 prevents PVR.

Nutlin-3a (RG7112/RO5045337) is a small molecule that is currently in clinical trials for certain types of cancer. Specifically, Nutlin-3 [\((+)-4-[4,5\text{-}\text{Bis}(4\text{-}\text{chlorophenyl})\text{-}2\text{-}((2\text{-}\text{isopropoxy}-4\text{-}\text{methoxy}-\text{phenyl})\text{-}4,5\text{-}\text{dihydro}\text{-}\text{imidazole}\text{-}1\text{-}\text{carbonyl}]\text{-}piperazin\text{-}2\text{-}\text{one}\) is a \(c\alpha\)-imidazoline analog which inhibits the interaction between mdm2 and tumour suppressor p53.

Nutlin-3 is arbitrarily referred to as enantiomer a because it appears as the first peak from chiral purification of racemic nutlin-3. It acts by preventing a decline in the level of p53, which is a transcription factor that is mutated in many, but not all tumors that occur in humans. By inhibiting the interaction between mdm2 and p53, Nutlin-3 stabilizes p53, and selectively induces a growth-inhibiting state called "senescence" in cancer cells.

Specifically, in those tumors in which p53 remains intact (normal/wild type p53), Nutlin-3a prevents proliferation, and promotes the apoptosis/senescence of the tumor cells.

Nutlins are ds-imidazoline analogs which inhibit the interaction between mdm2 and tumour suppressor p53. Nutlin-3 [\((+)-4-[4,5\text{-}\text{Bis}(4\text{-}\text{chlorophenyl})\text{-}2\text{-}((2\text{-}\text{isopropoxy}-4\text{-}m
methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl]-piperazin-2-one\]) has the following chemical structure:

![Chemical Structure](image)

Cis-isomers of nutlin-3 are:

![Cis-isomers](image)

Trans-isomers of this compound are:

![Trans-isomers](image)

Nutlin-3a is arbitrarily referred to as enantiomer "a" because it appears as the first peak from chiral purification of racemic nutlin-3. Nutlin-3a acts by preventing a decline in the level of p53, which is a transcription factor that is mutated in many, but not all tumors that occur in humans. By inhibiting the interaction between mdm2 and p53, Nutlin-3a stabilizes p53, and selectively induces a growth-inhibiting state called "senescence" in cancer cells. Specifically, in those tumors in which p53 remains intact (normal/wild type p53), Nutlin-3a prevents proliferation, and promotes the apoptosis/senescence of the tumor cells.

Analogs of nutlin-3 may be a compound of formula (I):

![Analogs](image)

wherein
R<sup>1</sup> is independently selected from F, Cl, Br, and I;
R<sup>2</sup> is independently selected from F, Cl, Br, and I;
R<sup>3</sup> is independently selected from hydrogen, Ci-C<sub>6</sub> alkyl, and Ci-C6-alkoxy;
s is 0, 1, 2, 3, 4, or 5;
t is 0, 1, 2, 3, 4, or 5; and
u is 0, 1, 2, 3, 4, or 5;
or a pharmaceutically acceptable salt, solvate, or prodrug thereof.

Analogs of nutlin-3 may be a compound of formula (II):

![Chemical Structure](image)

(II), wherein

R<sup>1</sup> is independently selected from F, Cl, Br, and I;
R<sup>2</sup> is independently selected from F, Cl, Br, and I; and
R<sup>3</sup> is independently selected from hydrogen, Ci-C<sub>6</sub> alkyl, and Ci-C6-alkoxy;
or a pharmaceutically acceptable salt, solvate, or prodrug thereof.

In one aspect, R<sup>1</sup> of formula (I) or (II) is Cl. In one aspect, R<sup>2</sup> of formula (I) or (II) is Cl. In another aspect, R<sup>3</sup> is methoxy or isopropyloxy.

As used herein, "alkyl" or "C1-C6 alkyl" is intended to include Ci, C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub>, C<sub>5</sub> or C<sub>6</sub> straight chain (linear) saturated aliphatic hydrocarbon groups and C<sub>3</sub>, C<sub>4</sub>, C<sub>5</sub> or C<sub>6</sub> branched saturated aliphatic hydrocarbon groups. For example, C1-C6 alkyl is intended to include C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub>, C<sub>5</sub> and C<sub>6</sub> alkyl groups. Examples of alkyl include, moieties having from one to six carbon atoms, such as, but not limited to, methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl, t-butyl, n-pentyl, s-pentyl or n-hexyl.

In certain embodiments, a straight chain or branched alkyl has six or fewer carbon atoms (e.g., Ci-C<sub>6</sub> for straight chain, C<sub>3</sub>-C6 for branched chain), and in another embodiment, a straight chain or branched alkyl has four or fewer carbon atoms.

The term "alkoxy" includes substituted and unsubstituted alkyl groups covalently linked to an oxygen atom. Examples of alkoxy groups include methoxy, ethoxy, isopropyloxy, propoxy, butoxy, and pentoxy groups.
"Isomer" means compounds that have identical molecular formulae but differ in the sequence of bonding of their atoms or in the arrangement of their atoms in space. Isomers that differ in the arrangement of their atoms in space are termed "stereoisomers". Stereoisomers that are not mirror images of one another are termed "diastereoisomers", and stereoisomers that are non-superimposable mirror images of each other are termed "enantiomers" or sometimes optical isomers. A mixture containing equal amounts of individual enantiomeric forms of opposite chirality is termed a "racemic mixture".

A carbon atom bonded to four non-identical substituents is termed a "chiral center". "Chiral isomer" means a compound with at least one chiral center. Compounds with more than one chiral center may exist either as an individual diastereomer or as a mixture of diastereomers, termed "diastereomeric mixture". When one chiral center is present, a stereoisomer may be characterized by the absolute configuration (R or S) of that chiral center.

Absolute configuration refers to the arrangement in space of the substituents attached to the chiral center. The substituents attached to the chiral center under consideration are ranked in accordance with the Sequence Rule of Cahn, Ingold and Prelog. (Cahn et al., Angew. Chem. Inter. Edit. 1966, 5, 385; errata 511; Cahn et al., Angew. Chem. 1966, 78, 413; Cahn and Ingold, J. Chem. Soc. 1951 (London), 612; Cahn et al., Experientia 1956, 12, 81; Cahn, J. Chem. Educ. 1964, 41, 116).

"Pharmaceutically acceptable salt" of a compound means a salt that is pharmaceutically acceptable and that possesses the desired pharmacological activity of the parent compound.

"Solvate" means solvent addition forms that contain either stoichiometric or non-stoichiometric amounts of solvent. Some compounds have a tendency to trap a fixed molar ratio of solvent molecules in the crystalline solid state, thus forming a solvate. If the solvent is water the solvate formed is a hydrate; and if the solvent is alcohol, the solvate formed is an alcoholate. Hydrates are formed by the combination of one or more molecules of water with one molecule of the substance in which the water retains its molecular state as H2O.

As used herein, the term "analog" refers to a chemical compound that is structurally similar to another but differs slightly in composition (as in the replacement of one atom by an atom of a different element or in the presence of a particular functional group, or the replacement of one functional group by another functional group). Thus, an analog is a compound that is similar or comparable in function and appearance, but not in structure or origin to the reference compound. The analogs described herein also retain similar function to
Nutlin-3a, in which the analog also reduces or inhibits the interaction or association between p53 and Hdm2, or increases intraocular p53 levels.

As used herein, "blocking the interaction or association" or "inhibiting or reducing binding" refers to preventing or reducing the direct or indirect association of one or more molecules, peptides, or proteins; or preventing or reducing the normal activity of one or more molecules, peptides, or proteins. The interaction, association, or binding is covalent, non-covalent, or ionic.

The present invention also provides functional derivatives of analogs of Nutlin-3a. As used herein, "functional analogs" of Nutlin-3a refers to small molecules, antibodies, polypeptides, or polynucleotides that inhibit or reduce the interaction or association between p53 and Hdm2. Preferably, the functional analog binds in the p53-binding pocket of Hdm2 and inhibits or reduces interaction, association or binding between p53 and Hdm2. Functional analogs of Nutlin-3a can be identified by screening methods known in the art. Suitable screening assays may utilize techniques known in the art such as two hybrid assay, fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), protein-fragment complementation (PCA), or co-immunoprecipitation assay which detect protein-protein interactions, to detect interaction of p53 and Hdm2, or inhibition or reduction of p53-Hdm2 binding.

In particular, a screening assay to identify functional analogs was described in Vassilev et al. (2004, Science, 303:845-848), hereby incorporated by reference in its entirety. For example, the screening assay comprises assaying (i) stabilization and accumulation of p53 protein, (ii) activation of Hdm2 expression, or (iii) activation of other p53-regulated genes and the p53 pathway. For example, the skilled artisan, using the amino acid sequences disclosed herein for p53 and Hdm2 could use recombinant DNA methods well known in the art to construct expression vectors and in vitro translate the p53 and Hdm2 proteins.

Incubation of the proteins with putative analogs of Nutlin or a library of agents to be screened can be performed with optimal conditions determined by the skilled artisan. Co-immunoprecipitation, immunoaffinity purification, western blotting or other methods well known in the art are then used to assess the binding between p53 and Hdm2, or the inhibition or reduction of binding by the introduction of the putative Nutlin analog. In other embodiments, stabilization and accumulation of p53 protein levels can be determined in vitro, through immunoblotting techniques utilizing p53-specific antibodies. In another embodiment, the activation of other p53-regulated genes and the p53 pathway, such as MDM2, apoptosis
genes PUMA and NOXA, cell cycle regulators p21, and p53 itself can be assessed by
determining or quantifying the expression levels by mRNA or protein.

**Pharmaceutical Compositions**

For administration to a subject such as a human or other mammal (e.g., companion,
zoological or livestock animal), the Nutlin or analog thereof is desirably formulated into a
pharmaceutical composition containing the active agent in admixture with one or more
pharmaceutically acceptable diluents, excipients or carriers. Examples of such suitable
excipients for can be found in U.S. Publication 2009/0298785 (incorporated by reference
herein in its entirety), the *Handbook of Pharmaceutical Excipients*, 2nd Edition (1994), Wade
and Weller, eds. Acceptable carriers or diluents for therapeutic use are well-known in the
pharmaceutical art, and are described, for example, in *Remington: The Science and Practice
Philadelphia, Pa. Examples of suitable carriers include lactose, starch, glucose, methyl
cellulose, magnesium stearate, mannitol, sorbitol and the like. Examples of suitable diluents
include ethanol, glycerol and water.

The choice of pharmaceutical carrier, excipient or diluent can be selected with regard
to the intended route of administration and standard pharmaceutical practice. The
pharmaceutical composition can contain as, or in addition to, the carrier, excipient or diluent
any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilizing
agent(s).

Examples of suitable binders include starch, gelatin, natural sugars such as glucose,
anhydrous lactose, free-flow lactose, beta-lactose, corn sweeteners, natural and synthetic
gums, such as acacia, tragacanth or sodium alginate, carboxymethyl cellulose and
polyethylene glycol.

Examples of suitable lubricants include sodium oleate, sodium stearate, magnesium
stearate, sodium benzoate, sodium acetate, sodium chloride and the like.

Preservatives, stabilizers, dyes and even flavoring agents can be provided in the
pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid
and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents can be also used.

A person of ordinary skill in the art can easily determine an appropriate dosage to
administer to a subject without undue experimentation. Typically, a physician will determine
the actual dosage that will be most suitable for an individual subject based upon a variety of
factors including the activity of the specific compound employed, the metabolic stability and
length of action of the compound, the age, body weight, general health, diet, mode and time
of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy. To determine a suitable dose, the physician or veterinarian could start doses levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. This is considered to be within the skill of the artisan and one can review the existing literature on a specific agent to determine optimal dosing.

The compositions described herein comprising a Nutlin or an analog thereof can be administered to a subject via intravitreally of subconjunctivally. In some embodiments, the composition is administered in the form of a liquid (e.g., drop or spray) or gel suspension. Alternatively, the composition is applied to the eye via liposomes or infused into the tear film via a pump-catheter system. Further embodiments embrace a continuous or selective-release device, for example, membranes such as, but not limited to, those employed in the OCUSERT System (Alza Corp., Palo Alto, Calif.) In an alternative embodiment, the p53 activator is contained within, carried by, or attached to a contact lens, which is placed on the eye. Still other embodiments embrace the use of the composition within a swab or sponge, which is applied to the ocular surface.

In some cases, the composition further comprises a pharmaceutically acceptable carrier, e.g., a pharmaceutically acceptable salt. Suitable ocular formulation excipients include FDA approved ophthalmic excipients, e.g., emulsions, solutions, solution drops, suspensions, and suspension drops, a list of which is provided in Table 1. Other suitable classifications include gels, ointments, and inserts/implants. Table 1 provides maximum percentages, when available, for the various formulation types.

Exemplary excipients for use in optimizing ocular formulations include alcohol, castor oil, glycerin, polyoxyl 35 castor oil, Tyloxapol, polyethylene glycol 8000 (PEG-8000), ethanol, glycerin, cremaphor, polyethylene glycol (pG), polypropylene glycol (ppG), and polysorbate 80. In some cases, citrate buffer and sodium hydroxide are included to adjust pH. Preferably, the formulation for ocular delivery of nutlin-3a comprises 5% cremaphor, 10% pG, 15% pPG, and 70% phosphate buffered saline (PBS).
Table 1

<table>
<thead>
<tr>
<th>Excipient/Co-solvent</th>
<th>Exemplary Percentage According to the FDA Excipient Database</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><a href="http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm">http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm</a></td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>0.2 (solution), 0.9% (drops)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>0.5% (solution), 1.4% (solution, drops)</td>
</tr>
<tr>
<td>Alcohol, dehydrated</td>
<td>0.5% (solution), 0.5% (solution, drops)</td>
</tr>
<tr>
<td>Ammonium hydroxide</td>
<td>N/A (solution; solution, drops)</td>
</tr>
<tr>
<td>Anhydrous trisodium citrate</td>
<td>N/A (solution)</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>0.1% (solution)</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>2% (solution), 0.9% (drops)</td>
</tr>
<tr>
<td>Benzododecinium</td>
<td>0.012% (solution)</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.1% (emulsion), 37.2% (solution), 1.9% (solution, drops), 1% (suspension), 0.6% (suspension drops)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>2% (solution)</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.02% (solution, drops)</td>
</tr>
<tr>
<td>Carbomer 1342</td>
<td>0.05% (emulsion)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.5% (solution), 0.2% (solution, drops)</td>
</tr>
<tr>
<td>Carbomer 934P</td>
<td>0.45% (suspension, drops)</td>
</tr>
<tr>
<td>Carbomer Homopolymer Type B</td>
<td>0.5% (suspension), 0.45% (suspension, drops)</td>
</tr>
<tr>
<td>Carboxymethylcellulose sodium</td>
<td>0.5% (solution, drops)</td>
</tr>
<tr>
<td>Castor oil</td>
<td>5% (emulsion)</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>0.5% (suspension)</td>
</tr>
<tr>
<td>Chlorobutanol</td>
<td>0.5% (solution), 0.2% (solution, drops)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>N/A (powder, for suspension)</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.2% (solution), 0.05% (solution, drops)</td>
</tr>
<tr>
<td>Citric acid monohydrate</td>
<td>0.05% (solution; solution, drops)</td>
</tr>
<tr>
<td>Diethanolamine</td>
<td>N/A (solution)</td>
</tr>
<tr>
<td>Divinylbenzene styrene copolymer</td>
<td>0.75% (suspension, drops)</td>
</tr>
<tr>
<td>Edetate disodium</td>
<td>10% (solution), 0.1% (solution, drops), 0.13% (suspension, drops), 0.101% (suspension, drops)</td>
</tr>
<tr>
<td>Edetate sodium</td>
<td>0.02% (emulsion), 0.1% (solution), 0.02% (suspension)</td>
</tr>
<tr>
<td>Gelman gum</td>
<td>0.6% (solution)</td>
</tr>
<tr>
<td>Glycerin</td>
<td>2.2% (emulsion), 3% (solution), 2.6% (solution, drops), 2.5% (suspension; drops)</td>
</tr>
<tr>
<td>Glyceryl stearate</td>
<td>0.5% (solution)</td>
</tr>
<tr>
<td>Hydrocarbon gel, plasticized</td>
<td>N/A (suspension)</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>1.06% (solution), 0.17% (solution, drops)</td>
</tr>
<tr>
<td>Hydroxyethyl cellulose</td>
<td>0.5% (solution; solution, drops), 0.25% (suspension, drops), 0.35% (suspension, drops)</td>
</tr>
<tr>
<td>Hydroxyethyl methylcellulose</td>
<td>0.5% (solution)</td>
</tr>
<tr>
<td>Hypermellose 2910</td>
<td>0.5% (solution; suspension; suspension, drops)</td>
</tr>
<tr>
<td>Hypermelloses</td>
<td>0.5% (solution), 0.5% (solution, drops; suspension, 0.6% (suspension, drops)</td>
</tr>
<tr>
<td>Laurakonium chloride</td>
<td>0.005% (solution, drops)</td>
</tr>
<tr>
<td>Lauroyl sarcosine</td>
<td>0.03% (suspension, drops)</td>
</tr>
<tr>
<td>Light mineral oil</td>
<td>N/A (suspension)</td>
</tr>
</tbody>
</table>

23
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium chloride</td>
<td>0.03% (powder, for solution), 0.0065% (solution, drops)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>23% (solution), 4.6% (solution, drops), 2.4% (suspension), 4% (suspension, drops)</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>0.5% (solution)</td>
</tr>
<tr>
<td>Methylparaben</td>
<td>0.05% (solution; solution, drops; suspension; suspension, drops)</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>0.1% (suspension)</td>
</tr>
<tr>
<td>Nitric acid</td>
<td>N/A (solution)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>N/A (solution)</td>
</tr>
<tr>
<td>Nonoxynol-9</td>
<td>0.125% (solution)</td>
</tr>
<tr>
<td>Nonoxynol-40</td>
<td>0.05% (solution), 0.01% (solution, drops)</td>
</tr>
<tr>
<td>Octylphenol polymethylene</td>
<td>N/A (solution)</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>N/A (solution, drops)</td>
</tr>
<tr>
<td>Polidronium chloride</td>
<td>0.0005% (solution, drops)</td>
</tr>
<tr>
<td>Poloxamer 188</td>
<td>0.1% (solution; solution, drops)</td>
</tr>
<tr>
<td>Poloxamer 407</td>
<td>0.2% (solution), 0.16% (solution, drops), 0.101% (suspension, drops)</td>
</tr>
<tr>
<td>Polycarbophil</td>
<td>0.9% (solution), 0.859% (suspension, drops)</td>
</tr>
<tr>
<td>Polyethylene glycol 300</td>
<td>N/A (solution)</td>
</tr>
<tr>
<td>Polyethylene glycol 8000</td>
<td>2% (solution)</td>
</tr>
<tr>
<td>Poloxyl 35 castor oil</td>
<td>5% (solution; solution, drops)</td>
</tr>
<tr>
<td>Polyoxyl 40 hydrogenated castor oil</td>
<td>0.5% (solution, drops)</td>
</tr>
<tr>
<td>Polyoxyl 40 stearate</td>
<td>7% (solution), 0.5% (suspension)</td>
</tr>
<tr>
<td>Polypropylene glycol</td>
<td>15% (solution)</td>
</tr>
<tr>
<td>Polysorbate 20</td>
<td>0.05% (suspension)</td>
</tr>
<tr>
<td>Polysorbate 80</td>
<td>4% (emulsion), 0.2% (solution), 1% (solution, drops), 0.1% (suspension, suspension, drops)</td>
</tr>
<tr>
<td>Polyvinyl alcohol</td>
<td>1.4% (solution; solution, drops; suspension; suspension, drops)</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>4% (powder, for solution)</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>22.2% (solution), 0.14% (solution, drops)</td>
</tr>
<tr>
<td>Potassium phosphate, monobasic</td>
<td>0.2% (solution), 0.065% (solution, drops), 0.44% (suspension)</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>0.47% (solution)</td>
</tr>
<tr>
<td>Povidone K29/32</td>
<td>1.8% (solution)</td>
</tr>
<tr>
<td>Povidone K30</td>
<td>2% (solution), 0.6% (suspension)</td>
</tr>
<tr>
<td>Povidone 90</td>
<td>1.2% (solution)</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>10% (solution), 0.75% (solution, drops), 5% (suspension), 1% (suspension, drops)</td>
</tr>
<tr>
<td>Propylparaben</td>
<td>0.015% (solution; solution, drops), 0.01% (suspension; suspension, drops)</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.05% (emulsion), 0.35% (solution), 1.279% (solution, drops)</td>
</tr>
<tr>
<td>Sodium bisulfite</td>
<td>0.1% (solution; solution, drops); 0.06% (suspension)</td>
</tr>
<tr>
<td>Sodium borate</td>
<td>0.543% (solution), 1.1% (solution, drops), 0.0285% (suspension, drops)</td>
</tr>
<tr>
<td>Sodium borate decahydrate</td>
<td>0.15% (solution), 0.095% (solution, drops)</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>1% (solution)</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>55% (solution), 0.9% (solution, drops), 0.85% (suspension), 0.68% (suspension, drops)</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>2% (solution), 2.2% (solution, drops), 0.3% (suspension), 0.45% (suspension, drops)</td>
</tr>
</tbody>
</table>
Preferably, the compositions are delivered by intravitreal injection or subconjunctival administration.

As described in detail below, suppressing expression of p53 was a required event in two assays of PVR, namely, PDGFRα-mediated contraction of cells in a collagen gel and retinal detachment in an animal model of PVR. Furthermore, as described in detail below, preventing the decline in the level of p53 with agents such as Nutlin-3a protected from retinal
detachment, which is the most vision-compromising component of PVR. Finally, as described herein, Nutlin-3a is effective in the clinical setting, as the small molecule prevented human PVR vitreous-induced contraction of cells isolated from a patient PVR membrane.

As described below, Nutlin-3a was administered in a series of intravitreal injections. While this approach completely prevented retinal detachment, 50% of the rabbits developed vitreal traction (stage 2) (Figure 2A). Since the last injection of Nutlin-3a was 23 days prior to the end of the experiment, the level of Nutlin-3a may have dropped below the therapeutic range. The recently-developed ocular formulation of Nutlin-3a, which can be administered as a subconjunctival injection (Brennan RC et al., 2011 Cancer Res, 71:4205-4213), is also suitable to achieve Nutlin-3a-mediated PVR prophylaxis.

As described in detail below, Nutlin-3a treatment very effectively prevented retinal detachment, and also slowed formation of membranes (stage 1) (Figure 2A), both of which are of clinical benefit. It was surprising that membranes were able to form in Nutlin-3a animals, because Nutlin-3 activates the p53 pathway (Vassilev LT et al., 2004 Science, 303:844-848), which counters cellular events that are intrinsic to membrane formation such as proliferation and survival (Lei H et al., 2010 Exp Eye Res, 90:376-381). However, this observation is consistent with reports that elevating p53 does not always cause apoptosis and/or cell cycle arrest (Giono LE et al., 2007 Mol Cell Biol, 27:4166-4178; Mendrysa SM et al., 2003 Mol Cell Biol, 23:462-472; Mendrysa SM et al., 2006 Genes Dev, 20:16-21). For instance, p53 is transiently elevated in mitotic cells of normal tissue in healthy animals, and fails to engage the p53 pathway because p53 is held in check by post-translational modifications (Loewer A et al., 2010 Cell, 142:89-100).

The results described herein reveal that cellular responses associated with PVR do not have the same requirements. Contraction and proliferation require a decline in the level of p53 and a second PDGFRα-mediate event(s), whereas protection from apoptosis and senescence proceed when only p53 is suppressed (Figure 6). Furthermore, while all 4 responses were completely dependent on expression of PDGFRα, contraction required the lowest level. This low threshold for PDGFRα expression and ability of contraction to proceed when other cellular response are impaired provide a plausible explanation for why therapies targeting other cellular responses have not been successful (Wiedemann P et al., 1998 Am J Ophthalmol, 126:550-559; Asaria RH et al., 2001 Ophthalmology, 108:1179-1183; Schiff WM et al., 2007 Arch Ophthalmol, 125:1161-1167).

As described in detail below, epiretinal membranes formed in rabbits injected with cells that were unable to suppress p53 efficiently (sh PDGFRα), which was required for RV-
mediated proliferation and viability, cellular events that are thought to be essential for membrane formation. Previous reports have shown that proliferation-incompetent cells induce PVR provided that they are injected at a sufficiently high level (Fastenberg DM et al., 1982 Am J Ophthalmol, 93:565-572). Thus, membranes may have formed in rabbits injected with sh PDGFRα cells because enough of them were injected.

The examples below demonstrate that both molecular and pharmacological approaches indicate that reducing the level of p53 was permissive for retinal detachment, a process that involves contraction of the retina-associated membrane. A simple explanation for this phenomenon is that p53 suppresses the expression of genes that are required for retinal detachment. For instance, p53 may inhibit production of those extracellular matrix proteins that are required for contraction of the membrane (Iotsova V et al., 1996 Cell Growth Differ, 7:629-634). However, such an explanation appears inadequate for the in vitro contraction assays, which contained ample extracellular matrix proteins that are conducive for contraction. p53 may down regulate expression of integrins such as β1 (Qiu J et al., 2011 Mol Cell Biochem, 357:125-133), whose interaction with extracellular matrix proteins is essential for contraction. Alternatively, there may be a connection to EMP2 (epithelial cell membrane protein) and FAK (focal adhesion kinase), which are essential for contraction of collagen gels and strongly implicated in PVR (Morales SA et al., 2009 Invest Ophthalmol Vis Sci, 50:4949-4956; Morales SA et al., 2007 Exp Eye Res, 85:790-798; Morales SA et al., 2009 Invest Ophthalmol Vis Sci, 50:462-469; Morales SA et al., 2011 Invest Ophthalmol Vis Sci, 52:5465-5472; Telander DG et al., 2011 Curr Eye Res, 36:546-552).

Finally, just as p53 suppresses cell cycle progression (Levine AJ et al., 1991 Nature, 351:453-456), the results presented herein indicate that p53 is a checkpoint of retinal detachment. In contrast to genetic lesion of the p53 pathway that are present in approximately 50% of solid tumors (Hainaut P and Hollstein M, 2000 Adv Cancer Res, 77:81-137, epigenetic, environmental factors that result in non-canonical activation of PDGFRα drive p53-dependent blinding diseases such as PVR.

**EXAMPLES**

**Example 1: Materials and Methods**

The materials and methods used in the examples described herein are set forth below.

**Major Reagents and Cell Culture**

The phospho-Y742 PDGFRα antibody was raised against the phospho-peptide [KQADTTQpYVPMLDMK (SEQ ID NO: 3), where the lower case "p" represents the
phosphorylated Tyrosine residue] (Lei H et al., 2010 Am J Pathol, 177:132-140). The Ras GTP-activating protein (RasGAP) antibody was crude rabbit antiserum against a GST fusion protein including the SH2-SH3-SH2 region of the human RasGAP (Rosenkranz S et al., 1999 J Biol Chem, 274:28335-28343). Antibodies against PDGFRa, phospho-Akt (S473), Akt, and p53 were purchased from Cell Signaling Technology (Beverly, MA). Secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, and goat anti-mouse immunoglobulin G) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent substrate for detection of horseradish peroxidase was from Pierce Protein Research Products (Rockford, IL). ApoAlert annexin VFITC apoptosis kit and in situ β-galactosidase assay kit were purchased from Clontech Laboratories, Inc. (Mountain View, CA) and Agilent Technologies, (Santa Clara, CA), respectively. Nutlin-3a was purchased from Cayman Chemical (Ann Arbor, MI). Normal rabbit vitreous (RV) was prepared from frozen rabbit eyeballs as previously described (Lei H et al., 2009 J Biol Chem, 284:6329-6336). The level of PDGFs in RV is either very low, or below the level of detection (Lei H et al., 2007 Invest Ophthalmol Vis Sci, 48:2335-2342; Pennock S et al., 2011 Am J Pathol, 179:2931-2940).

RPEM cells are RPE cells derived from a human epiretinal membrane, as previously described (Wong CA et al., 2002 Can J Ophthalmol, 37:211-220). Primary rabbit conjunctival fibroblasts (RCFs) were obtained and cultured as described previously (Ikuno Y et al., 2002 Invest Ophthalmol Vis Sci, 43:2406-2411). RCFs that stably expressed the shRNA targeting vector specific for GFP, PDGFRa, p53 or PDGFRa and p53 were designated sh GFP, sh PDGFRa sh p53 and sh PDGFRa/p53, respectively. F cells are immortalized mouse embryo fibroblasts derived from PDGFR knock-out mice that do not express either of the two PDGFR genes. Fa and Fβ cells are F cells in which PDGFRa or PDGFRβ has been re-expressed (Andrews A et al., 1999 Invest Ophthalmol Vis Sci, 40:2683-2689).

Knockdown of PDGFRa and p53

Oligos (GCCAGCTCTTATTACCTCTA (SEQ ID NO: 4)) for PDGFRa, (CGGGCGTAAACGCTTCGAGAT (SEQ ID NO: 5)) for p53 and (ACAACAGCCCAACGTCTATA (SEQ ID NO: 6)) for GFP in a hairpin-pLKO.1 retroviral vector respectively, the packaging plasmid (pCMVdR8.91), the envelope plasmid (VSV-G/pMD2.G) and 293T packaging cells used. The shRNA lentiviruses were prepared as described previously (Lei H et al., 2011 Mol Cell Biol, 31:1788-1799). The viruses were used to infect RCF cells. Successfully infected cells were selected on the basis of their ability
to proliferate in media containing puromycin (1 μg/ml). The resulting cells were characterized by western blot analysis using antibodies against PDGFRα, p53 and RasGAP (loading control).

**Western Blot**

Cells were grown to 90% confluence in serum-containing medium, and then incubated for 24 hr in medium without serum. Cells were stimulated (as detailed for each experiment), washed twice with ice-cold phosphate buffered saline (PBS), and lysed in extraction buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/ml aprotinin, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride).

Lysates were clarified by centrifugation at 13,000 x g, 4°C for 15 min. Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and then subjected to western blot analysis using indicated antibodies. Signal intensity was determined by densitometry and analyzed with the Quantity One (Bio-Rad) software.

**Collagen I Contraction Assay**

Cells were trypsinized, washed and resuspended in 1.5 mg/ml of neutralized collagen I (INAMED, Fremont, CA) (pH 7.2) at a density of 1 x 10⁶ cells/ml for RPEM or 5 x 10⁴ cells/ml for RCFs. The mixture was aliquoted into wells of a 24-well plate that had been preincubated overnight with 5 mg/ml bovine serum albumin in PBS. The collagen solution was solidified by incubating at 37°C for 90 min, and overlaid with medium containing the desired agents. The media were replaced every day, and the gel diameter was measured on day 3. The gel area was calculated using the formula πr², where r is the radius of the gel.

**Proliferation and Apoptosis Assays**

Proliferation and apoptosis was assayed as previously described (Lei H et al., 2009 J Biol Chem, 284:6329-6336). Briefly, RCFs were seeded into 24-well plates at a density of 50,000 cells/well in DMEM + 10% FBS. After 6 hrs the cells had attached; the medium was aspirated, the cells were rinsed twice with PBS and the cells were cultured in serum-free DMEM with or without RV (1:3 dilution). The media were replaced every day. On Day 3, the cells were counted in a hemocytometer. At least three independent experiments were performed. To monitor apoptosis, RCFs were seeded into 6 cm-dishes at a density of 1 x 10⁵ cells per dish in DMEM + 10% FBS. After the cells had attached the dishes, they were treated as described above in the proliferation Assay. On Day 3, the cells were harvested and stained with FITC-conjugated Annexin V and propidium iodide according to the instructions provided with the apoptosis kit (BD Biosciences, Palo Alto, CA). The cells were analyzed by
flow cytometry in Coulter Beckman XL. At least three independent experiments were performed.

Senescence Assay

RCF cells were plated into a 12-well plate (10,000 cells/well) in DMEM (high glucose) supplemented with 10% FBS. After 6 hrs the medium was changed into DMEM with or without RV (1:3 dilution), and replenished every 24 hrs. On day 3, the β-galactosidase activity was assessed according to the manufacturer's instructions provide with the in situ β-galactosidase assay kit.

Rabbit Model for PVR

PVR was induced in Dutch Belted rabbits, purchased from Covance (Denver, PA), as previously described (Lei H et al., 2009 Invest Ophthalmol Vis Sci, 50:3394-3403). Briefly, a gas vitrectomy was performed by injecting 0.1 ml of perfluoropropane (C3F8) (Alcon, Fort Worth, TX) into vitreous. One week later, the right eye of rabbits was injected in one of two ways. For the experiment injecting RCFs expressing shRNAs, 0.1 ml of DMEM containing $1 \times 10^5$ RCFs that were modified as outlined in the legend were injected along with 0.1 ml rabbit platelet-rich plasma. For the Nutlin-3a experiment, all rabbits were injected with 0.1 ml of DMEM containing $1 \times 10^5$ unmodified RCFs, 0.1 ml rabbit platelet-rich plasma and either not injected a third time, or injected with vehicle, or 0.1 ml of 200 μM Nutlin-3a. The vehicle or Nutlin-3a injection was repeated on day 3 and 5. The retinal status was evaluated with an indirect ophthalmoscope fitted with a +30 D fundus lens on day 1, 3, 5, 7, 14, 21 and 28. PVR was graded according to the Fastenberg scale of classification 41: stage 0, no disease; stage 1, epiretinal membrane; stage 2, vitreoretinal traction without retinal detachment; stage 3, localized retinal detachment (1-2 quadrants); stage 4, extensive retinal detachment (2-4 quadrants without complete detachment); stage 5, complete retinal detachment. On day 28, animals were sacrificed, and eyes were enucleated and frozen at -80°C.

Immunohistochemistry

Rabbit eyeballs were fixed in 10% formalin for 48 hr and embedded in paraffin after dehydration. Subsequently, 4 μm paraffin sections were prepared, dewaxed in xylene and rehydrated in ethanol, diluted ethanol and deionized water. Antigen retrieval that was performed by boiling the slides for 20 min in a citrate-based buffer (Vector Laboratories Inc., Burlingame, CA). The endogenous peroxidase activity was blocked by incubation with 1% H202 in methanol for 10 min and the endogenous avidin and biotin binding sites were blocked by incubation with avidin and biotin blocking buffers (Vector Laboratories). The
resulting sections were first incubated in blocking buffer containing 3% goat serum, and then
in primary antibody (diluted 1:200 in blocking buffer, anti-p53 from ABcam [Cambridge,
MA]) overnight at 4°C. Incubation with secondary antibody (biotinylated goat anti-mouse;
ABcam) was for one hour at room temperature. Finally, the ABC reagent (Vector
Laboratories) was added for 45 min and the sections were stained with DAB (Thermo
Scientific, Rockford, IL). The sections were observed and photographed under a microscope.

Statistics

The experimental data were analyzed using an unpaired t test and one way ANOVA
and/or post tests. A p value of less than 0.05 was considered statistically significant.

Example 2: Suppressing p53 was essential for RV-induced contraction, and retinal
detachment

RV contains a variety of non-PDGFs that indirectly activate PDGFRa and thereby
chronically stimulate Akt (Lei H et al., 2011 Mol Cell Biol, 31:1788-1799), which
phosphorylates and activates Mdm2 (Zhou BP et al., 2001 Nat Cell Biol, 3:973-982) that
mediates a decline in the level of p53 (Ogawara Y et al., 2002 J Biol Chem, 277:21843-
21850; Gottlieb TM, et al., 2002 Oncogene, 21:1299-1303; Haupt Y et al., 1997 Nature,
387:296-299). Nutlin-3a antagonizes the interaction of Mdm2 and p53, and thereby prevents
Mdm2-mediated reduction of p53 (Vassilev LT et al., 2004 Science, 303:844-848). Because
of these properties, it was determined whether Nutlin-3a would prevent RV-mediated
reduction in the level of p53, contraction of cells in collagen gels and protect rabbits from
developing PVR. Primary rabbit conjunctival cells (RCFs) were utilized in these experiments
because they robustly contract collagen gels and induce PVR.

Nutlin-3a effectively blocked the RV-mediated decline in the level of p53 and
contraction of collagen gels (Figure 1). As expected, it had no effect on RV-induced
phosphorylation of PDGFRa or activation of Akt (Figure 1A), events that are upstream of the
known action of Nutlin-3a. The minimum dose to prevent the RV-induced reduction of p53
was 2 micromolar (Figure 8A), whereas the maximum tolerated dose was 30 micromolar
(Figure 8B). Moreover, multiple intravitreal injection of Nutlin-3a of up to 20 micromolar
did not produce overt signs of retinal toxicity (Figure 9, and data not shown). These results
set the stage to test if Nutlin-3a could prevent retinal detachment in an animal model of PVR.

As shown in Figure 2, 100% of the animals in both control groups (uninjected, empty
circles; or injected with vehicle, filled circles) developed complete retinal detachment (stage
5) by day 28. In contrast, 0% of the Nutlin-3a-treated animals (squares in Figure 2A)
succumbed to even partial retinal detachment (stage 3 or 4), although they did form membranes (stage 1 and 2 in Figure 2A; Figure 9). Importantly, the p53 level was higher in epiretinal membranes isolated from Nutlin-3a-injected animals as compared with vehicle-injected controls (Figure 2B). These observations indicate that Nutlin-3a treatment maintained a high level of p53 expression in cells of the epiretinal membrane and prevented retinal detachment.

A molecular approach to assess the importance of reducing p53 for RV-mediated contraction and retinal detachment led to a similar conclusion. The overall strategy of this second approach was to reduce the PVR potential of RCFs by silencing expression of PDGFRα, and then to test if it could be rescued by silencing expression of p53. Lentiviral-mediated delivery of shRNAs directed toward either PDGFRα, or p53 suppressed expression by at least 80% (Figure 3A). As shown in Figure 3B, RV-mediated suppression of p53 in sh PDGFRα cells was substantially reduced, although not completely eliminated. Similarly, RV-induced contraction of cells was largely, although not completely diminished in sh PDGFRα cells (Figure 3C).

To investigate whether the incomplete suppression of RV-mediated contraction was due to the residual expression of PDGFRα, the efficacy of Imatinib to interfere with RV-induced contraction was examined. Imatinib completely blocked RV-induced contraction in sh GFP cells (Figure 10A), which indicated that one of the Imatinib targets (abl, c-kit PDGFRα and PDGFRβ [Druker BJ et al., 2001 N Engl J Med, 344: 1031-1037] was essential. The previously characterized panel of cells that do or do not express PDGFRs (Andrews A et al., 1999 Invest Ophthalmol Vis Sci, 40:2683-2689) provided the opportunity to identify the relevant Imatinib target. F cells, which are immortalized fibroblasts from mice lacking both PDGFR genes (but harboring all other Imatinib targets) contracted weakly to RV, and this response was unaffected by Imatinib (Figure 10B). Expressing PDGFRβ in these cells did not improve RV-induced contraction or generate sensitivity to Imatinib (Figure 10B). Finally, expression of PDGFRα improved this RV-stimulated response, which was erased by Imatinib (Figure 10B). These observations indicate that PDGFRα was the relevant target of Imatinib, and that the modest RV-induced contraction seen in sh PDGFRα cells (Figure 3C) was due to residual expression of PDGFRα.

As expected from previous studies assessing the importance of PDGFRα for experimental PVR (Andrews A et al., 1999 Invest Ophthalmol Vis Sci, 40:2683-2689; Lei H et al, 2009 Invest Ophthalmol Vis Sci, 50:3394-3403; Ikuno Y et al., 2000 Invest Ophthalmol Vis Sci, 41:3107-3116), there was a significantly statistic reduction in the PVR potential of sh
PDGFRα cells (Figure 4). The sh PDGFRα cells failed to induce retinal detachment, although they retained their ability to form membranes, which exerted traction of the retina (Figure 4; compare empty and filled circles). Molecularly suppressing p53 in sh PDGFRα cells fully restored their ability to induce retinal detachment (Figure 4; compare filled circles with squares). Thus, two different experimental approaches indicated that reducing the level of p53 was essential for RV-mediated contraction and retinal detachment in an animal model of PVR.

Example 3: PDGFRα did more than reduce p53 to promote contraction

To assess if reducing p53 was the only PDGFRα-mediated event required for contraction in response to RV, this outcome was compared in sh GFP and sh p53 cells. If it was, then contraction of sh 53 cells would be RV-independent. As shown in Figure 11, this was not the case. While basal contraction of sh 53 cells was enhanced, they responded well to RV. These observations indicated that PDGFRα did more than reduce the level of p53 in order to mediate RV-dependent contraction.

The same question was addressed for 3 additional cellular responses associated with PVR, proliferation and protection from apoptosis and senescence. Like contraction, proliferation of sh p53 cells was responsive to RV (Figure 5A), whereas protection from apoptosis and senescence were fully engaged in unstimulated cells and RV did not enhance these responses further (Figure 5B and C). These findings indicate that reducing p53 was sufficient to trigger some of the RV-stimulated cellular responses that are associated with PVR (protection from apoptosis and senescence), whereas contraction and proliferation required an event(s) in addition to reducing the level of p53 (Figure 6).

Residual expression of PDGFRα in shPDGFRα cells provided an opportunity to compare cellular responses associated with PVR for their dependence on the level of expression of PDGFRα. RV was unable to promote proliferation of sh PDGFRα cells or protect them from apoptosis or senescence (Figure 5), which indicated that these responses required expression of PDGFRα in excess of 20% of the control level. In contrast, contraction remained responsive to RV (albeit weakly) in sh PDGFRα cells (Figure 3C).

Thus, contraction required less PDGFRα expression than did proliferation or protection from apoptosis and senescence.

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Example 4: Relevance to clinical PVR

To begin to assess the clinical relevance of these findings the impact of Nutlin-3a on HV-mediated signaling events and contraction of RPE cells isolated from a human PVR membrane was considered. As shown in Figure 7A, Nutlin-3a prevented the precipitous fall in the level of p53 observed in HV-treated control cells, without impacting upstream signaling events. Furthermore, Nutlin-3a inhibited HV-stimulated contraction of RPE-containing collagen gels (Figure 7B). In addition, p53 was undetectable in epiretinal membranes from PVR patients (Figure 12). These findings indicate that Nutlin-3a had the potential to protect patients from developing PVR.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.
What is claimed is:

1. A composition comprising an agent that
   a. inhibits an intra-ocular reduction of the level of p53 associated with proliferative
      vitreoretinopathy (PVR), and/or
   b. prevents p53 from interacting with human double min 2 (Hdm2),

wherein said composition is for inhibiting or reducing the severity of PVR in a subject
suffering from or at risk of developing PVR.

2. The composition of claim 1, wherein said agent comprises Nutlin-3a
   (RG7 112/RO5045337) or an analog thereof.

3. The composition of claim 1, wherein said agent comprises a small molecule.

4. The composition of claim 1, wherein said agent comprises a polynucleotide, a
   polypeptide, or an antibody.

5. The composition of claim 2, wherein said Nutlin-3a, or analog thereof, is administered at
   a concentration of 0.1 μM, 0.5 μM, 1.0 μM, 2.0 μM, 5 μM, 10 μM, 20 μM, 30 μM, or 50
   μM.

6. The composition of claim 2, wherein said Nutlin-3a, or analog thereof, is present in a
   concentration of 0.1-10% (mg/ml).

7. The composition of claim 1, wherein said composition is administered intravitreally or
   subconjunctivally.

8. The composition of claim 1, wherein said reduction is a platelet-derived growth factor
   receptor a (PDGFRα)-mediated reduction.

9. The composition of claim 1, wherein said subject suffering from or at risk of developing
   PVR has undergone rhegmatogenous retinal detachment surgery.

10. The composition of claim 1, wherein administration of said composition prevents retinal
    detachment in said subject.

11. The composition of claim 1, wherein administration of said composition reduces the
    formation of epiretinal membranes in said subject.
12. The composition of claim 1, wherein administration of said composition inhibits the contraction of retinal pigment epithelial (RPE) cells in said subject.

13. The composition of claim 1, wherein said composition is administered every 48 hours, every 24 hours, every 12 hours, or every 6 hours.

14. The composition of claim 1, wherein said composition is administered for 1 day, 3 days, 7 days, 14 days, 30 days, 60 days, 90 days, 120 days, or 365 days.

15. The composition of claim 1, wherein said composition further comprises a pharmaceutically acceptable carrier.

16. The composition of claim 1, wherein the form of said composition is a solid, a paste, an ointment, a gel, a liquid, an aerosol, a mist, a polymer, a film, an emulsion, or a suspension.

17. The composition of claim 1, wherein said subject is a human.

18. The composition of claim 1, wherein said subject has not been diagnosed with a retinoblastoma.

19. The composition of claim 1, wherein the subject is older than 24 months of age.

20. The composition of claim 1, wherein said agent is Nutlin-3a and the structure of said Nutlin-3a comprises
21. A pharmaceutical composition comprising a Nutlin-3a compound, or analog thereof, and an ophthalmic excipient for inhibiting or reducing the severity of proliferative vitreoretinopathy (PVR).

22. A method for inhibiting or reducing the severity of proliferative vitreoretinopathy (PVR) comprising:

identifying a subject suffering from or at risk of developing PVR; and
administering a composition comprising an agent that reduces the intra-ocular reduction of the level of p53, thereby inhibiting or reducing the severity of PVR.

23. A method for inhibiting or reducing the severity of proliferative vitreoretinopathy (PVR) comprising:

identifying a subject suffering from or at risk of developing PVR; and
administering a composition comprising an agent that prevents p53 from interacting with human double min 2 (Hdm2).

24. The method of claim 23 or 24, wherein said agent that inhibits the intra-ocular reduction of the level of p53 is a polynucleotide, a polypeptide, an antibody, or a small molecule.

25. The method of claim 23 or 24, wherein said small molecule is Nutlin-3a (RG7112/RO5045337), or an analog thereof.

26. The method of claim 25, wherein said Nutlin-3a, or analog thereof, is administered at a concentration of 0.1 µM, 0.5 µM, 1.0 µM, 2.0 µM, 5 µM, 10 µM, 20 µM, 30 µM, or 50 µM.

27. The method of claim 25, wherein said Nutlin-3a, or analog thereof, is present in a concentration of 0.1-10% (mg/ml).

28. The method of claim 23 or 24, wherein said composition is administered intravitreally or subconjunctivally.

29. The method of claim 23 or 24, wherein said reduction is a platelet-derived growth factor receptor a (PDGFRa)-mediated reduction.
30. The method of claim 23 or 24, wherein said subject suffering from or at risk of developing PVR has undergone rhegmatogenous retinal detachment surgery.

31. The method of claim 23 or 24, wherein the subject is older than 24 months of age.

32. The method of claim 23 or 24, wherein said administration prevents retinal detachment in said subject.

33. The method of claim 23 or 24, wherein said administration reduces the formation of epiretinal membranes in said subject.

34. The method of claim 23 or 24, wherein said administration inhibits the contraction of retinal pigment epithelial (RPE) cells in said subject.

35. The method of claim 23 or 24, wherein said composition is administered every 48 hours, every 24 hours, every 12 hours, or every 6 hours.

36. The method of claim 23 or 24, wherein said composition is administered for 1 day, 3 days, 7 days, 14 days, 30 days, 60 days, 90 days, 120 days, or 365 days.

37. The method of claim 23 or 24, wherein said composition further comprises a pharmaceutically acceptable carrier.

38. The method of claim 23 or 24, wherein the form of said composition is a solid, a paste, an ointment, a gel, a liquid, an aerosol, a mist, a polymer, a film, an emulsion, or a suspension.

39. The method of claim 23 or 24, wherein said subject is a human.

40. The method of claim 23 or 24, wherein said subject has not been diagnosed with a retinoblastoma.
41. The method of claim 23 or 24, wherein the agent is Nutlin-3a, and wherein the structure of said Nutlin-3a comprises

![Chemical structure](image)

42. A pharmaceutical composition comprising a Nutlin-3a compound, or analog thereof, and an ophthalmic excipient.
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<th>Vehicle</th>
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<tr>
<td>RV</td>
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**FIG. 1A**

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<th></th>
<th>Vehicle</th>
<th>Nutlin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gel area (mm²)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 1B**
FIG. 2A

FIG. 2B
FIG. 3A

FIG. 3B
FIG. 3C

Collagen gel area (mm²)

- 180
- 160
- 140
- 120
- 100
- 80
- 60
- 40
- 20
- 0

RV

-  sh GFP
-  +

contraction (%): 67.7

-  sh PDGFRα
-  +

contraction (%): 13.8

-  sh PDGFRα/p53
-  +

contraction (%): 55.9

FIG. 4

PVR (Fastenberg scale)

-  5
-  4
-  3
-  2
-  1
-  0

Day

1  3  5  7  14  21  28

sh GFP
sh PDGFRα
sh PDGFRα/p53
FIG. 5A
FIG. 6

non-PDGFs

ROS/SFKs

Reducing p53

Protection from apoptosis and senescence

#1 + #2

Contraction Proliferation

?
**Fig. 7A**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Nutlin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-PDGFRα</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-Akt</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Akt</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>p53</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RasGAP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**Fig. 7B**

```
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<th>Vehicle</th>
<th>Nutlin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-PDGFRα</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-Akt</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Akt</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>p53</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RasGAP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.95</td>
</tr>
</tbody>
</table>
```

* The asterisks (*) indicate significant differences.
### FIG. 8A

<table>
<thead>
<tr>
<th>Nutlin-3 RV</th>
<th>-</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>10.0 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-PDGFRα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>PDGFRα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>p-Akt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RasGAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold</td>
<td>1.0</td>
<td>0.26</td>
<td>0.28</td>
<td>0.30</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

### FIG. 8B

- **Vehicle**
- **5 μM**
- **10 μM**
- **20 μM**
- **30 μM**
- **50 μM**
FIG. 10A

FIG. 10B
FIG. 11A

<table>
<thead>
<tr>
<th></th>
<th>sh GFP</th>
<th>sh PDGFRα</th>
<th>sh PDGFRα/p53</th>
<th>sh p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>RasGAP</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PDGFRα/RasGAP:</td>
<td>1.00</td>
<td>0.18</td>
<td>0.18</td>
<td>1.00</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53/RasGAP:</td>
<td>1.00</td>
<td>0.18</td>
<td>0.19</td>
<td>0.19</td>
</tr>
</tbody>
</table>

FIG. 11B

<table>
<thead>
<tr>
<th></th>
<th>sh GFP</th>
<th>sh PDGFRα</th>
<th>sh PDGFRα/p53</th>
<th>sh p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RasGAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53/RasGAP:</td>
<td>1.00</td>
<td>0.23</td>
<td>1.00</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>0.18</td>
<td>0.24</td>
<td>0.16</td>
</tr>
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</table>

FIG. 11C

<table>
<thead>
<tr>
<th></th>
<th>sh GFP</th>
<th>sh PDGFRα</th>
<th>sh PDGFRα/p53</th>
<th>sh p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Contraction (%)</td>
<td>67.5</td>
<td>13.1</td>
<td>55.8</td>
<td>63.8</td>
</tr>
</tbody>
</table>

Gel area (mm²)

* NS
<table>
<thead>
<tr>
<th>Box No. 1</th>
<th>Nucleotide and/or amino acid sequence(s) (Continuation of item 1c of the first sheet)</th>
</tr>
</thead>
</table>

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)
      - [ ] on paper
      - [x] in electronic form

   b. (time)
      - [x] in the international application as filed
      - [ ] together with the international application in electronic form
      - [ ] subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Form PCT/ISA/210 (continuation of first sheet (1)) (July 2009)
A. CLASSIFICATION OF SUBJECT MATTER
IPC (8) - A61K 31/496, 31/4164; A61P 27/02 (2013.01)
USPC - 514/254.07, 20.8; 424/9.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/496, 31/497, 31/4164, 31/517; A61P 27/02(201) 3.01
USPC - 514/254.07, 254.05, 254.01 , 252.13, 252.12, 252.1, 247, 183, 1.1, 1, 20.8, 19.2, 19.3; 4249.1 , 9.2

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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<tbody>
<tr>
<td>Y</td>
<td>PENNOCK, S et al. A Novel Strategy To Develop Therapeutic Approaches To Prevent Proliferative Vitreoretinopathy. The American Journal of Pathology. 28 October 2011, Vol. 179, pp 2931-2940; page 2932, Table 1; page 2932, left column, second paragraph to right column, first paragraph, Growth Factors, Antibodies, and Neutralization Reagents section; page 2936, figure 3; page 2936, left column, first to second paragraph. DOI: 10.1016/j.ajpath.2011.08.043</td>
<td>4, 8-12, 19, 29/22, 29/23, 30/22, 30/23, 31/22, 31/23, 32/22, 32/23, 33/22, 33/23, 34/22, 34/23</td>
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</tbody>
</table>

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier application or patent but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 17 December 2013 (17.12.2013)

Date of mailing of the international search report: 0 3 JAN 2014

Name and mailing address of the ISA/US: Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer: Shane Thomas
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774
<table>
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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
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<td>Y</td>
<td>LEI, H et al. Pathological Signaling Via Platelet-Derived Growth Factor Receptor a Involves Chronic Activation of Akt And Suppression Of p53. Molecular and Cellular Biology. 28 February 2011, Vol 31, pp 1788-1799; page 1792, right column, second paragraph to third paragraph; page 1795, figure 5. DOI: 10.128/MCB.01321-10</td>
<td>8, 29/22, 29/23</td>
</tr>
<tr>
<td>A</td>
<td>US 2010/0239499 A 1 (DRENSER, K) September 23, 2010; abstract; Claims 14, 15, 22</td>
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