VITAMIN D COMPOUNDS AND METHODS FOR REDUCING OCULAR HYPERTENSION (OHT)

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Field of Classification Search
CPC ... A61K 31/59; A61K 31/592; A61K 31/593 See application file for complete search history.

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ABSTRACT

The present invention relates to compounds and methods for reducing intraocular pressure and treating ocular hypertension in a subject.

9 Claims, 17 Drawing Sheets
(56) References Cited

OTHER PUBLICATIONS


* cited by examiner
Fig. 1

IOP (mean=8) of 5ul PG in one eye vs. nontreated eye

*different from d1 baseline

13%±4%

15%±5%

IOP Prop

IOP no trt
Fig. 2A

IOP (Mean=8) 5 topical applications of a 5ug 1,25 in 5ui PG in OD eye vs. vehicle (propylene glycol = PG) in OS eye

29% ± 8%

* * different from d1 baseline

$ different from opposite eye corrected

corrected for d1 BL
IOP (Mean=5) 5 topical applications of a 5μg 1,25 in 5μl propylene glycol in OD eye vs. vehicle (propylene glycol) in OS eye

* Different from d1 baseline
$ Different from opposite eye corrected for d1 BL

Fig. 2B
IOP (mean = 4) 5 topical applications of a 5μg AGR in 5μl propylene glycol in OS eye
vs. vehicle (5μl propylene glycol) in OD eye

* different from d1 baseline

Fig. 3
Mean IOP (n=8) 5 topical applications of a 6µg 2MD in 5µl propylene glycol vs. vehicle (5µl propylene glycol)

*different from d1 baseline

Fig. 4
Mean (n=4) serum Ca^{2+} Level in monkeys during 5 topical applications of a 5μl propylene glycol in OS eye vs. untreated OD eye

Fig. 5
Mean (n=8) serum Ca2+ level in monkeys during 5 topical applications of a 5µg 1,25-Dihydroxyvitamin D3 in 5µl propylene glycol in one eye vs. 5ml propylene glycol

Fig. 6
Mean serum Ca2+ Level (n=4) in monkeys after 5th topical application of a 5µg AGR in 5µl propylene glycol in OS vs. 5ml propylene glycol in OD eye
Mean (n=8) serum Ca2+ Level in monkeys after 5th topical application of a 6µg 2MD in 5µl propylene glycol in OS vs. 5ml propylene glycol in OD

Fig. 8
% of IOP reduction (the highest, statistically significant) in the treated eye of cynomolgus monkeys (n=6-8) within 8 hour period after the 5th topical application of 1,25 in Propylene Glycol (2 doses/day)

Fig. 9A
% of IOP reduction (the highest) in the treated eye of cynomolgus monkeys (n=4) within 8 hours period after the 5th topical application of VitD analog in Propylene Glycol (2 doses/day) (purple bars= compds listed in Ichih's patent)

VitD analog in 5 unilateral topical applications treatment
% of IOP reduction (the highest) in the treated eye of cynomolgus monkeys (n=4) within 8 hours period after the 5th topical application of ViTD analog in Propylene Glycol (2 doses/day) after subraction of PG (purple bars = compds listed in Itoh's patent)

<table>
<thead>
<tr>
<th>% of IOP reduction</th>
<th>Pr-OH</th>
<th>1,25 (5 µg)</th>
<th>1,25 (5,5 µg)</th>
<th>2M (5 µg)</th>
<th>2M (5,5 µg)</th>
<th>AGR (5 µg)</th>
<th>ZP (5 µg)</th>
<th>25R-2MD (5 µg)</th>
<th>25R-2HDP (5 µg)</th>
<th>25R-2HDP (5 µg)(n=0)</th>
<th>VHRD (10 µg)</th>
<th>1α-CH3(OH)D3 (5 µg)</th>
<th>25R-CH3(OH)D3</th>
</tr>
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<tbody>
<tr>
<td></td>
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</tbody>
</table>

NB: low original IOP

ViTD analog in 5 unilateral topical applications treatment

n=8

n=8

n=8

n=3
Fig. 10A
FIG. 10B
Fig. 11B
Fig. 12
VITAMIN D COMPOUNDS AND METHODS FOR REDUCING OCULAR HYPERTENSION (OHT)

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/035,192, filed Mar. 10, 2008, the entirety of which is hereby incorporated by reference for all purposes.

STATEMENT OF FEDERALLY SPONSORED RESEARCH

Not Applicable.

FIELD OF THE INVENTION

The present invention relates to compounds and methods for reducing intraocular pressure and treating ocular hypertension in a subject.

BACKGROUND OF THE INVENTION

Elevated intraocular pressure (IOP) is a component in at least two visual system disorders. The first disorder is primary open angle glaucoma (POAG), which combines elevated IOP with a progressive optic neuropathy and results in characteristic excavation of the optic nerve head and corresponding visual field defects. The second disorder is ocular hypertension (OHT), in which IOP is elevated but no glaucomatous damage to the optic nerve head is observed and the detectable visual field does not change. Elevated IOP is a critical risk factor in the development of glaucomatous optic neuropathy [Armaly, 1980] and other visual field disorders. For example, between 4% [Kass, 2002] and 20% [Ontoso, 1997] of people with OHT will develop visual field defects within five years.

Although elevated IOP is a component in POAG, some other forms of glaucoma do not involve elevated IOP. Normal tension glaucoma (NTG) is a clinical entity characterized by similar damage of the optic nerve head and similar visual field defects, but without an elevated IOP. POAG is arbitrarily distinguished from NTG using a cut-off point of IOP of 21 mmHg [Vass, 2007].

Ocular hypertension is the strongest known risk factor for POAG. Intraocular pressure (IOP) is determined by aqueous production in the eye (α and β-adrenergic blockers, carbonic anhydrase inhibitors (CAis), cholinergics and prostaglandin (PG) compounds). The IOP is lowered either by decreasing the production of aqueous humor in the eye (α- and β-adrenergic blockers, carbonic anhydrase inhibitors and Na+/K+-ATPase inhibitors) or by improving its outflow either through the conventional pathway (through the canal of Schlemm such as cholinergics, MMP activators and protein kinase inhibitors) or through the uveoscleral outflow pathway (PGs) [Clark, 2003; Institute, 2006; Orihashi, 2005; Marquis, 2005]. Over the course of time, most patients will use more than one medication, singly and in varying combinations, experimenting with differing classes of compounds with varying mechanisms of action. All of the above-mentioned treatment agents have one or more serious and undesirable side effects [Kaufman, 2006].

Therefore, a need exists for a new class of effective IOP-lowering compounds which have minimal or beneficial side effects. The search for new, more effective and more selective compounds with fewer side effects for the treatment of ocular hypertension and glaucoma may also contribute to understanding the molecular mechanisms involved in the regulation of intraocular pressure.

SUMMARY OF THE INVENTION

The present invention provides a method of reducing ocular hypertension in a subject, the method comprising administering to at least one eye a therapeutically effective amount of a vitamin D compound according to the following formula:

(Formula 1)
methods of the present invention provide many advantages. Further, the compounds do not have the serious and unexpected side effects common in conventional ocular hypertension treatments. Further still, the compounds of the present invention are stable and easily formulated.

In a preferred version, the compound of the present invention is administered in an amount ranging from about 0.2 µg to about 1 mg per day as a topical preparation, such as eye drops.

In a further preferred version, the vitamin D compound is selected from the group consisting of 1α,25-dihydroxyvitamin D3 (E- and Z-isomers); 26-homo-1α,25-dihydroxyvitamin D3; 26,27-Dimethyl-1α,25-dihydroxyvitamin D3; or 25-hydroxyvitamin D3.

A kit is also provided, comprising a compound according to Formula 1, and instructions for use. The compounds and methods of the present invention provide many advantages. For instance, the compounds can be used to reduce ocular hypertension in both eyes while only treating one eye. Further, the compounds do not have the serious and unexpected side effects common in conventional ocular hypertension treatments. Further still, the compounds of the present invention are stable and easily formulated.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1** depicts an IOP response (mean=8) after the 5th dose of twice daily treatments with 5 µl propylene glycol in one eye vs. the untreated opposite eye.

**FIG. 2** depicts an IOP response after the 5th dose of twice daily treatments with 5 µg 1,25-Dihydroxyvitamin D3 in 5 µl propylene glycol in one eye and vehicle (5 µl propylene glycol) in the opposite eye.

**FIG. 3** depicts an IOP response after the 5th dose of twice daily treatments with 6 µg 2MD in 5 µl propylene glycol in one eye and vehicle (5 µl propylene glycol) in the opposite eye.

**FIG. 5** depicts a mean serum Ca²⁺ Level (n=4) in monkeys during 5 topical applications of 5 µl propylene glycol in one eye vs. the untreated opposite eye.

**FIG. 6** depicts a mean serum Ca²⁺ Level (n=8) in monkeys during topical applications #1-5 of 5 µg 1,25-Dihydroxyvitamin D3 in 5 µl propylene glycol in one eye vs. 5 µl propylene glycol in the opposite eye.

**FIG. 7** depicts a mean serum Ca²⁺ Level (n=4) in monkeys after the 5th topical application of 5 µg AGR in 5 µl propylene glycol in one eye vs. 5 µl propylene glycol in the opposite eye.

**FIG. 8** depicts a mean serum Ca²⁺ Level (n=8) in monkeys after the 5th topical application of 6 µg 2MD in 5 µl propylene glycol in one eye vs. 5 µl propylene glycol in the opposite eye.

**FIG. 9A** depicts the dose dependence of the IOP decrease after 5 topical unilateral applications of 1α,25-dihydroxyvitamin D3 in monkey eye. **FIG. 9B** is a bar graph depicting the percentage of IOP reduction after application of different vitamin D compounds. **FIG. 9C** is a bar graph indicating the percentage of IOP reduction after application of different vitamin D compounds after subtracting the propylene glycol (PG) effect (10%). If a deltaIOP is negative, the treatment has decreased IOP which is the desired effect.

**FIG. 10** depicts the expression of representative genes modulated by vitamin D that are involved in the regulation of IOP (rat intestine data (filled bars), mouse calvarial cells data (open bars). **FIG. 10A** shows genes down-regulated by 1,25-(OH)2D3: CA I, carbonic anhydrase I; ACE, angiotensin I-converting enzyme; ACTA1, actin alpha 1; ACTG2, actin gamma 2; ATP1A1, Na+/K+ transporting ATPase, alpha 1 polypeptide; AQP1, aquaporin 1; CEACAM1, CEA-related cell adhesion molecule 1; FN1, fibronectin 1; CD44, Hyaluronate receptor or cell adhesion molecule (CD44) and TIMP3, tissue inhibitor of metalloproteinases 3. **FIG. 10B** shows genes up-regulated by 1,25-(OH)2D3: PGER4, pros-taglandin E receptor subtype 4; MMP3, MMP11, MMP13, MMP14, matrix metalloproteinases 3, 11, 13, 14. The fold change is the average of 2-3 microarray experiments.

**FIG. 11** depicts IOP-changes, % (mean±SEM) in (A) Control (vehicle, 5 µl propylene glycol) and (B) vitamin D (1,25-(OH)2D3 in 5 µl of propylene glycol) treated eyes of normotensive cynomolgus monkeys after unilateral topical administration of 0.1 µg (triangles, n=7); 1 µg (squares, n=7) and 5 µg (circles, n=8) 1,25-(OH)2D3 (see Example 3). Pretreatment IOP on day 1 (dl) (mean±SEM) was 17.5±0.5 in to-be-0.1 µl vitamin D-treated eyes and 17.0±0.5 in to-be-control eyes; 19±0.9 in to-be-1 µg vitamin D-treated eyes and 20.0±0.9 in to-be-control eyes; 19.2±0.7 mmHg in to-be-5 µg vitamin D-treated eyes 18.5±0.9 mmHg in to-be-control eyes. (#) (*) Significantly different from respective day one baseline for 1 µg or 5 µg respectively 1,25-(OH)2D3 treatment experiment by the two-tailed paired t-test (p<0.05), d.—day.

**FIG. 12** depicts outflow facility ratios plotted against time (Mean±SEM, n=8, combined groups A and B, see Table 2) measured within 30 min intervals (normalized with respect to an initial 90 min baseline). Open squares correspond to perfusion with vehicle (propylene glycol) in Control eye and solid dots correspond to perfusion with vitamin D (1,25-(OH)2D3 in propylene glycol) in contralateral eye of normotensive cynomolgus monkeys (see Example 3). No significant difference was found between eyes when the data for the entire 90 minutes period was analyzed or when 30
minutes increments were analyzed. Significantly different from 1.0 by the two-tailed paired t-test: *p<0.05.

DETAILED DESCRIPTION OF THE INVENTION

I. In General

In the specification and in the claims, the terms “including” and “comprising” are open-ended terms and should be interpreted to mean “including, but not limited to...”. These terms encompass the more restrictive terms “consisting essentially of” and “consisting of.”

As used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. As well, the terms “a” (or “an”), “one or more” and “at least one” can be used interchangeably herein. It is also to be noted that the terms “comprising,” “including,” “characterized by,” and “having” can be used interchangeably.

Abbreviations used for Vitamin D Compounds are as follows: PrGl represents Propylene Glycol; 1,25 represents 1α,25-dihydroxyvitamin D₃; 2MD represents 2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D₃; AGR represents 2-(3'-hydroxypropylidene)-19-nor-1α,25-dihydroxyvitamin D₃ (E-isomer); BH represents 2-methylene-19-nor-(20S)-1-hydroxy-bishomopregnacalciferol (2MbisP); 20R-2MD represents 2-methylene-19-nor-(20R)-1α,25-dihydroxyvitamin D₃; ZP represents 1α,25-dihydroxy-19-nor-vitamin D₂ (Zemplar or Paricalcitol); E or Z represents 17-20 dehydro-2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D₃ (E- or Z-isomers); 26-Homo represents 26-homo-1α,25-dihydroxyvitamin D₃; 26,27-Diethyl represents 26,27-Dimethyl-1α,25-dihydroxyvitamin D₃; VitD₃ represents Vitamin D₃; 1α-(OH)D₃ represents 1α-hydroxyvitamin D₃; and 25(OH)D₃ represents 25-hydroxyvitamin D₃.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications and patents specifically mentioned herein are incorporated by reference in their entirety for all purposes including describing and disclosing the chemicals, instruments, statistical analyses and methodologies which are reported in the publications which might be used in connection with the invention. All references cited in this specification are to be taken as indicative of the level of skill in the art. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

II. The Invention

Since the beginning of the last century, vitamin D has been established as the primary regulator of calcium and phosphorous homeostasis in mammals and the major compound for prevention and treatment of rickets. Decades of research has revealed that vitamin D (its hormonal form, 1α,25-dihydroxyvitamin D₃ or 1,25-(OH)₂D₃) is able to prevent and cure a broad spectrum of diseases including cancers, diabetes, autoimmune diseases, hypertension and more [DeLuca, 2008]. However, here we show for the first time that vitamin D (1,25-(OH)₂D₃) is a very powerful and promising compound for reducing ocular hypertension. As described below, unilateral topical application of vitamin D to the eye greatly reduces the intraocular pressure in both treated and control eyes, thus exhibiting an unprecedentedly strong bilateral hypotensive effect without changing the aqueous humor formation or drainage rates of the eye.

The present invention is a method of reducing ocular hypertension (OHT) in a patient, the method comprising administering to at least one eye of subject exhibiting an elevated ocular pressure in at least one eye a therapeutically effective amount of a vitamin D compound of the following formula:

wherein R is

wherein R₁ and R₂ are H, methyl or 3'-hydroxypropylidene, or taken together as =CH₂ or methylene; wherein R₃ and R₄ are selected from H, alkyl (1-3 carbons), alkoxy, and can be the same or different from each other; wherein X is a hydroxyl or protected hydroxyl group; wherein A is oxygen or carbon, with the proviso that if A is oxygen, then R₄ is absent; and wherein C and D are H or taken together as =CH₂, wherein upon administration with the compound according to Formula I, the intracocular pressure is reduced, preferably by at least 15%.

By “ocular hypertension” we mean intraocular pressure that is consistently higher than normal, typically exceeding 21 mmHg.

By “reducing” we mean reducing the ocular hypertension of the subject by at least 5%, at least 10%, and preferably by at least 15% to 50% per eye.

By reducing the OHT in a subject, the compounds and methods of the present invention provide a novel treatment for glaucoma and other disorders exhibiting an elevated intraocular pressure. For purposes of the present invention, “treating” or “treatment” describes the management and care of a subject for the purpose of combating the disease, condition, or disorder. The terms embrace both preventative, i.e., prophylactic, and palliative treatment. Treating includes the administration of a compound of present invention to
prevent the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

In a preferred embodiment, one would evaluate the success of the treatment described above in several ways. Typically, one would measure the intraocular pressure of the affected eye or eyes and calculate a percentage OHT reduction [Kass, 2002]. By “measure” we mean determine the IOP (“tonometry”) through any method known to the art, including but not limited to digital tonometry (Indentation method), Maklakov tonometer (Impression-Applanation Tonometry), Tonomat instrument (Impression-Applanation Tonometry), Wolfe Tonometer (Indentation tonometer), Goldmann Tonometry (Applanation Tonometry), and/or a non-contact tonometer (Indentation tonometry). Alternatively, one may directly measure changes in vision.

By “subject” we mean mammals and non-mammals. “Mammals” means any member of the class Mammalia including, but not limited to, humans, non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, and swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice, and guinea pigs; and the like. Examples of non-mammals include, but are not limited to, birds, and the like. The term “subject” does not denote a particular age or sex.

By “administering” we mean any means for introducing a colchicines neoglycoside into the body, preferably into the systemic circulation. Examples include but are not limited to oral, buccal, sublingual, pulmonary, transdermal, transmucosal, as well as subcutaneous, intraperitoneal, intravenous, and intramuscular injection.

By “therapeutically effective amount” we mean amount of a compound that, when administered to a subject for treating a disease, is sufficient to effect such treatment for the disease. The “therapeutically effective amount” will vary depending on the compound, the disease state being treated, the severity or the disease treated, the age and relative health of the subject, the route and form of administration, the judgment of the attending medical or veterinary practitioner, and other factors. In a preferred embodiment, a therapeutically effective amount means an amount of vitamin D compound sufficient to reduce ocular hypertension between at least 10% and 50% in each eye. Reducing OHT by at least 20% will slow the progression of glaucoma in most patients suffering from glaucoma. Lowering OHT by at least 20% also produces a 50% protective benefit in patients with ocular hypertension but no optic disc or visual field deterioration [Kass; 2002; Kanner; 2006; National Eye Institute website at www.nei.nih.gov].

By “vitamin D compound” we mean any compound or derivative of the vitamin D formula described above, including 

1α,25-dihydroxyvitamin D 3 (1,25-(OH) 2 D 3 ); 2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D 3 (E-isomer); 1α,25-dihydroxy-19-nor-vitamin D 3 ; 2-methylene-19-nor-(20S)-1-hydroxy-bishomopregnacalciferol; 2-(3‘-hydroxypropylidine)-19-nor-1α,25-dihydroxyvitamin D 3 (E-isomer); 17-20 dehydro-2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D 3 (E- and Z-isomers); 26-homo-1α,25-dihydroxyvitamin D 3 ; 26,27-dimethyl-α,25-dihydroxyvitamin D 3 ; Vitamin D 3 ; 25-hydroxyvitamin D 3 .

The invention also provides a method of preventing glaucoma in a subject at risk of developing glaucoma, comprising administering to at least one eye of the at risk subject a therapeutically effective amount of a vitamin D compound of the following formula:

wherein R 1 and R 2 are H, methyl or 3’-hydroxypropylidine, or taken together as —CH 2 or methylene; wherein R 3 and R 4 are selected from H, alkyl (1-3 carbons), alkoxy, and can be the same or different from each other; wherein X is a hydroxyl or protected hydroxyl group; wherein A is oxygen or carbon, with the proviso that if A is oxygen, then R 4 is absent; and wherein C and D are H or taken together as —CH 2 , wherein after administering the compound of formula I, the subject does not develop glaucoma.

By “glaucoma” we mean an eye disease that damages the optic nerve and impairs vision (sometimes progressing to blindness).

By “at risk for developing” glaucoma we mean any subject with a family history of ocular hypertension, or any subject exhibiting any risk factors of ocular hypertension, including poor eyesight, poor physical health, and the like.

Applicants note that the result of unilocular treatment is a bilateral IOP response. Therefore, treatment may be in both eyes or in either eye and result in successful treatment of OHT. For instance, OHT in a first eye may be reduced by treating either the first eye exhibiting the OHT, or in some cases, by treating only the second eye. In a preferred method, the eye exhibiting OHT is treated with the compounds of the present invention to reduce the OHT by at least 10%.

However, in a situation where a first eye is exhibiting OHT but direct treatment of that eye with the compounds of the present invention is not advisable (because, for instance, the eye is thoroughly bandaged and not receptive to eye drops), the OHT in the first eye may be reduced by treating the second eye (which may or may not be exhibiting OHT) with the compounds of the present inventions. The OHT in the first untreated eye typically experiences approximately 20% less reduction in OHT as compared to the treated eye.
One of skill in the art will understand how to accommodate treatment to compensate for this bilateral response.

Administration and Dose.

The composition of the present invention is intended to therapeutically treat conditions of the eye itself or the tissue surrounding the eye. The composition of the present invention may be incorporated in the topical delivery systems of this invention in therapeutically active amounts, usually in amounts ranging from about 0.2 µg-1 mg per day, preferably 5 µg, most preferably 10 µg (+/-10%) per day.

The composition may be applied to the patient daily. In one preferred embodiment, the composition may be applied to the patient one to two times daily, for each eye to be directly treated. The composition may be applied to the entire surface of the eye in a therapeutically effective amount, the exact amount depending on the factors such as age and general health condition of the patient to whom the composition of the present invention is being administered must be considered. Thus, a patient under age 10 will be therapeutically treated conditions of the eye itself or the tissue present invention which may be less than that used for this invention in therapeutically active amounts, usually in

materials which themselves are available in the art and can readily enable the physician to maximize the treatment regime for a particular patient.

The composition of the present invention can be administered to the eye by known means of administering other medicaments to the eye. For example, the composition, suitably formulated, can be administered in the form of eye drops or with ocular inserts. Suitable formulations may also incorporate standard eye vehicles which are physiologically acceptable to the eye. Such vehicles can be solutions or ointments, as desired. Further, the composition of the present invention can be formulated in unit dosage form with non-active opthalmologically-acceptable carriers well known in the art, or with other active medicaments where treatment of other conditions of the eye, for example, infection, allergy or inflammation, is prescribed.

The term “unit dosage form” as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical diluent, carrier or vehicle. The specifications for the novel unit dosage forms of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitation inherent in the art of compounding such an active material for therapeutic use in humans. Examples of suitable unit dosage forms in accord with this invention are tablets, capsules, ocular inserts, dropperfuls, segregated multiples of any of the foregoing, and other forms as are known in the art. The composition of the present invention may be easily prepared in unit dosage form with the employment of pharmaceutical materials which themselves are available in the art and can be prepared by established procedures.

The composition of the present invention may be applied to eyes without further formulation as eye drops. The composition of the present invention may also be formulated in solutions, ointments, creams, gels, sprays or any other form together with pharmaceutically acceptable carriers for topical application. The composition of the present invention may be also applied alone, in either diluted or concentrated form, without further formulation as a topical pharmaceutical agent. Solutions, i.e., dilute aqueous preparations containing the composition of the present invention and preservatives but without substantial concentrations of thickeners, can be sprayed upon the affected surface as by an aerosol pump. This type of delivery may be of value for treating larger areas, or for use with subjects having trouble administering eye drops.

The composition of the present invention may also be used in a pharmaceutical formulation containing antimicrobials, including antibiotics, antifungals, and other anti-viral compounds, which may complement or supplement the activity of the basic composition. Suitable antibiotics include tetracycline, polymyxin B or other common antibiotics used in topical compositions, especially over-the-counter formulations. Examples of useful antifungals include tolnaftate and micatin. Examples of anti-virals include interferon, either natural or recombinant, as well as nucleoside analogs, e.g., acyclovir. Counter-irritants such as camphor and menthol, drying agents such as benzyl alcohol, resorcinol and phenol, and astringents such as zinc sulfate and tannic acid can also be added to the composition as can other types of agents such as emollients, preservatives, antioxidants, color additives, lubricants or moisturizers.

The composition of the present invention may be prepared in almost any relatively inert topical carrier. Generally, the composition could take several forms, e.g., a polymer, a hydrogel, a cream, a gel, an ointment, a wax and/or a solution, capable of effectively retaining the physiologically active compounds of the present invention. Each of these formulations may contain the composition of the present invention as well as microorganism growth inhibitors (preservatives) and other additives above noted. Many such carriers are routinely used and can be obtained by reference to standard pharmaceutical texts. Examples include polyethylene glycols (PEG), polypropylene glycol copolymers, and some water soluble gels. A preferred carrier is an emulsified cream, but other common carriers such as certain petrolatum or mineral oil-based ointments in which the composition of the present invention is dispersible can be substituted.

Gels, i.e., thickened aqueous polymer or alcoholic solutions, containing the composition of the present invention and stabilizers may be clear and/or colored with suitable dyes. Suitable thickeners may include carboxymethylcellulose, polyvinylpyrrolidone or polyacrylic acid salts. Hydrogels may be used to provide a delayed-release of the physiologically active compounds of the present invention to the eye [Eremeev, 2006].

Ointments employed in practicing the present invention may be prepared utilizing known pharmaceutical techniques with conventional vehicles. For instance, hydrophilic or hydrophobic ointments may also be employed as carriers. However, hydrophobic ointments, such as petrolatum jelly, which are based upon hydrocarbon and wax derivatives may not be as efficacious as the hydrophilic ointments because they may impede penetration into the skin. Hydrophilic ointments such as those based upon propylene glycol, polyalylkene glycols, and the propylene glycol copolymers are therefore preferred for ointment formulations. Propylene glycol, as a base, is preferable to polyethylene glycol. Wax formulations may also be employed in some situations where ease of application is a primary objective.

The composition of this invention can be formulated in any other suitable manner. For example, dicyclonac sodium may be dissolved and added by sterile filtration to a preparation containing sodium chloride, hydroxypropyl methyl cellulose and surfactant. This mixture may then be adjusted to the appropriate pH by known techniques, for example by
the addition of sodium hydroxide. Other methods will be apparent to one skilled in the art.

The composition of the present invention may also contain surfactants and, if desired, adjuvants, including additional medicaments, buffers, antioxidants, toxicity adjusters, preservatives, thickeners or viscosity modifiers, and the like. Additives in the formulation may desirably include sodium chloride, EDTA (disodium edetate), and/or BAK (benzalkonium chloride) or sorbic acid. Additional additives may include antioxidants, fragrance, color, water, preservatives (either antioxidants or antimicrobials), lubricants, moisturizers, or drying agents.

The composition may be formulated as an aqueous suspension. In general, aqueous suspensions suitable for topical ophthalmic administration may be formulated and administered in accordance with techniques familiar to persons skilled in the art. The finished suspensions are preferably stored in opaque or brown containers to protect them from light exposure, and under an inert atmosphere. These aqueous suspensions can be packaged in preservative-free, single-dose non-reclosable containers. This permits a single dose of the medicament to be delivered to the eye as a drop or ribbon, with the container then being discarded after use. Such containers eliminate the potential for preservative-related irritation and sensitization of the corneal epithelium, as has been observed to occur particularly from ophthalmic medicaments containing mercurial preservatives. Multiple dose containers can also be used, if desired, particularly since the relatively low viscosities of the aqueous suspensions of this invention permit constant, accurate dosages to be administered dropwise to the eye as many times each day as necessary.

Aqueous suspensions of the present invention may be formulated so that they retain the same or substantially the same viscosity in the eye that they had prior to administration to the eye. Alternatively, suspensions of the present invention may be formulated so that there is increased gelation upon contact with tear fluid. For instance, when a formulation containing DURASITE™ is administered to the eye at a lower pH, the DURASITE™ system swells upon contact with tears. This gelation or increase in gelation leads to entrapment of the suspended drug particles, thereby extending the residence time of the composition in the eye.

Aqueous solutions used in accordance with this invention may be formulated, for example, in accordance with the procedures set forth in Chapter 83 of Remington’s Pharmaceutical Sciences, 14th Edition, Mack Publishing Company. Such ophthalmic solutions are sterile and may contain a bacteriological preservative to maintain sterility during use. The quaternary ammonium bacteriostats such as benzalkonium chloride are satisfactory for this purpose. An antioxidant may also be employed if desired. By way of example, suitable antioxidants include sodium bisulfite, N-acetylcysteine, and/or sodium metabisulfite. Other water soluble ophthalmically acceptable antioxidants known to the pharmaceutical art.

In one embodiment, the composition of the present invention incorporates insoluble polymers to provide a gel or liquid drops which release the drug over time. The composition may contain water soluble polymers or water insoluble polymers as the suspending agent. Examples of such soluble polymers are cellulose polymers like hydroxypropyl methylcellulose. Water insoluble polymers are preferably crosslinked carboxy-containing polymers. Suitable carboxy-containing polymers for use in the present invention and method for making them are described in U.S. Pat. No. 5,192,535 to Davis et al. which is hereby incorporated by reference and relied upon. These polymer carriers include lightly crosslinked carboxy-containing polymers (such as polycarboxphil), dextran, cellulose derivatives, polyethylene glycol 400 and other polymeric demulcents such as polyvinylpyrrolidone, polysaccharide gels and GELRITE™. A carboxy-containing polymer system such as DURASITE™, containing polycarboxphil, a sustained release topical ophthalmic delivery system that releases the drug at a controlled rate, may also be used.

Aqueous mixtures of this invention may also contain amounts of suspended lightly cross-linked polymer particles ranging from about 0.1% to about 6.5% by weight, and preferably from about 0.5% to about 4.5% by weight, based on the total weight of the aqueous suspension. They will preferably be prepared using pure, sterile water, preferably deionized or distilled, having no physiologically or ophthalmologically harmful constituents, and will be adjusted to a pH of from about 4.0 to about 6.8, and preferably from about 5.5 to about 6.5, using any physiologically and ophthalmologically acceptable pH adjusting acids, bases or buffers, e.g., acids such as acetic, boric, citric, lactic, phosphoric, hydrochloric, or the like, bases such as sodium hydroxide, sodium phosphate, sodium borate, sodium citrate, sodium acetate, sodium lactate, THAM (trishydroxymethylamino methane), or the like and salts and buffers such as citrate/dextrose, sodium bicarbonate, ammonium chloride and mixtures of the aforementioned acids and bases.

When formulating the aqueous suspensions, the osmotic pressure may be adjusted to from about 10 milliosmolar (mOsM) to about 400 mOsM, using appropriate amounts of physiologically and ophthalmologically acceptable salts. Sodium chloride is preferred to approximate physiologic fluid, and amounts of sodium chloride ranging from about 0.01% to about 1% by weight, and preferably from about 0.05% to about 0.45% by weight, based on the total weight of the aqueous suspension, will give osmolalities within the above-stated ranges. Equivalent amounts of one or more salts made up of cations such as potassium, ammonium and the like and anions such as chloride, citrate, ascorbate, borate, phosphate, bicarbonate, sulfate, thiosulfate, bisulfate, sodium bisulfate, ammonium sulfate, and the like can also be used in addition to or instead of sodium chloride to achieve osmolalities within the above-stated ranges. Sugars like mannitol, dextrose, glucose or other polyols may be added to adjust osmolality.

The amounts of insoluble lightly cross-linked polymer particles, the pH, and the osmotic pressure chosen from within the above-stated ranges will be correlated with each other and with the degree of cross-linking to give aqueous suspensions having viscosities ranging from about 500 to about 100,000 centipoise, and preferably from about 5,000 to about 30,000, or about 5,000 to about 20,000 centipoise, as measured at room temperature (about 25°C) using a Brookfield Digital LVT Viscometer equipped with a number 25 spindle and a 13R small sample adapter at 12 rpm. Formulations of the present invention should have a viscosity that is suited for the selected route of administration. Viscosity up to about 30,000=drop. About 30,000 to about 100,000 centipoise is an advantageous viscosity range for ophthalmic administration in ribbon form. When water soluble polymers are used, such as hydroxypropyl methylcellulose, the viscosity will typically be about 10 to about 400 centipoises, more typically about 10 to about 200 centipoises or about 10 to about 25 centipoises.
In one preferred embodiment, the kit comprises a vitamin D compound according to the present invention formulated, delivered and stored for use in physiologic conditions. The following examples are, of course, offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and the following examples and fall within the scope of the appended claims.

**III. EXAMPLES**

The Examples described below show that the vitamin D compounds of the present invention can reduce intraocular pressure (IOP) and ocular hypertension (OHT) in subjects suffering therefrom. These examples provide the basis for the further development of vitamin D compounds for treating and preventing disorders such as glaucoma, Given that vitamin D is the endogenously synthesized “magic pill” or “sunshine” vitamin [Holick, 2008] able to prevent and to cure a number of diseases, its use for the treatment of ocular hypertension and glaucoma may provide other positive, beneficial side effects.

**Example 1**

Treatment of OHT with 1.25 (OH)\(_2\)D\(_3\), AGR and 2MD

The Examples below disclose compounds and methods used to reduce OHT by reducing primate intraocular pressure (IOP).

Materials and Methods.
1.25-Dihydroxyvitamin D\(_3\) (1.25(OH)\(_2\))D\(_3\)), AGR and 2MD compounds were ≥98% pure.

Animals. Anesthesia.
Ocular normotensive adult cynomolgus monkeys (Macaca fascicularis), of either sex, weighing 3-7 kg were anesthetized with i.m. ketamine HCl (3-25 mg/kg, supplemented with 1-10 mg/kg) for IOP and topical drop administration. All monkeys were free of any ocular abnormalities according to slit lamp biomicroscopy at the time measurements were taken. The monkeys were provided by the Primate Center at the University of Wisconsin-Madison. All experiments were done in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Treatments and IOP Measurements.
The intraocular pressure (IOP) was determined with a “minified” Goldmann applanation tonometer [Kaufman, 1980] using HALF AND HALFTM creamer solution (Borden) as the tear film indicator [Croft, 1997] with the monkey lying prone in a head holder and the eyes positioned 4 to 8 cm above the heart. All monkeys were examined by slit-lamp before the first IOP measurement in each protocol. For each eye, two or three IOP measurements were averaged as a baseline.

Under ketamine anesthesia (KETAJECT, Phoenix Pharmaceutical, St. Joseph, Mo. (3-25 mg/kg i.m., supplemented with 1-10 mg/kg i.m. as needed) baseline IOP was determined, usually between 7:30 and 9 am (2 readings, 5 minutes apart; if IOP baseline measurements were not within 2-3 mm Hg of each other, a 3rd reading was taken 5 min later). Baseline IOP was at least 15 mmHg if possible. A baseline blood sample was taken from the femoral artery or vein, or sometimes the brachial vein (1-2 ml). This was allowed to clot and then spun down (3000 rpm for 10 min, Damon/IEC NH-SII centrifuge) and the serum removed and frozen at -20 C for no more than 1 week. Baseline systolic, diastolic and mean arterial pressure as well as heart rate were recorded with the Dinamap monitor from a cuff placed around the arm or the leg.

After baseline measurements, the monkey was placed supine with the eye pointing up and the eyelid held open. A 5 µl drop of test material or vehicle was delivered to opposite eyes. The eyelid was held open for at least 30 sec and the eye was maintained in the upward position for an additional 30 sec. The monkey was then returned to its cage and allowed to wake up. In the afternoon, at least 6 hours after the morning treatment, another treatment was administered. On the second day, dosing was repeated in the am and pm.

On the 3rd day, prior to the 5th treatment, baseline IOP, biomicroscopy and MAP were determined. Preliminary studies indicated an effect was observed after these treatments but not after a single treatment. Following the 5th dose, IOP was measured at 1, 2, 3, 4, 5, 6, 7, 8, 12 (if possible), 24, and sometimes 48 hr. Biomicroscopy was done at 1, 3, 6, 24 and sometimes 48 hr. MAP on day 3 (some protocols) was determined at 1, 2, 3, 4, 5, 6, 7, 8, 24 and sometimes 48 hr if possible. Blood samples after the 5th treatment were collected at 6, 24, and sometimes 48 hr. For some protocols, IOP was also measured prior to each treatment; MAP was measured prior to the morning treatment; blood was collected prior to the afternoon treatment. Subsequent screening protocols will not include the MAP and blood collections and will only measure IOP at baseline and prior to and for 1-6 hr after the 5th treatment.

Data Analysis.
Empirically, we found that approximately 8-10 experiments are required for any drug dose in order to obtain a reliable quantitative, statistically testable estimate of the response. Formal sample size calculations have corroborated this impression, as hereafter described. Generally speaking, we wished to identify mean physiologic responses that were >25% of the baseline value (adjusted for non-drug or non-stimulus-related baseline drift) and >1.5 SD of the mean response. The following standard equation for sample size

\[ n = \frac{2\sigma^2}{\delta^2} \]

where \( \sigma \) is the pooled standard deviation of the response, \( \delta \) is the difference from baseline, and \( n \) is the number of observations required.
calculation was used: $N=2 \times \frac{(Z_{1-0.025} + Z_{1-0.025})^2}{\delta^2}$, where $Z_{1-0.025} = 1.960$ for one-sided and two-sided 5% significance, respectively; $Z_{1-0.025} = 0.841$ or 1.282 for 80% and 90% power, respectively; $\delta$ = population standard deviation; $\sigma$ = the difference (i.e., response) in the parameter being measured ($\delta$ and $\sigma$ must have the same units). From that equation, it was determined that 5.5 experiments were required to detect differences of 1.5 standard deviations in a paired test at a one-sided 5% significance level with 80% power, while 9.3 experiments were needed to detect such a difference at a two-sided 5% significance level with 90% power.

Data are expressed as the mean±s.e.m. Significance was determined by the two-tailed paired t-test for ratios compared to 1.0 or differences compared to 0.0.

Results

Vitamin D Compounds as Ocular Hypotensive Agents (FIG. 1)

The effects of propylene glycol vehicle alone on IOP were compared to diurnal IOP in the untreated opposite eye. Prior to the first treatment, baseline IOP in treated and control eyes was 18.8±1.2 and 19.3±1.3 mmHg, respectively (n=8). Prior to the 5th treatment, there was no difference in IOP in either eye compared to baseline. After the 5th treatment, IOP gradually decreased by 4-15% over the next 8 hr in both eyes. IOP in the treated eye was consistently, but not significantly, less than in the control eye at nearly all time points. The diurnal decline in IOP has been previously reported [Gabelt, 1994]. There was no ocular inflammation at any time-point.

1,25-Dihydroxyvitamin D₃ (FIGS. 2A and 2B).

Prior to the first treatment, baseline IOP in treated and control eyes was 18.9±0.7 and 18.5±0.9 mmHg, respectively (n=8). Prior to the 5th treatment, IOP in both treated and control eye was approximately 1-4 mmHg (~17%, p<0.05, n=8) less than at baseline. After the 5th treatment, IOP continued to decrease by an additional 1-2 mmHg (total reduction of ~20-30%, p<0.02) from hr 1-4 followed by a gradual recovery toward baseline by 24-48 hr. IOP in the treated eye was consistently, but not significantly less than in the control eye at nearly all time points. There was no ocular inflammation at any time-point.

AGR (FIG. 3).

Prior to the first treatment, baseline IOP in treated and control eyes was 16.5±1.3 and 15.9±1.6 mmHg, respectively (n=4). Prior to the 5th treatment, IOP in both treated and control eye was no different than at baseline. After the 5th treatment, IOP was not significantly decreased in the treated eye except at the 3 hr time point. There was no change in IOP in the control eye and no difference between the eyes at any time-point. There was no ocular inflammation at any time-point.

2MD (FIG. 4).

Prior to the first treatment, baseline IOP in treated and control eyes was 18.9±1.0 and 18.8±0.8 mmHg, respectively (n=8). Prior to the 5th treatment, IOP in both treated and control eye was no different than at baseline. After the 5th treatment, IOP gradually decreased over the next 8 hours similar to the diurnal decline seen with vehicle alone. There may have been a small drug effect at 6 and 7 hours. There was no ocular inflammation at any time-point.

Serum Ca²⁺ Level after the Ocular Topical Applications of Vitamin D Compounds

There were no significant serum Ca²⁺ level increase past treatments (FIG. 5-8), indicating that vitamin D₃ compounds probably do not enter into systemic circulation.
mg), a dopamine D_3-preferring receptor agonist, decreased the intraocular pressure (TOP) bilaterally in a dose-dependent manner. The primary site of D_3 receptor-mediated action of 7-OH-DPAT is located on postganglionic sympathetic nerve endings in the ciliary body of rabbit. Suppression of activity of the peripheral sympathetic nervous system plays a role in the suppression of aqueous humor flow by 7-OH-DPAT [Chu, 2000].

Contralateral response was also observed after Selective Laser Trabeculoplasty procedure in treatment of glaucoma patients.

Example 2

Measuring IOP Reduction Following Treatment with Vitamin D Compounds

Monkeys Treatments and Intraocular Pressure (TOP) Measurements.

Adult cynomolgus monkeys (Macaca fascicularis) of either sex were anesthetized with intramuscular ketamine HCl (10 mg/kg initial, 5 mg/kg supplemental). Baseline pretreatment IOP was determined by Goldmann applanation tonometry [Kaufman, 1980] with cream used as a tear film indicator [Croft, 1997]. Two baseline IOP measurements were taken 5 minutes apart. Monkeys were then treated topically with 5 µl of 1,25-dihydroxyvitamin D_3 (1,25-vehicle (propylene glycol) to the opposite eye twice a day 30 minutes and at hours 3 and 6 (24 and 48 hr where possible). Measurement was determined on serum, diluted 1:40 with 1 g/L LaCl_3 (Halloran and DeLuca, 1981) using a 3110 atomic absorption spectrometer (Perkin Elmer, Norwalk, Conn.).

Experimental Design for Rat Microarrays Study.

Vitamin D-deficient rats were given one bolus intravenous dose of 730 ng of 1,25-(OH)_2D_3/kg of body weight in ethanol or ethanol vehicle (control). Rats were anesthetized with isoflurane and decapitated 1, 3, 6, 10 and 24 h after injection of the dose or vehicle. There were three rats in each group for each time point. Blood was collected at the same time for determination of changes in serum calcium concentration. For each rat, the first 15 cm of intestine (duodenum) was removed, slit longitudinally and scraped with a glass slide. The mucosa was placed in a vial with GTC extraction buffer supplemented with 2% of β-mercaptoethanol (PolyATtract System 1000, Promega Corp., Madison, Wis.), homogenized at high speed with PowerGen 700 (Fisher Scientific, Pittsburgh, Pa.), flash frozen in liquid N_2 and stored at −80° C. Experiments were done in duplicate.

Rat mRNA Preparation.

For each time point, Poly(A+) RNA was isolated from pooled homogenized mucosa of three 1,25-(OH)_2D_3/kg vehicle-treated rats. The mRNA was isolated using the PolyATtract System 1000 (Promega Corp., Madison, Wis.). The quality, integrity and quantity of the Poly(A+) RNA were determined by agarose gel electrophoresis, UV absorption spectrophotometry and the use of Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, Calif.).
Cells were plated in the 2x6-well plates (5x10^5 cells/well) and cultured with medium changes performed on days 1 and 4. On day 4 cells on 1 plate were treated with 1,25(OH)_2D_3 (10 nM final concentration). Second plate was used as the control. After 24 h of incubation with 1,25(OH)_2D_3, cells were harvested and total RNA was isolated with Trizol reagent (Invitrogen Life Technologies, Carlsbad, Calif.). The mRNA was further purified using a RNaseasy kit (Qiagen, Chatsworth, Calif.). The quality, integrity and quantity of the total RNA were assessed by agarose gel electrophoresis and UV absorption spectrophotometry. Experiments were done in triplicates.

Microarray Probe Preparation.

Double-stranded cDNA was synthesized from 3 µg of rat polyadenylated poly(A)+ RNA or 13 µg mouse total RNA using the Superscript Choice system (Invitrogen Life Technologies, Carlsbad, Calif.), all according to the Affymetrix Gene Expression manual (Affymetrix, Inc., Santa Clara, Calif.). Following phenol/chloroform extraction and ethanol precipitation, a biotin-labeled in vitro transcription reaction was performed using the cDNA template and BioArray High Yield In Vitro Transcription kit (Enzo Life Sciences, Farmingdale, N.Y.). The cRNA was fragmented at 0.7-1.1 µg/µl final concentration in 1x fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). The size of cRNA before (0.5 kb and longer) and after (35-200 base fragments) fragmentation was checked by agarose gel electrophoresis.

Microarray Hybridization Procedure.

The hybridization reaction and the automated hybridization procedure were performed by the Gene Expression Center at the Biotechnology Center at the University of Wisconsin-Madison as described (Kutuzova, 2004). Each probe was tested on an Affymetrix Test3 Array and the quality of the cDNA and cRNA was determined by a 3'/5' ratio of housekeeping genes within the array (ubiquitin, glyceraldehyde 3-phosphate dehydrogenase, β-actin, and hexokinase). If the sample passed the quality control on the Affymetrix Test3 Array, it was hybridized to Affymetrix human oligonucleotide arrays (Rat Expression 40 Arrays). (Affymetrix GeneChip Expression Analysis Technical Manual; http://www.affymetrix.com/support/technical/manual/expressionmanual.affx). Expression data were analyzed using the Affymetrix Microarray Suite software version 5.0 (MAS 5.0). Comparison tables for each time point for 1,25-(OH)_2D_3 vs. vehicle-treated rats were generated in EXCEL (Microsoft). For each comparison, e.g. 1,25-(OH)_2D_3 treated relative to control (vehicle treated), and for each cDNA represented in the array, a ratio (e.g. 1.25-(OH)_2D_3/control) and an absolute difference of intensities for 1,25-(OH)_2D_3 and vehicle treated were calculated. Microarray data validation was done by Quantitative Real Time PCR (Q-PCR) as described previously (Kutuzova, 2004).

Monkeys Treatments and Intraocular Pressure (IOP) Measurements.

Baseline pretreatment IOP was determined by Goldmann applanation tonometry [Kaufman, 1980] with cream used as a tear film indicator [Croft, 1997]. Two baseline IOP measurements were taken 5 minutes apart. Monkeys were then treated topically with 5 µl of 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3) (0.1-5 µg) to one eye and vehicle (propylene glycol) to the opposite eye twice a day for 5 treatments total. Drops were administered to the central cornea, one min apart, while the monkeys were in a supine position with their eyelids held open for at least 30 sec post drops. IOP was also measured prior to the afternoon treatment. On the third day, IOP was measured prior to the morning treatment. Following the fifth treatment, IOP was measured hourly for 8 hours and, in some cases, also at 12, 24, and 48 hours. Slit lamp examination (to determine the presence of biomicroscopic cells or flare) was performed prior to the 1st IOP measurement and at hours 3 and 6 (24 and 48 hr where appropriate). Monkeys were allowed to rest for at least 2 weeks between studies. There were 8 monkeys for each treatment group.

Aqueous Humor Formaition Study.

Aqueous humor formation rate was determined by ocular scanning fluorophotometry (Fluorotron Master, OcuMetrics Inc, Mountain View, Calif.) as previously described [Rasmussen, 2007]. Fluorescein drops were administered at least 30 minutes after the fourth treatment (see above) with vitamin D or vehicle. On day 3, prior to the fifth treatment, IOP and biomicroscopy were done. Following the fifth treatment scans were taken hourly, beginning 1 hour after treatment, until 6 duplicates scans were collected. Baseline scans were collected for 6 hours within 2 weeks before and at least 2 weeks after the treatment study. Post treatment aqueous humor formation rates were compared to the average of the pre and post baseline scans and to the vehicle treated eyes by the paired t-test for ratios different from 1.0. There were 8 monkeys for each treatment group.

Outflow Facility Study.

Outflow facility was determined in pentobarbital-anesthetized monkeys [Gibelt, 2004] by two-level constant pressure perfusion of the anterior chamber with Barány’s perfusand [Bárány, 1964] (n=8). Four monkeys (group A) received the single bolus injection of 1 µl of 1 µg vitamin D 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3) into the anterior chamber of one eye (Treated eye) or 1 µl of propylene glycol into the anterior chamber of fellow eye (Control eye). Four monkeys (group B) were treated topically with 5 µg of vitamin D in 5 µl of propylene glycol or vehicle (5 µl of propylene glycol) twice daily for two days.

Following baseline outflow facility measurements on the third day, the fifth treatment was administered as a single bolus injection of 1 µl of 1 µg vitamin D 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3) into the anterior chamber of one eye (Treated eye) or 1 µl of propylene glycol into the anterior chamber of fellow eye (Control eye). Following injections, the treatment bolus was allowed to wash in for 5 min with flow from the reservoirs. Then the contents of the anterior chamber were mixed by blowing cold air on the cornea to create convection. Reservoirs were closed for 75 minutes, then reopened and outflow facility measured for 60-90 minutes. Data were averaged for the entire 60-90 minute period and for 30-minute intervals and then were compared to baseline and to the vehicle treated eyes. Ratios were compared by the two-tailed paired t-test for ratios different from 1.0.

Results

Vitamin D Modulates the Expression of Genes Involved in Regulating IOP.

We used rat and mouse microarrays for identification of a novel vitamin D target genes that we selected as described in [Kutuzova, 2004]. Comprehensive microarray data analysis showed that 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3) altered expression of genes known to be involved in the regulation of IOP. The largest relevant changes found included strong reductions in mRNA expression for carbonic anhydrase I (CA1), angiotensin 1 converting enzyme (ACE) and actin alpha (ACTA1) (FIG. 10A). Significantly down-regulated by 1,25-(OH)_2D_3 were actin gamma (ACTG2), Na+/K+ ATPase alpha 1 (ATP1A1), aquaporins 1 (AQP1),
carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM), fibronectin 1 (FN1), CD44 and tissue inhibitor of metalloproteinase 3 (TIMP3) (FIG. 10A). Significant increases were found in the expression of prostaglandin E receptor 4 for PGE2 (PTGER4) and matrix metalloproteinases 5 (MMP3), 11 (MMP11) and 13 (MMP13) (FIG. 10B).

In our study, vitamin D decreased expression of several genes (vasoactive intestinal peptide, topoisomerase I, MMP2) (the data are not shown) that were found consistently up-regulated in the human trabecular meshwork (TM) during a pressure-induced homeostatic response [Vititow, 2004].

Topical Application of 1α,25-Dihydroxyvitamin D3 Strongly Reduces IOP in Nonhuman Primates Bilaterally.

Pretreatment IOP on day 1 (d1) (mean±SEM) was 18.9±0.7 mmHg in eyes to-be-treated with 5 µg of vitamin D and 18.5±0.9 mmHg in to-be-control eyes. Prior to the fifth treatment on day 3 (d3), IOP had significantly decreased by approximately 20% (3 mmHg) in both eyes (p<0.05) (FIG. 11A, B). Following the fifth topical treatment with vitamin D compound of the present invention or vehicle, IOP decreased bilaterally by an additional 7% (1.5 mmHg) in control eyes and by 10% (2.5 mmHg) in vitamin D treated eyes over the next 1-4 hours before gradually returning to near pretreatment baseline after 48 hours (FIG. 11). There appeared to be a slightly greater IOP reduction in the 5 µg vitamin D treated eye as compared to the control eye (30% vs. 27%) but there were no significant differences between the two eyes except for the time period of 12 h or longer (FIG. 11). In a separate experiment, the vehicle (propylene glycol) alone with no treatment of the contralateral eye had little or no effect on IOP (data not shown).

The IOP Reduction by Vitamin D was Dose-Dependent.

Unilateral topical treatment with 1 µg of vitamin D decreased IOP bilaterally but to a lesser extent than treatment with 5 µg of 1,25-(OH)2D3 (20% vs. 30%) with stronger IOP reduction in the fellow control eyes than in the treated eyes (FIG. 11). Unilateral 0.1 µg of 1,25-(OH)2D3 did not have any significant effect on IOP in either eyes (FIG. 11).

Vitamin D does not Change the Serum Calcium Level in Monkeys.

Since vitamin D functions to maintain blood serum calcium level [DeLuca, 2008], we monitored the serum calcium levels during a pressure-induced homeostatic response [Vititow, 2004].

Aqueous humor formation was measured by fluorophotometry during the interval 1-6 hr after the 5th topical bid treatment with vitamin D or vehicle to opposite eyes (see Materials and Methods). Units for aqueous humor formation are µl/min. Data are Mean±Sem. Rx, treatment (Vitamin D or vehicle) n=6.

Baseline outflow facilities were studied in two groups (A and B) of monkeys (Materials and Methods). Group A (n=4) was treated with single bolus intracameral injection of 1 µg vitamin D (1,25-(OH)2D3) in 1 µI propylene glycol in one eye and 1 µI vehicle, propylene glycol in the control eye (Table 2, A). Group B (n=4) was treated topically with 5 µg of vitamin D in 5 µI of propylene glycol or vehicle (5 µI of propylene glycol) twice daily for two days. Then, following baseline outflow facility measurements on the third day, the fifth treatment was administered as a single bolus injection of 1 µI of 1 µg vitamin D 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) into the anterior chamber of one eye (Treated eye) or 1 µI of propylene glycol into the anterior chamber of fellow eye (Control eye).

TABLE 2

<table>
<thead>
<tr>
<th>Group A. 1 µg Intracameral (n = 4)</th>
<th>Outflow facility (µl/min/mmHg)</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated eye</td>
<td>Control eye</td>
<td>Treated/Control</td>
</tr>
<tr>
<td>0.24 ± 0.08</td>
<td>0.29 ± 0.07</td>
<td>0.90 ± 0.34</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.37 ± 0.00</td>
<td>0.82 ± 0.02</td>
</tr>
<tr>
<td>Vitamin/D/Baseline</td>
<td>1.80 ± 0.30</td>
<td>1.04 ± 0.13</td>
</tr>
<tr>
<td>Group B. 5 µg Topical (4 treatments); 1 µg Intracameral (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated eye</td>
<td>Control eye</td>
<td>Treated/Control</td>
</tr>
<tr>
<td>0.31 ± 0.12</td>
<td>0.38 ± 0.23</td>
<td>1.11 ± 0.17</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.51 ± 0.26</td>
<td>0.91 ± 0.27</td>
</tr>
<tr>
<td>Vitamin/D/Baseline</td>
<td>1.39 ± 0.45</td>
<td>0.84 ± 0.18</td>
</tr>
</tbody>
</table>

There were no changes in the aqueous humor formation rates in vehicle control or in 5 µg vitamin D treated eyes compared to the baseline or to each other post treatment at any time interval when IOP was strongly decreased bilaterally (Table 1, FIG. 11).
known to occur in all non-human species [Scott, 2007]. Still, 50
way for vitamin D-induced Ca²⁺ absorption and immunomodulation suggesting a novel pathway for vitamin D-induced Ca²⁺ absorption [Kutuzova, 2004]. The comprehensive microarray data analysis in rats and mice presented herein shows the novel vitamin D-modulated genes that are known to be involved in the regulation of IOP. Many changes in gene expression that we observed after the vitamin D treatment are relevant to the regulation of aqueous humor formation and drainage. Our microarray studies also show that vitamin D modulated expression of genes may negate the events associated with dexamethasone treatment of trabecular meshwork cells, thereby providing a treatment for steroid-induced glaucoma in susceptible individuals [Rozsa, 2006].

In addition to the microarray studies mentioned above, we investigated the effect of vitamin D on IOP, aqueous humor formation and outflow facility in nonhuman primates following topical and/or intracameral administration. It has long been suggested that extracellular matrix (ECM) components of the ocular drainage pathways are crucial determinants of resistance to aqueous humor outflow and consequently of the IOP [Kaufman, 1984]. ECM molecules and factors that affect their metabolism, synthesis, and response to changing environments are important components of susceptibility to ocular hypertension. Enhancing trabecular outflow can be achieved by disrupting the actin cytoskeleton. Compounds with cytoskeletal effects offer therapeutic possibilities for substantial long-term IOP reduction. Most current IOP-reducing agents either suppress aqueous humor production or increase outflow through the ciliary muscle, thus reducing aqueous humor flow through the TM. All currently known IOP lowering agents have more or less severe side effects [Kaufman, 2006].

Conventional treatments for lowering IOP, such as BETAGAN® (levobunolol) or XALATAN® (latanoprost), can cause side effects including transient ocular burning and stinging, blepharoconjunctivitis, decreases in heart rate and blood pressure, iridocyclitis, headache, transient ataxia, dizziness, lethargy, urticaria, macular edema, pruritus, a decreased corneal sensitivity, upper respiratory tract infections/flu and/or a rash or allergic reactions. None of these side effects have been seen in the compounds of the present invention.

Vitamin D is able to prevent and cure a broad spectrum of diseases such as rickets, cancers, diabetes and autoimmune diseases [Delucu, 2004, 2008]. Another biological function of vitamin D is to regulate genes responsible for detoxification of endo- and xenobiotics [Kutuzova, 2007]. The active form of vitamin D is 1,25 dihydroxyvitamin D₃ or calcitriol (1,25(OH)₂D₃), a seco-steroid hormone, that in association with high affinity vitamin D receptor (VDR) and following heterodimerization with the retinoid X receptor acts as a ligand-activated transcription factor and binds to specific DNA—vitamin D response elements (VDREs), transactivating or transrepressing a large variety of genes [Jones, 1998]. From our microarray study in rats (in vivo) [Kutuzova, 2004] and mice (in vitro) treated with the active form of vitamin D (1,25(OH)₂D₃) we discovered that vitamin D-modulated genes of the cell cytoskeleton, extracellular matrix, cell adhesion and genes of other proteins and enzymes that are known to be involved in IOP regulation (FIG. 10).

Cytoskeleton dynamics has been implicated in trabecular meshwork function and aqueous humor outflow regulation since actin-depolymerizing drugs increased outflow facility and decreased IOP. Agents, which disrupt the actin cytoskeleton lower IOP and increase outflow facility in vivo. We show here for the first time that vitamin D strongly down-regulates the expression of the major cytoskeleton proteins (actins, alpha and gamma), decreases expression of proteins involved in cell adhesion (CEACAM and CD44) and fibronectin I—one of the major ECM proteins involved in

### Table 2-contd

<table>
<thead>
<tr>
<th>Cumulative 90 min outflow facility in monkey eyes after topical and/or intracameral application of vitamin D (1,25-(OH)₂D₃) (Treated eye) or vehicle (propylene glycol) (Control eye).</th>
<th>Treated eye</th>
<th>Control eye</th>
<th>Treated/Control:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.28 ± 0.07</td>
<td>0.34 ± 0.11</td>
<td>1.00 ± 0.18</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.44 ± 0.13</td>
<td>0.58 ± 0.20</td>
<td>0.87 ± 0.15</td>
</tr>
<tr>
<td>Vitamin D/Baseline</td>
<td>1.59 ± 0.19*</td>
<td>1.77 ± 0.16*</td>
<td>0.94 ± 0.11</td>
</tr>
</tbody>
</table>

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Outflow facility units are µl/min/mmHg; ratios are unitless.

Data = Mean ± SEM.

Outflow facility measurements treatment were begun 75 minutes after Vitamin D administration and continued for 90 minutes.

Topical administration of 5 µg of vitamin D or vehicle for 2 days (4 treatments) with intracameral treatment same as in Group A on the third day (see Materials and Methods for details).

No significant difference was found between eyes when the data for the entire 90 minutes period was analyzed or when 30 minutes increments were analyzed. Significantly different from 1.0 by the two-tailed paired t-test: *p < 0.05.
ECM organization and cell interaction (FIG. 10A). Actin disruptions can lead to alterations in cellular adhesions resulting in relaxation of the trabecular meshwork to enhance the area available for fluid outflow [Tian, 2008]. CEACAM has not been investigated in the outflow pathways but reductions in cell adhesion molecules in general would be expected to enhance outflow through the trabecular meshwork [Kuespert, 2006]. Reductions in fibronectin I and in CD44 that we observed after the vitamin D treatment might also lead to enhanced fluid outflow by decreasing the outflow resistance as the result of disruption of the cellular adhesions and reductions in contractility molecules [Wordinger, 2007; Acott, 2008; Tan, 2006].

Vitamin D increased expression of matrix metalloproteinases (FIG. 10B) and decreased expression of their inhibitors (FIG. 10A). Matrix metalloproteinases (MMPs) and their inhibitors remodel ECM material. Elevated levels of matrix metalloproteinases can remodel the extracellular matrix resulting in enhancement of fluid outflow and in the reduction of IOP [Tan, 2006].

The other class of genes down-regulated by vitamin D that we present here for the first time, and which are also known to be involved in IOP reduction, are transporters and channels: aquaporin 1 (Aqp1) and sodium-potassium ATPase (ATP1A1) (FIG. 10A). Aqp1 is the water channel and is expressed at sites of aqueous fluid production and outflow. Mice deficient in aquaporin water channel genes have lower aqueous humor inflow and lower IOP than normal controls [Zhang, 2002]. Therefore, inhibiting aquaporins could be utilized for glaucoma therapy. ATP1A1 in the non-pigmented ciliary epithelium is involved in aqueous humor formation [Riley, 1986]. Inhibiting the ciliary process ATP1A1 by cardiac glycosides (e.g. ouabain) or vanadate significantly reduces the rate of aqueous humor formation and IOP in experimental animals [Podos, 1984; Dismuke, 2009] and humans [Podos, 1989].

Previously [Kutuzova, 2004] we identified other genes whose expression was drastically suppressed by vitamin D and that are relevant to IOP reduction including angiotensin I converting enzyme (ACE) and carbonic anhydrase (CAI) (FIG. 10A). Carbonic anhydrase inhibitors are widely employed for glaucoma therapy to lower IOP by suppressing aqueous humor formation [Mincione, 2007; Supuran, 2008].

ACE is known to be a key part of the renin angiotensin system that regulates blood pressure by converting angiotensin I (AngI) to angiotensin II (AngII), which then increases vasopressin release. ACE can also inactivate the vasodilator bradykinin. Both of these effects elevate arterial blood pressure. ACE inhibitors are widely used for the treatment of hypertension. There is also evidence that the eye contains a renin-angiotensin system and that it may be involved in the regulation of IOP. The presence of ACE activity, the concentrations of angiotensinogen and angiotensin II, and the density of angiotensin-II AT1 receptors in ocular tissues and fluids have been demonstrated in several species, including humans [Wallow, 1993; Cullinane, 2002; Vaajanan, 2008]. The recent studies in hypertensive rats suggested the strong positive correlation between the blood pressure and IOP [Vaajanan, 2008].

A strong correlation between blood pressure and IOP was established also in a human comprehensive study suggesting a common mechanism or common genes that may be controlling pressure both in the eye and in the vascular system [Klein, 2005; Duggal, 2007]. Topical and oral administration of ACE inhibitors has been shown to lower IOP in animal models and in humans; they are currently under development as glaucoma therapeutic agents [Constad, 1988; Costagliola, 1995]. Epidemiological and clinical studies of many years established an inverse relationship between vitamin D and blood pressure in human population [Li, Y. C., 2003]. Vitamin D is a potent suppressor of the renin-angiotensin system and can reduce blood pressure [Li, 2004]. The strong inhibition of ACE expression by vitamin D described previously [Kutuzova, 2004] could be one of many genetic factors responsible for the vitamin D lowering effect of both arterial blood pressure and IOP.

The significant vitamin D induced increase in the expression of prostaglandin E receptor 4 (EP4) for prostaglandin E2 (FIG. 10B) could also contribute to IOP reduction, since ocular hypotensive effect of prostaglandin E2 (PGE2) analogs is mediated by multiple EP receptors present in the eye [Takamatsu, 2000]. Prostaglandins induce matrix metalloproteinases that degrade the ECM in the TM to enhance outflow. Therefore the increased expression of the EP4 receptor stimulated by vitamin D could also contribute to IOP reduction.

The current study demonstrates that topically applied vitamin D is indeed able to substantially lower IOP in non-human primates and thus vitamin D and the whole class of its compounds have the potential to be used as glaucoma therapeutics. The only known previous study supporting the potential use of vitamin D to lower IOP took place more than 50 years ago, when a single intramuscular injection of vitamin D3 (not vitamin D2) was administered to several patients with glaucoma and IOP reduction was observed in some patients [Guist, 1953].

However, these data were not statistically significant, have never been repeated and thus the question on the reproducibility of the results remained open. Moreover vitamin D3, or ergocalciferol, often plant-sourced, is not the endogenous human form of vitamin D (which is vitamin D3) and has far less effect in the body. The other indirect evidence supporting our idea that vitamin D plays the role in IOP regulation and thus in POAG comes from epidemiological studies showing the prevalent susceptibility of African-descent population to POAG as compared to Caucasian populations [Miao, 2008; Lucas, 2008]. Individuals with African ancestry are known to have approximately two-fold lower levels of serum vitamin D (25(OH)D) compared with individuals of European ancestry [Harris, 2006; Zadshir, 2005] due to the fact that pigmentation reduces vitamin D production in the skin. Lower vitamin D status may account for this population being more prone to high blood pressure, diabetes [Harris, 2006] and a higher prevalence of peripheral arterial disease [Reis, 2008].

We showed that IOP is significantly lowered in nonhuman primates following the topical 1,25 dihydroxyvitamin D3 or calcitriol application in a dose-dependent manner with prolonged effects lasting more than 12 hours (FIG. 11). The reduction in IOP occurred bilaterally after unilateral topical application even at lower doses (FIG. 11A). The mechanism of the bilateral IOP decrease by some agents is not clearly understood or explained. One possible mechanism for the contralateral effect is systemic absorption of the topically applied drug through the nasolacrimal mucosa into the blood circulation to the contralateral eye (Piltz, 2000) e.g. detectable plasma levels of the calcium channel blocker flunarizine were reported in rabbits after its topical administration [Maltese, 2003].

Another possibility is that the compound acts through the CNS or peripheral nervous systems [Trzeciakowski, 1987]. Some investigators have emphasized that the nervous system must be considered as the most important regulator of IOP, since changes in IOP were recorded after stimulation of...
sensory, sympathetic and parasympathetic (both oculomotor and facial) nerve fibers [TenTusscher, 1994]. The cannabinoids, opioids and prostaglandins also decrease IOP bilaterally but not to the same extent as vitamin D [Rasmussen, 2007; Kaufman 2008]. Ca++ channel blockers, α2- and β-adrenergic antagonists also cause bilateral IOP decrease, which is usually less prominent in the control eye than in the treated eye [Wang, 2008; Gabelt, 1994; Piltz, 2000]. This suggests that the compounds of the present invention may be useful in treating disorders of the nervous system (CNS/PNS) such as depression, brain cancer, Alzheimer’s, Parkinson and the like.

As the first step in the investigation we studied the aqueous humor formation process. It has become clearly obvious from our experiments that vitamin D does not change the aqueous humor formation (Table 1). Next we studied the aqueous humor outflow facility in vitamin D treated monkey eyes and showed that both vehicle and vitamin D treated eyes experienced the identical increase in outflow facilities (Table 2, FIG. 12). The moderate degree of outflow facility increase, stimulated by vitamin D in both, vehicle and vitamin D treated eyes, is likely the result of “washout” phenomenon common in all species except humans, and in which perfusion of an eye at physiological pressure results in a volume-dependent increase in the measured facility of aqueous humor outflow [Scott, 2007]. Similar results were observed for outflow facilities bilateral increase that was considered as “washout” in monkeys treated with kappa opioid agonist bremazocine [Rasmussen, 2007].

In our study vitamin D did not change either the aqueous humor flow or the outflow facilities and thus did not effect the aqueous humor dynamics in nonhuman primates. This is in stark contrast to all other known ocular hypotensive agents, suggesting that vitamin D’s mechanism of lowering IOP may be different from that of other ocular hypotensive agents. Given the variety of vitamin D target genes presented here involved in IOP regulation, there is a strong evidence to suggest that vitamin D has the potential to lower IOP via several mechanisms.

It should be noted that the above description, attached figures and their descriptions are intended to be illustrative and not limiting of this invention. Many themes and variations of this invention will be suggested to one skilled in the art. Various changes may be made without departing from the spirit and scope of the invention. Therefore, the invention is intended to embrace all known or later-developed alternatives, modifications, variations, improvements, and/or substantial equivalents of these exemplary embodiments.

REFERENCES


We claim:

1. A method of reducing ocular hypertension in a human subject, the method comprising topically administering to at least one eye a therapeutically effective amount of a vitamin D compound according to the following formula, wherein the compound is administered in an amount ranging from about 0.2 µg to about 1 mg per day:

2. The method of claim 1 wherein R is

3. The method of claim 1 wherein R is

4. The method of claim 1 wherein R is
wherein R₁ and R₂ are H, methyl or 3'-hydroxypropylidine, or taken together as =CH₂ or methylene; wherein R₃ and R₄ are selected from H, alkyl (1-3 carbons), alkoxy, and can be the same or different from each other; wherein R₃ and R₄ are selected from H, alkyl (1-3 carbons), alkoxy, and can be the same or different from each other; wherein X is a hydroxy or protected hydroxyl group; wherein A is oxygen or carbon, with the proviso that if A is oxygen, then R₄ is absent; and wherein C and D are H or taken together as =CH₂; and; 
c) measuring the intraocular pressure of the first and second eye; wherein the ocular hypertension in the first and second eye is reduced by at least 15%.

8. A method of reducing intraocular pressure in a human subject's eyes, the method comprising the steps of:
   a) determining a baseline intraocular pressure of a first eye; 
   b) determining a baseline intraocular pressure of a second eye; 
   b) topically administering to the first eye a therapeutically effective amount of a vitamin D compound according to the following formula, wherein the compound is administered in an amount ranging from about 0.2 µg to about 1 mg per day:

   (Formula I)

   wherein X is a hydroxy or protected hydroxyl group; wherein A is oxygen or carbon, with the proviso that if A is oxygen, then R₄ is absent; and wherein C and D are H or taken together as =CH₂; and; 
c) measuring the intraocular pressure of the first and second eye; wherein the ocular hypertension in the first and second eye is reduced by at least 15%.

7. A method of reducing intraocular pressure in a human subject's eyes, the method comprising the steps of:
   a) determining a baseline intraocular pressure of a first eye; 
   b) determining a baseline intraocular pressure of a second eye; 
   b) topically administering to the first and second eye a therapeutically effective amount of a vitamin D compound according to the following formula, wherein the compound is administered in an amount ranging from about 0.2 µg to about 1 mg per day:

   (Formula I)

   wherein X is a hydroxy or protected hydroxyl group; wherein A is oxygen or carbon, with the proviso that if A is oxygen, then R₄ is absent; and wherein C and D are H or taken together as =CH₂; and; 
c) measuring the intraocular pressure of the first and second eye; wherein the ocular hypertension in the first and second eye is reduced by at least 15%.

6. The method of claim 1 wherein the topical preparation is eye drops.

5. The method of claim 1 wherein the topical preparation is eye drops.

4. The method of claim 1 wherein the vitamin D compound is selected from the group consisting of 1α,25-dihydroxyvitamin D₃; 2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D₃; 1α,25-dihydroxy-19-nor-vitamin D₃; 2-(3'-hydroxypropylidene)-19-nor-1α,25-dihydroxyvitamin D₃ (E-isomer); 17-20 dehydro-2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D₃ (E- and Z-isomers); 26-homo-1α,25-dihydroxyvitamin D₃, 26,27-Dimethyl-1α,25-dihydroxyvitamin D₃, 25-hydroxyvitamin D₇
9. The method of claim 8 wherein the compound is selected from 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃); 2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D₃; 1α,25-dihydroxy-19-nor-vitamin D₂; 2-(3’-hydroxypropyldiene)-19-nor-1α,25-dihydroxyvitamin D₃ (E-isomer); 17-20 dehydro-2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D₃ (E- and Z-isomers); 26-homo-1α,25-dihydroxyvitamin D₃; 26,27-Dimethyl-1α,25-dihydroxyvitamin D₃; 25-hydroxyvitamin D₃.