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European Patent Office
Postbus 5818
2280 HV Rijswijk
NETHERLANDS
Tel: +31 70 340 2040
Fax: +31 70 340 3016

Lang, Christian
LangPatent Anwaltskanzlei IP Law Firm
Ingolstädter Straße 5
80807 München
ALLEMAGNE

Formalities Officer
Name: Bonomelli, Luca
Tel: +31 70 340 - 3552
or call
+31 (0)70 340 45 00

Application No.	Ref.	Date
16 838 461.8 - 1111	P12250EPPC	13.11.2020
Applicant Kaohsiung Medical University		

Communication under Rule 71(3) EPC

1. Intention to grant

You are informed that the examining division intends to grant a European patent on the basis of the above application, with the text and drawings and the related bibliographic data as indicated below.

A copy of the relevant documents is enclosed.

1.1 In the text for the Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

Description, Pages

1-15 as published

Sequence listings, SEQ ID NO

1-23 as published

Claims, Numbers

1-8 filed in electronic form on 06-10-2020

Drawings, Sheets

1/5-5/5 as published

With the following amendments to the above-mentioned documents proposed by the division

Description, Pages 2-5, 7, 8

Registered letter

Claims, Numbers 1, 6, 8

Comments

DESCRIPTION
Page 2: Mention of relevant prior art in the description (Rule 42(1) EPC)
Pages 3, 4: Embodiment no longer covered by (amended) claims (Art. 84 EPC)
Page 5: Inconsistency between claim and description removed (Art. 84 EPC)
Pages 7, 8: Description adapted to amended claims (Art. 84 EPC)
CLAIMS
Claims 1,6: ocular cells are changed to retinal pigment epithelium (RPE) cell because no literal basis for the term ocular cells could be found in the application (Art. 123(2) PCT).
Claim 1: the wording related to the sequence has been amended to avoid lack of clarity and consistency with claims 2 and 3.
Page 1, Claim 8: Inconsistency between claims removed (Art. 84 EPC)

1.2 Bibliographic data

The title of the invention in the three official languages of the European Patent Office, the international patent classification, the designated contracting states, the registered name(s) of the applicant(s) and the other bibliographic data are shown on **EPO Form 2056** (enclosed).

2. Invitation

You are invited, **within a non-extendable period of four months** of notification of this communication,

2.1 to EITHER approve the text communicated above and verify the bibliographic data (Rule 71(5) EPC)

(1) by filing a translation of the claim(s) in the other two official languages of the EPO

	Fee code	EUR
(2a) by paying the fee for grant including the fee for publication:	**007**	960.00
minus any amount already paid (Rule 71a(5) EPC):		0.00
	Total amount:	960.00
(3) by paying additional claims fees under Rule 71(4) EPC; number of claims fees payable: 0	**016**	0.00
minus any amount already paid (Rule 71a(5) EPC):		0.00
	Total amount:	0.00

Important: If the translations of the claims and fees have already been filed and paid respectively in reply to a previous communication under Rule 71(3) EPC, e.g. in the case of resumption of examination after approval (see Guidelines C-V, 6), **agreement as to the text to be granted** (Rule 71a(1) EPC) must be expressed within the same time limit (e.g. by approving the text and verifying the bibliographic data, by confirming that grant proceedings can go ahead with the documents on file and/or by stating which translations of the claims already on file are to be used).

Note 1: See "Notes concerning fee payments" below.
Note 2: Any overpaid "minus" amounts will be refunded when the decision to grant (EPO Form 2006A) has been issued.
Note 3: For the calculation of the grant fee under Article 2(2), No. 7, RFees (old fee structure), the number of pages is determined on the basis of a clean copy of the application documents, in which text deleted as a result of any amendments by the examining division is not shown. Such clean copy is made available via on-line file inspection only.

2.2 OR, in the case of disapproval, to request reasoned amendments or corrections to the text communicated above or keep to the latest text submitted by you (Rule 71(6) EPC).

In this case the translations of the claims and fee payments mentioned under point 2.1 above are NOT due.

The terms "amendment(s)" and "correction(s)" refer only to amendments or corrections of the application documents and not of other documents (e.g. bibliographic data, the designation of the inventor, etc.).

If filing amendments, you must identify them and indicate the basis for them in the application as filed. Failure to meet either requirement may lead to a communication from the examining division requesting that you correct this deficiency (Rule 137(4) EPC).

2.3 Bibliographic data

Where you request a change or correction of bibliographic data in response to the Rule 71(3) communication, this will **not** cause the sending of a further communication under Rule 71(3) EPC. You will still have to pay the fees and file translations in reply to the Rule 71(3) communication in the case of 2.1 above, unless you also file a reasoned request for amendments or corrections in response to the Rule 71(3) communication (see case 2.2 above).

3. Loss of rights

If neither of the two possible actions above (see points 2.1 or 2.2) is performed in due time, the European patent application will be deemed to be withdrawn (Rule 71(7) EPC).

4. Further procedure

4.1 In the case of point 2.1 above

4.1.1 The decision to grant the European patent will be issued, and the **mention of the grant** of the patent will be published in the European Patent Bulletin, if the requirements concerning the translation of the claims and the payment of all fees are fulfilled and there is agreement as to the text to be granted (Rule 71a(1) EPC).

Note on payment of the renewal fee:
If a renewal fee becomes due before the next possible date for publication of the mention of the grant of the European patent, publication will be effected only after the renewal fee and any additional fee have been paid (Rule 71a(4) EPC).

Under Article 86(2) EPC, the obligation to pay renewal fees to the European Patent Office terminates with the payment of the renewal fee due in respect of the year in which the mention of the grant of the European patent is published.

Note on payment of the designation fee(s):
If the designation fee(s) become(s) due after the communication under Rule 71(3) EPC, the mention of the grant of the European patent will not be published until these fees have been paid (Rule 71a(3) EPC).

4.1.2 After publication, the **European patent specification** can be downloaded free of charge from the EPO publication server https://data.epo.org/publication-server.

4.1.3 Filing of translations in the contracting states

As regards translation requirements prescribed by the contracting states under Article 65(1) EPC, please consult the website of the European Patent Office
www.epo.org ➞Law & practice ➞Legal texts, National law relating to the EPC
www.epo.org ➞Law & practice ➞All Legal texts ➞London Agreement

In the case of a valid extension or validation

As regards translation requirements prescribed by the extension or validation states, please consult the website of the European Patent Office
www.epo.org ➞Law & practice ➞Legal texts, National law relating to the EPC

Failure to supply a prescribed translation in a contracting state, or in an extension or validation state may result in the patent being deemed to be void *ab initio* in the state concerned (Art. 65(3) EPC).

4.2 In the case of 2.2 above

If the present communication under Rule 71(3) EPC is based on an auxiliary request and, within the time limit, you maintain the main request or a higher ranking request which is not allowable, the application will be refused (Art. 97(2) EPC).

If the examining division gives its consent to the requested amendments or corrections, it will issue a new communication under Rule 71(3) EPC; otherwise, it shall resume the examination proceedings (Rule 71(6) EPC).

5. Filing of a divisional application

Any divisional application relating to this European patent application must be filed directly with the European Patent Office in Munich, The Hague or Berlin and will be in the language of the proceedings for the present application, or if the latter was not in an official language of the EPO, the divisional application may be filed in the language of the present application as filed (see Article 76(1) and Rule 36(2) EPC). Any such divisional application must be filed while the present application is still pending (Rule 36(1) EPC; Guidelines A-IV, 1.1.1).

6. Notes concerning fee payments

6.1 Making payments

For payments made via deposit account, please note that as from 1 December 2017 debit orders will only be carried out if filed in an electronically processable format (xml), using an accepted means of filing as laid down in the Arrangements for deposit accounts (ADA), published in the Supplementary publication in the Official Journal.

All relevant information related to the modes of payment of fees to the EPO can be retrieved from the EPO website at "**Making Payments**".

6.2 Information concerning fee amounts

Procedural fees are usually adjusted every two years, on even years, with effect from 1 April. Therefore, before making a payment, parties should verify the amounts actually due on the date of payment using the applicable version of the Schedule of fees and expenses, published as a Supplement to the Official Journal of the EPO, available on the EPO website (**www.epo.org**) at **www.epo.org/schedule-of-fees**. The "Schedule of fees" table allows the viewing, downloading and searching of individual fee amounts, both current and previous.

6.3 Note to users of the automatic debiting procedure

The fee for grant, including the fee for publication, and any additional claims fees due under Rule 71(4) EPC will be debited automatically on the date of filing of the translations of the claims, or on the last day of the period of this communication. However, if the designation fee(s) become(s) due as set out in Rule 71a(3) EPC and/or a renewal fee becomes due as set out in Rule 71a(4) EPC, these should be paid separately by another permitted way of payment in order not to delay the publication of the mention of the grant. The same applies in these circumstances to the payment of extension and validation fees. The same applies in these circumstances to the payment of extension and validation fees.

Examining Division:

Chairman:	Lejeune, Robert
2nd Examiner:	Macchia, Giovanni
1st Examiner:	Romano, Alper

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Bonomelli, Luca
For the Examining Division
Tel. No.: +31 70 340 - 3552 **Branch at The Hague**

Enclosures: Text intended for grant

EPO Form 2056

SEQUENCE LISTING

<110> KAOHSIUNG MEDICAL UNIVERSITY

<120> MICRORNA-328 ANTI-SENSE COMPOSITION AND THERAPEUTIC USE

<130> 16548WO

<150> US 62/210,340
<151> 2015-08-26

<160> 23

<170> PatentIn version 3.4

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European Patent Office
Office européen des brevets

European Patent Office
Postbus 5818
2280 HV Rijswijk
NETHERLANDS
Tel: +31 70 340 2040
Fax: +31 70 340 3016

Annex to EPO Form 2004, Communication pursuant to Rule 71(3) EPC

Bibliographical data of European patent application No. 16 838 461.8

For the intended grant of the European patent, the bibliographical data are set out below, for information:

Title of invention:	- MICRORNA-328-ANTISENSE-ZUSAMMENSETZUNG UND THERAPEUTISCHE VERWENDUNG - MICRORNA-328 ANTI-SENSE COMPOSITION AND THERAPEUTIC USE - COMPOSITION DE MICRO-ARN-328 ANTISENS ET UTILISATION THÉRAPEUTIQUE
Classification:	INV. C12N15/113 A61K31/711 A61K31/712 A61K31/7125 ADD. A61P27/10
Date of filing:	28.07.2016
Priority claimed:	US / 26.08.2015 / USP201562210340
Contracting States* for which fees have been paid:	AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Extension States* for which fees have been paid:	
Validation States* for which fees have been paid:	
Applicant(s):**	Kaohsiung Medical University No. 100, Shiqhuan 1st Road, Sanmin District, Kaohsiung City 807 TW
Inventor(s):	JUO, Suh-Hang H. No. 100 Shiquan 1st Rd. Sanmin Dist. Kaohsiung Taiwan 807 TW

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
Postbus 5818
2280 HV Rijswijk
NETHERLANDS
Tel: +31 70 340 2040
Fax: +31 70 340 3016

HSI, Edward
No. 100
Shiquan 1st Rd.
Sanmin Dist.
Kaohsiung
Taiwan 807
TW

LIANG, Chung-Ling
No. 100
Shiquan 1st Rd.
Sanmin Dist.
Kaohsiung
Taiwan 807
TW

*)	If the time limit for the payment of designation fees according to Rule 39(1) EPC has not yet expired and the applicant has not withdrawn any designation, **all Contracting States/Extension States/Validation States** are currently still deemed to be designated. See also Rule 71a(3) EPC and, if applicable, the above Note to users of the automatic debiting procedure.
**)	If two or more applicants have designated different Contracting States, this is indicated here.

MICRORNA-328 ANTI-SENSE COMPOSITION AND THERAPEUTIC USE

TECHNICAL FIELD

The present invention relates to anti-sense microRNA-328 in a form of oligodeoxyribonucleotide or locked nucleic acid (LNA)-modified and phosphorothioate bond-modified oligonucleotides, and their therapeutic use in treating ocular diseases such as myopia.

BACKGROUND OF THE INVENTION

Myopia causes the eye to elongate, which in turn stretches and thins the retina and the sclera of the eye. Accordingly a myopic eye has a longer axial length than the normal eye. Noticeably, the axial length can vary among individuals. In animal studies, one eye is induced to myopia and the other eye is used as the control. The difference of axial length between the induced myopic eye and the control eye can be used to indicate the severity of myopia. In addition, the change of difference of axial length can also be used as a readout to assess the therapeutic effect for anti-myopic treatment. The elongation of eyeball is considered the major underlying mechanism to cause myopia complications such as retinal detachment, macular degeneration. That also explains why the correction of refraction without preventing axial length elongation (such as eyeglasses) cannot prevent myopia complications.

The paired box 6 (PAX6) gene belongs to a highly conserved family of transcription factors containing the paired and homeobox DNA-binding domains. PAX6 is involved in the development of the central nervous system and the eye. It plays significant roles during the induction of lens and retina differentiation, and has been considered the master gene in eye development. In humans, mutations in PAX6 are associated with a variety of human ocular diseases including aniridia, foveal hypoplasia, presenile cataract, and aniridia-related keratopathy (reviewed by Tsonis and Fuentes). In addition to the biological plausibility, a genome-wide linkage study revealed a strong linkage of refractive error to the PAX6 locus. Accordingly, PAX6, has been proposed as a candidate gene for the development of myopia. A low level of PAX6 may be a risk factor for myopia.

MicroRNAs (miRNAs) are noncoding, single-stranded RNA molecules of about 21-23 nucleotides in length. In animals, a mature miRNA is complementary to the 3' untranslated region (UTR) of one or more messenger RNAs (mRNAs). The annealing of a miRNA to its

target mRNA causes an inhibition of protein translation, and/or cleavage of the mRNA. miRNAs can regulate cell growth, differentiation, and apoptosis.

Chen et al (Invest. Ophthalmol. Vis. Sci., 53:2732-2739, 2012) report that microRNA-328 (miR-328) may influence myopia development by mediating the PAX6 gene 2 . CN102533755 discloses antisense oligonucleotide targeting miR-328 in treatment of miR-328 overexpressing tumours especially glioma. 2

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 shows relative miR-328 expressed levels in RPE cells after transfection with miR-328 anti-sense DNA (15mer-30mer) and control. Mean and standard deviation are shown (n= 3).

FIG. 2 shows relative miR-328 expressed levels in RPE cells after transfection with miR-328 anti-sense DNA modified by locked nucleic acids and phosphorothioated bonds (401-406) and control. Mean and standard deviation are shown (n= 3).

FIG. 3 shows the delta axial length of mice between right eye (myopia) and left eye in mice treated with saline control (NS), DNA 16mer, DNA 17mer, LNA 403, LNA404, and LNA 405.

FIG. 4 shows the delta axial length of mice between right eye (myopia) and left eye in mice treated with saline control (saline), 1% atropine (atropine), DNA 16mer, and LNA 403.

FIG. 5 shows the delta axial length of mice between right eye (myopia) and left eye in mice treated with saline control (saline), 1% atropine, 10nM, 100nM, and 1μM of DNA 16mer.

FIG. 6 shows the delta axial length of rabbit between right eye (myopia) and left eye in rabbit treated with saline control (saline), 10μM, and 50μM of DNA 16mer.

DETAILED DESCRIPTION OF THE INVENTION

Definition

A “locked nucleic acid” (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation, which is often found in the A-form duplexes. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired.

An “oligodeoxyribonucleotide”, as used herein, refer to a deoxyribonucleic acid (DNA) having 5-50 bases, preferably 10-30 bases, or 15-30 bases long. The DNA is optionally modified on the bases or on the phosphodiester bond.

An “oligoribonucleotide”, as used herein, refer to a ribonucleic acid (RNA) having 5-50

bases, preferably 10-30 bases, or 15-30 bases long. The RNA is optionally modified.

An "oligonucleotide", as used herein, refer to an oligodeoxyribonucleotide, an oligoribonucleotide, or a hybrid thereof.

"Phosphorothioates" are a variant of normal DNA in which at least one of the non-bridging oxygens of the phosphodiester bonds is replaced by a sulfur. The sulfurization of the internucleotide bond dramatically reduces the action of endonucleases and exonucleases. Inclusion of phosphorothioate (PS) bonds increases oligonucleotide half-life in human serum; however, the introduction of PS bonds may lower the binding affinity of the oligonucleotides and may cause cytotoxicity.

The present disclosure ~~invention~~ is directed to oligonucleotide sequences that are anti-sense to miR-328. In one embodiment, the oligonucleotides are oligodeoxyribonucleotides that have specific lengths. In another embodiment, the oligonucleotides have LNA modifications and phosphorthioate modifications. The oligonucleotides of the present invention are useful to prevent or to treat ocular diseases such as myopia.

Human mature miR-328 has the sequence of CUGGCCCUCUCUGCCCUUCCGU (SEQ ID NO: 1).

In a first aspect ~~of the invention,~~ the inventors have designed anti-sense miR-328 oligodeoxyribonucleotides (15-22 mer in length) according to mature human miR-328, and designed anti-sense miR-328 oligodeoxyribonucleotides (23-30 mer in length) according to premature human miR-328 sequences. The inventors then obtained the miRNA-328 anti-sense DNAs having 15-30 bases (15-30mer) and tested their activities. The sequences of DNA 15mer-30mer are shown in Table 1.

Table 1. Antisense DNA sequence

DNA Antisense	Sequence	SEQ ID NO:
DNA 30mer	5'-ACGGAAGGGCAGAGAGGGCCAGGGGCTGTA-3'	17
DNA 29mer	5'-ACGGAAGGGCAGAGAGGGCCAGGGGCTGT-3'	16
DNA 28mer	5'-ACGGAAGGGCAGAGAGGGCCAGGGGCTG-3'	15
DNA 27mer	5'-ACGGAAGGGCAGAGAGGGCCAGGGGCT-3'	14
DNA 26mer	5'-ACGGAAGGGCAGAGAGGGCCAGGGGC-3'	13

DNA 25mer	5'-ACGGAAGGGCAGAGAGGGCCAGGGG-3'	12
DNA 24mer	5'-ACGGAAGGGCAGAGAGGGCCAGGG-3'	11
DNA 23mer	5'-ACGGAAGGGCAGAGAGGGCCAGG-3'	10
DNA 22mer	5'-ACGGAAGGGCAGAGAGGGCCAG-3'	9
DNA 21mer	5'-ACGGAAGGGCAGAGAGGGCCA-3'	8
DNA 20mer	5'-CGGAAGGGCAGAGAGGGCCA-3'	7
DNA 19mer	5'-GGAAGGGCAGAGAGGGCCA-3'	6
DNA 18mer	5'-GAAGGGCAGAGAGGGCCA-3'	5
DNA 17mer	5'-AAGGGCAGAGAGGGCCA-3'	4
DNA 16mer	5'-AGGGCAGAGAGGGCCA-3'	3
DNA 15mer	5'-GGGCAGAGAGGGCCA-3'	2

The inventors have discovered that out of the 16 anti-sense DNAs tested, only 16mer and 17mer inhibited miR-328 expression in vitro. Surprisingly, the anti-sense DNAs (15mer and 18-30mers) did not show any activity for inhibiting miR-328 expression in vitro. The 16mer and 17mer were safe in animal studies, and they exhibited an activity for treating myopia by decreasing an average axial length in the treated mice.

The present invention is directed to DNA 16mer, 5'-AGGGCAGAGAGGGCCA-3' (SEQ ID NO: 3) and DNA 17mer, 5'-AAGGGCAGAGAGGGCCA-3' (SEQ ID NO: 4).

In a second aspect ~~of the invention,~~ the inventors have designed LNA-modified, and phosphorothioate (PS) bond-modified antisense oligonucleotides, ranging from 17 to 22 mers according to mature human miR-328 sequence and gapmer principle (Kurreck et al, Nucleic Acids Res., 30: 1911–1918, 2002). The 15 and 16 mers of LNA-modified antisense oligonucleotides are not included, because the 5'-end terminal sequences of 15 and 16 mers show consecutive 3 G's and the self-complementary pattern, which can cause the oligonucleotide to form dimers. The inventors then obtained miRNA-328 anti-sense LNA modified oligonucleotides having 17-22 bases and tested their activities. The sequences of anti-sense miR-328 oligonucleotides that are LNA- modified and PS bond-modified are shown in Table 2. Their respective non-modified, native DNA sequences are shown in the computer-readable format of sequence listing as SEQ ID NOs: 18-23.

Table 2. LNA modified, and PS bond modifed Antisense oligonucleotide sequence

Antisense	Sequence (LNA-modified and PS bond-modified)	SEQ ID NO^ (non-modified version)
401 (22mer)	5'-+A*+C*+G*+G*A*A*G*G*G*C*A*G*A*G*A*G*G*G*+C*+C*+A*+G-3'	23
402 (21mer)	5'-+A*+C*+G*+G*A*A*G*G*G*C*A*G*A*G*A*G*G*+G*+C*+C*+A-3'	22
403 (20mer)	5'-+C*+G*+G*+A*A*G*G*G*C*A*G*A*G*A*G*G*+G*+C*+C*+A-3'	21
404 (19mer)	5'-+G*+G*+A*+A*G*G*G*C*A*G*A*G*A*G*G*+G*+C*+C*+A-3'	20
405 (18mer)	5'-+G*+A*+A*+G*G*G*C*A*G*A*G*A*G*G*+G*+C*+C*+A-3'	19
406 (17mer)	5'-+A*+A*+G*+G*G*C*A*G*A*G*A*G*G*+G*+C*+C*+A-3'	18

+: indicates Locked nucleic acid (LNA) modification; *: indicates phosphodiester bonds were replaced by phosphorothioate bonds. ^ indicates that SEQ ID NOs: 18-23 are the sequences of native (unmodified) oligonucleotides of their respective LNA-modified and PS-modified counterparts shown with "+" and "*" in the table.

In Table 2, every phosphodiester bond of the DNA sequences is modified to a phosphorothioate bond (PS). In Table 2, the central core of each DNA sequence is flanked by four LNA-modified nucleotides at both 5' and 3' ends.

The inventors have discovered that out of the 6 anti-sense LNA/PS bond modified oligonucleotides tested, only 403, 404, and 405 inhibited miR-328 expression in vitro. Other anti-sense LNA/PS bond modified DNAs did not show any activity for inhibition miR-328 expression in vitro. In addition to the in vitro activity, 403 exhibited an activity for treating myopia by decreasing an average axial length in the treated mice.

The present invention is directed to anti-sense LNA/PS bond modified 2 oligonucleotide 403. 2 ~~oligonucleotides 403, 404, 405; with 403 being preferred~~

The miRNA-328 antisense compositions of the present invention have good hybridization activity toward miRNA-328, have good solubility in water, and are stable (resistant to exonucleases).

PAX6, FMOD and COL1A1 are important genes in myopia pathogenesis and they are direct target genes of miR-328. These genes have been shown to play a role in myopia

development. The miRNA-328 antisense compositions of the present invention increased the expression levels of PAX6, FMOD and COL1A1 in vitro.

The miRNA-328 antisense compositions of the present invention are useful in preventing or treating ocular diseases. In particular, the miRNA-328 antisense compositions of the present invention are useful in preventing or treating myopia.

The present invention is directed to a pharmaceutical composition comprising miRNA-328 anti-sense oligonucleotides of the present invention and a pharmaceutically acceptable carrier. A preferred form for treating myopia is a topical solution or a topical ointment.

A topical solution containing anti-sense miRNA-328 can contain a physiologically compatible vehicle, as those skilled in the ophthalmic art can select using conventional criteria. The ophthalmic vehicles include, but are not limited to, saline solution, water polyethers such as polyethylene glycol, polyvinyls such as polyvinyl alcohol and povidone, cellulose derivatives such as methylcellulose and hydroxypropyl methylcellulose, petroleum derivatives such as mineral oil and white petrolatum, animal fats such as lanolin, polymers of acrylic acid such as carboxypolymethylene gel, vegetable fats such as peanut oil and polysaccharides such as dextrans, and glycosaminoglycans such as sodium hyaluronate and salts such as sodium chloride and potassium chloride.

The formulation optionally includes a preservative, such as benzalkonium chloride and other inactive ingredients such as EDTA. However, for chronic (over two weeks) use, preferred formulations are those without any preservatives due to the potential for damage to the corneal epithelium that may result from long term, frequent exposure to preservatives such as benzalkonium chloride. The formulations without preservatives are prepared in a unit dose and stored in a single-use container.

The pH of the formulation is adjusted by adding any physiologically and ophthamologically acceptable pH adjusting acids, bases or buffers to within the range of about 5 to 7.5, preferably 6 to 7. Examples of acids include acetic, boric, citric, lactic, phosphoric, hydrochloric, and the like, and examples of bases include sodium hydroxide, sodium phosphate, sodium borate, sodium citrate, sodium acetate, sodium lactate, tromethamine, THAM (trishydroxymethylamino-methane), and the like. Salts and buffers include citrate/dextrose, sodium bicarbonate, ammonium chloride and mixtures of the aforementioned acids and bases.

The osmotic pressure of the aqueous ophthalmic composition is generally from about 200

to about 400 milliosmolar (mOsM), more preferably from 260 to 340 mOsM. The osmotic pressure can be adjusted by using appropriate amounts of physiologically and ophthamologically acceptable ionic or non-ionic agents. Sodium chloride is a preferred ionic agent, and the amount of sodium chloride ranges from about 0.01% to about 1% (w/v), and preferably from about 0.05% to about 0.45% (w/v). 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 be used in addition to or instead of sodium chloride to achieve osmolality within the above-stated range. Further, non-ionic agents such as mannitol, dextrose, sorbitol, glucose and the like can also be used to adjust the osmolality.

MiRNA-328 anti-sense oligonucleotides of the present invention can be administered to the eyes of a patient by any suitable means, but are preferably administered in the form of drops, spray, gel, or ointment. In one embodiment, the oligonucleotide is in the form of drops, and is dropped onto the ocular surface. In another embodiment, the oligonucleotide is contained within a swab or sponge which can be applied to the ocular surface. In another embodiment, the oligonucleotide is contained within a liquid spray or ointment which can be applied to the ocular surface. In another embodiment, the oligonucleotide is injected directly into the lacrimal tissues or onto the eye surface. Alternatively, the oligonucleotide can be applied to the eye via liposomes. Further, the oligonucleotide can be infused into the tear film via a pump-catheter system. As an additional embodiment, the oligonucleotide can be contained within, carried by, or attached to contact lenses or other compatible controlled release materials, which are placed on the eye.

The concentration of the oligonucleotide included in a topical solution is an amount sufficient to prevent and/or to treat myopia. The oligonucleotide concentration is generally in the range of about 30nM-2.5mM, preferably about 100nM-10μM, about 1μM-100μM, or about 15 μM-1.5mM. "About", as used herein, refers to ± 10% of recited value.

The present disclosure is further directed to a method for preventing or treating myopia in a subject. The method comprises the step of administering to a subject in need thereof an effective amount of the anti-sense microRNA-328 oligonucleotide composition of the present invention. Topical route of administration is preferred. "An effective amount", refers to an

amount that is effective to prevent or to treat myopia, i.e., to prevent the axial length in the eye from increasing, or to reduce the axial length in the myopic eye.

The daily dose to treat or prevent myopia can be divided among one or several unit dose administrations. The daily dose, for example, can range from one drop (about 30-50 μl), one to four times a day, depending upon the age and condition of the subject. One regimen for miR-328 anti-sense DNA composition is one drop of the topical solution, 1 to 2 times a day. Alternatively, the topical solution can be administered one time per week.

When treating or preventing myopia, the present method can be combined with other methods known to a person skilled in the art.

The present disclosure ~~invention~~ also provides use of a deoxyribonucleotide sequence according to the present invention for the manufacture of a medication or pharmaceutical composition for preventing or treating myopia. In a preferred embodiment, the medication or pharmaceutical composition is a topical solution or a topical ointment.

The present disclosure ~~invention~~ also provides use of a locked nucleic acid-modified, and phosphorothioate bond-modified oligonucleotide sequence according to the present invention for the manufacture of a medication or pharmaceutical composition for preventing or treating myopia. In a preferred embodiment, the medication or pharmaceutical composition is a topical solution or a topical ointment.

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be construed as being limiting.

EXAMPLES

Example 1. Antisense oligonucleotides (DNA) synthesis and purification

Anti-sense miR-328 oligonucleotides that consist of DNA nucleotides were designed according to mature human miR-328 and human pre-miR-328 sequences. The DNA antisense oligonucleotides were synthesized using the DNA/RNA synthesizer called Dr. Oligo192 (Biolytic Lab Performance Inc.) according to manufacturer's instructions. Anti-sense oligonucleotides ranging 15mers to 30mers containing all deoxyribonucleotides were made. The products were purified by HPLC. The synthesis and purification of the antisense oligonucleotides were performed by the Genomics BioSci & Tech. Ltd. (Taiwan). The sequences

of DNA 15mer-30mer are shown in Table 1.

Example 2. Antisense oligonucleotides (LNA) synthesis and purification

LNA-modified, and PS bond modified antisense oligonucleotides ranged from 17 to 22 mers were designed according to mature human miR-328 sequence and gapmer principle; the modified sequences and the non-modified version are shown in Table 2. According to the design, the LNA-modified, and PS bond modified antisense sequences of 17-22 mers were synthesized and purified by Exiqon (Denmark).

Example 3. In vitro tests for Inhibition of miR-328 expression by antisense oligonucleotides

Cell Culture, Treatments, and Transfection

An RPE cell line called "ARPE-19" was grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium. The cell line was grown with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide (CO_2). To conduct the transfection experiments, cells were seeded into a 12-well plate at a density of 1×10^5 cells/well. After achieving 70% confluence in a well, antisense oligonucleotides (concentrations of 30, 50 and 100 nM) were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hours of incubation, cells were lysed for further studies.

Detect the inhibition of miR-328 expression by antisense oligonucleotides

Total RNA was extracted from cultured cells using Trizol according to the manufacturer's instructions. RNA purity was checked using A_{260}/A_{280} readings. miR-328 was first reversed transcribed to miR-328 cDNA using the following procedure: 5 ng of RNA was reverse transcribed with miR-328 specific primer and MultiScribe reverse transcriptase kit (Applied Biosystems). To measure the miR-328 expression, quantitative real-time PCR was performed on an ABI 7500 real-time PCR machine (Applied Biosystems) with miR-328 specific probe according to manufacturer's instructions (Applied Biosystems). The inhibitory effect by antisense oligonucleotides was evaluated by measuring the level of miR-328 cDNA by quantitative real-time PCR. The expression level of miR-328 was normalized to that of a small

non-coding nuclear RNA U6 as the internal control.

Results from in vitro tests for Inhibition of miR-328 expression by antisense oligonucleotides

a. DNA anti-sense oligonucleotides

Sixteen DNA antisense oligonucleotides, from 15 to 30 mers in length prepared according to Example 1, were examined for their inhibitory effects on the expression of miR-328 in RPE cells. The relative miR-328 expression levels are shown in FIG. 1. Only DNA16mer and DNA17mer inhibited miR-328 expression. The inhibitory effects of DNA16mer were 39%, 35% and 49% under the concentrations of 30, 50 and 100nM. For DNA17mer, the inhibitory effects were 43%, 41% and 48% under the concentrations of 30, 50 and 100nM. The rest of antisense oligonucleotides (15mer and 18-30mers) showed no inhibitory effect on miR-328 expression.

b. LNA-modified antisense oligonucleotides

Six LNA-modified antisense sequences, named LNA401 to LNA406, were examined for the inhibitory effect on miR-328 expression in RPE cells. The relative miR-328 expressed levels are shown in FIG. 2. Only LNA403, LNA404 and LNA405 inhibited miR-328 expression. The inhibitory effects of LNA403 were 97%, 97% and 90% under the concentrations of 30, 50 and 100nM, respectively. LNA404 and LNA405 had the same the inhibitory effects of 98%, 98% and 99% under the concentrations of 30, 50 and 100nM, respectively. The rest of LNA oligonucleotides showed no inhibitory effect on miR-328 expression.

Example 4. In vivo study to evaluation of efficacy of antisense oligonucleotides in treating nearsightedness

Animal model

The 21 days old C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan. All animal experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The procedure of myopia induction is described briefly as the following: Right eyes of the 23 days old mice were covered to induce myopia (i.e. nearsightedness) and the left eyes were uncovered. Then the right eyes of the myopia-induced mice were treated with 30μL of saline (i.e. control group), DNA16mer, DNA17mer, LNA403, LNA404, or LNA405, at 1μM on 30^{th}, 37^{th} and 44^{th} day. Mice were

sacrificed on 51th day and the eyes were collected. The isolated eyes were photographed under a dissection microscope and the axial lengths were measured using imageJ. Myopia (nearsightedness) causes elongation of axial length, which is the major pathological change in myopia. The difference of axial length (i.e. delta axial length) between the covered right eye and the non-covered left eye of the same mouse indicates the severity of myopia. The results are summarized in Table 3 and FIG. 3. Statistical evaluation was done by using Mann-Whitney U test. Analysis with p-values < 0.05 being considered significant.

Table 3. Delta axial length of mice treated with antisense oligonucleotides or saline

	Animal Number	**Delta axial length, mm mean (SD)**
Saline	26	0.018 (0.072)
DNA 16mer	25	-0.059 (0.061)*
DNA 17mer	26	-0.047 (0.073)*
LNA 403	27	-0.036 (0.079)*
LNA 404	17	-0.007 (0.100)
LNA 405	16	-0.017 (0.054)

* indicated significant difference with p value < 0.05, when compared to Saline group

DNA16mer and DNA17mer

The average delta axial length in mice treated with DNA16mer and DNA17mer were statistically significantly smaller than that in control animals. DNA16mer and DNA17mer significantly reduced the delta axial length by 0.077 mm and 0.065 mm compared to control group. The results showed that DNA16mer and DNA17mer were effective in treating myopia in mice.

LNA-modified antisense oligonucleotides

The average delta axial lengths in mice treated with LNA403-405 were smaller than that in control animals. LNA403 reduced the delta axial length by 0.054mm, LNA404 reduced the delta axial length by 0.025mm and LNA405 reduced the delta axial length by 0.035mm, when

compared to control group. However, only LNA 403 shows a statistical significance (p value <0.05). The results showed that LNA403 was effective in treating myopia in mice.

Example 5. Validation of DNA 16mer and LNA 403

Animal model

The 21 days old C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan. All animal experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The procedure of myopia induction is described briefly as the following: Right eyes of the 23 days old mice were covered for 4 weeks to induce myopia (i.e. nearsightedness) and the left eyes were uncovered. Then the right eyes of the myopia-induced mice were treated with 30μL of saline (i.e. negative control group), 1% atropine (i.e. positive control group), DNA16mer or LNA403 at 1μM on 30^{th}, 37^{th} and 44^{th} day. Mice were sacrificed on 51^{th} day and the eyes were collected. The isolated eyes were photographed under a dissection microscope and the axial lengths were measured using imageJ and a proprietary software for automatic measure. Myopia (nearsightedness) causes elongation of axial length, which is the major pathological change in myopia. The difference of axial length (i.e. delta axial length) between the covered right eye and the non-covered left eye of the same mouse indicates the severity of myopia. The results are summarized in Table 4 and FIG. 4. Statistical evaluation was done by using Mann-Whitney U test. Analysis with p-values < 0.05 being considered significant.

Table 4. Delta axial length of mice treated with atropine, antisense oligonucleotides or saline

	n	**Mean of delta AXL**	**SE**	**p**
Saline	26	0.008	0.008	
1% Atropine	28	-0.037	0.010	<0.0001
DNA 16mer	38	-0.055	0.008	<0.0001
LNA403	40	-0.052	0.011	0.0024

AXL: axial length of eyeball

To further compare the efficacy of DNA 16mer and LNA 403 with the well-documented anti-myopia drug (atropine), we tested these three types of eyedrop. It needs to be noticed that we used 1% atropine that is 10x higher concentration than clinical concentration, and 1% atropine has been shown to be the most effective than lower concentration. Since DNA 16mer and LNA403 were dissolved in normal saline, we used normal saline as the negative control. The above data showed that both DNA 16mer and LNA403 were more effective than 1% atropine in reducing elongation of eyeball.

Example 6. Confirmation of DNA 16mer by an independent third party

We also used a CRO to confirm our results of DNA 16mer.

Animal model

The procedure of myopia induction is: The 21 days old C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan. All animal experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The procedure of myopia induction is described briefly as the following: Right eyes of the 23 days old mice were covered for 4 weeks to induce myopia and the left eyes were uncovered. Then the right eyes of the myopia-induced mice were treated with 30μL of saline (i.e. negative control group), 1% atropine (i.e. positive control group), 10nM, 100nM, and 1μM of DNA 16mer on 30^{th}, 37^{th} and 44^{th} day. Mice were sacrificed on 51^{th} day and the eyes were collected. The isolated eyes were photographed under a dissection microscope and the axial lengths were measured using imageJ and a proprietary software for automatic measure. Myopia (nearsightedness) causes elongation of axial length, which is the major pathological change in myopia. The difference of axial length (i.e. delta axial length) between the covered right eye and the non-covered left eye of the same mouse indicates the severity of myopia.

The results of the CRO experiments are shown in Table 5 and FIG.5. The 3^{rd} party results completely replicate our data.

Table 5. Delta axial length of mice treated with atropine, antisense oligonucleotides or saline

	n	left eye axial, mm Mean(SEM)		right eye axial, mm Mean(SEM)		Delta axial length Mean(SEM)	
Saline	24	3.135	(0.027)	3.135	(0.019)	-0.001	(0.031)

1% Atropine	24	3.108	(0.018)	3.049	(0.020)	-0.060	(0.022)
DNA 16mer 10nM	24	3.139	(0.013)	3.083	(0.021)	-0.056	(0.020)
DNA 16mer 100nM	24	3.092	(0.017)	3.023	(0.017)	-0.069	(0.022)
DNA 16mer 1μM	24	3.110	(0.018)	3.052	(0.021)	-0.058	(0.021)

Example 7. Confirm DNA 16mer effect on a 2nd animal

Animal model

The procedure of myopia induction: The 3 days old pigmented rabbits were purchased from the DA-ZONG livestock farm, Taiwan. All animal experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. On the age of 7 days, the right eye of the rabbit was covered for 8 weeks to induce myopia, and the left eye left uncovered. The axial lengths (AXL) of both eyes were measured by A-scan (Sonomed®, PACSCAN 300A, USA) once a week since day 21. We defined myopia as right eye AXL is longer than left eye AXL by 0.2mm or longer on day 35. If a rabbit reached this defined criterion, the right eye of such a myopic rabbit was treated with 20μL of saline (i.e. control group) or DNA16mer (20μL) at 10μM or 50uM every other day since day 35 till day 63. The final AXL measured on day 61 was used to obtain to calculate the difference between right AXL and left AXL (i.e. delta AXL). If there is no therapeutic effect, the delta AXL will be a positive value.

The results indicate a dose-dependent response for reducing AXL in myopic eyes (see Table 6 and FIG. 6).

Table 6. Delta axial length of rabbits treated with antisense oligonucleotides or saline

	n	Delta axial length	
		Mean	**SEM**
Saline	15	0.051	0.056
DNA 16mer 10μM	9	-0.234	0.083
DNA 16mer 50μM	4	-0.370	0.068

The invention, and the manner and process of making and using it, are now described in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, to make and use the same. It is to be understood that the foregoing describes preferred embodiments of the present invention and that modifications may be made therein without departing from the scope

of the present invention as set forth in the claims. To particularly point out and distinctly claim the subject matter regarded as invention, the following claims conclude the specification.

P12250EPPC

CLAIMS

1. An antisense oligodeoxyribonucleotide targeting miR-328 in 2 retinal pigment epithelium (RPE) 2 ~~ocular~~ cells, wherein the 2 deoxyribonucleotide sequence of the oligodeoxyribonucleotide is 2 ~~oligodeoxyribonucleotide is a deoxyribonucleotide sequence of~~ SEQ ID NO: 3 or SEQ ID NO: 4.
2. The oligodeoxyribonucleotide according to claim 1, wherein the deoxyribonucleotide sequence of the oligodeoxyribonucleotide is SEQ ID NO: 3.
3. The oligodeoxyribonucleotide according to claim 1, wherein the deoxyribonucleotide sequence of the oligodeoxyribonucleotide is SEQ ID NO: 4.
4. A pharmaceutical composition for preventing or treating an ocular disease, comprising the oligodeoxyribonucleotide according to claim 1 and a pharmaceutically acceptable carrier.
5. The pharmaceutical composition according to claim 4, wherein the ocular disease is myopia.
6. A locked nucleic acid-modified, and phosphorothioate bond-modified oligonucleotide targeting miR-328 in 2 retinal pigment epithelium (RPE) 2 ~~ocular~~ cells, wherein the phosphorothioate bond-modified oligonucleotide has an oligonucleotide sequence of SEQ ID NO: 21, wherein from the 5' end to the 3' end of SEQ ID NO: 21, positions 1, 2, 3, 4, 17, 18, 19 and 20 are modified by locked nucleic acid and every phosphodiester bond of SEQ ID NO: 21 is modified to a phosphorothioate bond.
7. A pharmaceutical composition for preventing or treating an ocular disease, comprising the locked nucleic acid-modified, and phosphorothioate bond-modified oligonucleotide according to claim 6 and a pharmaceutically acceptable carrier.
8. The pharmaceutical composition according to claim 2 7, 2 ~~6,~~ wherein the ocular disease is myopia.

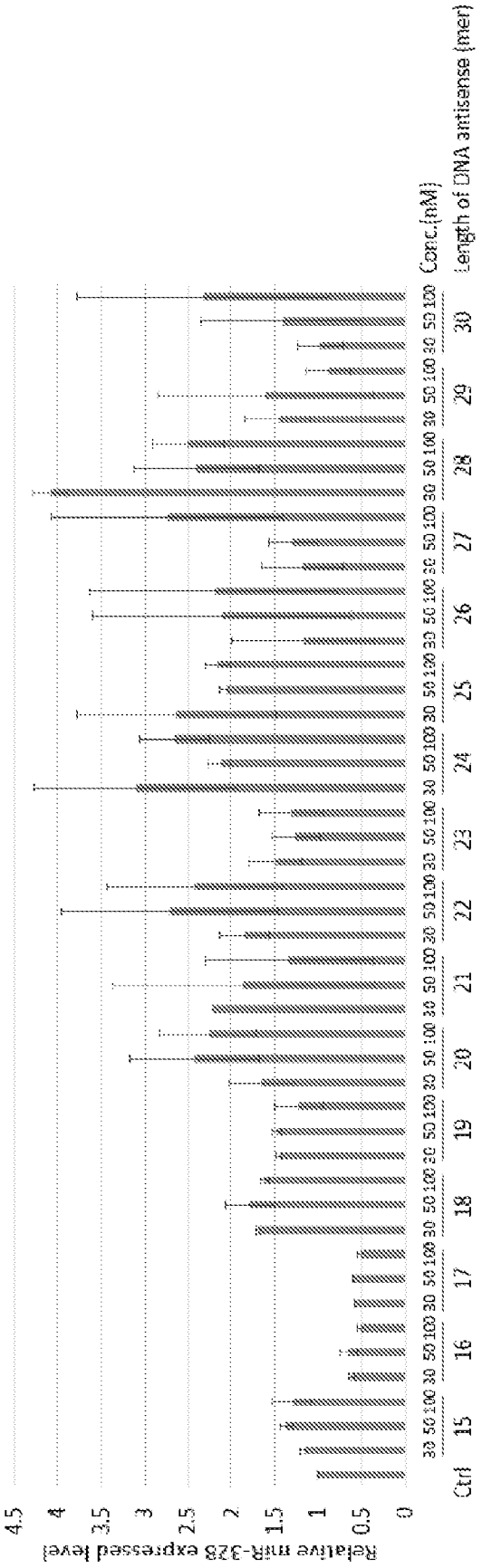
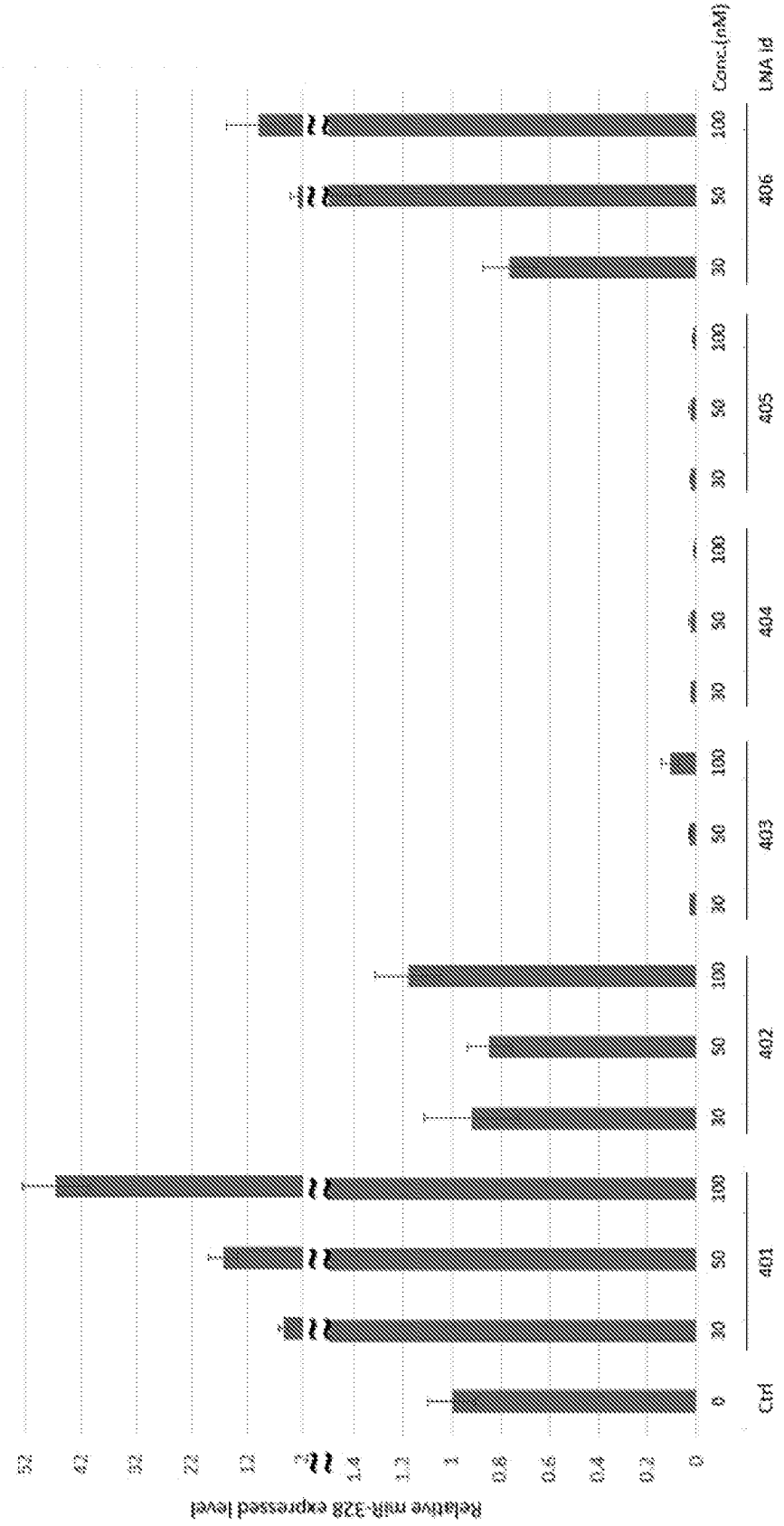

Relative miR-328 expressed level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Ctrl
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
Conc.(nM)
Length of DNA antisense (mer)

Figure 1


Relative miR-328 expressed level
52
42
32
22
12
2
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl
401
402
403
404
405
406
LNA id
Conc.(nM)
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20
50
100
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50
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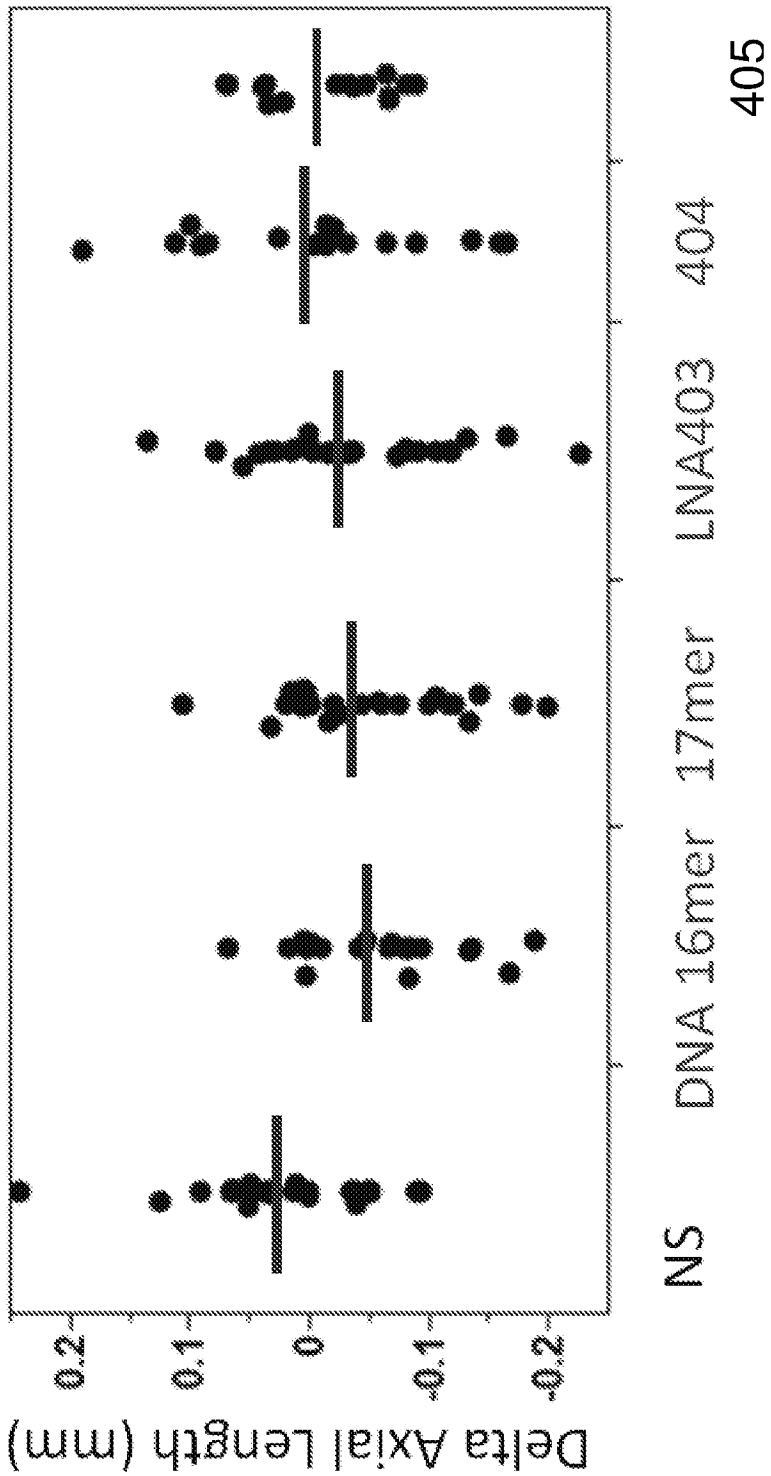
20
50
100
20
50
100
20
50
100

Figure 2


Delta Axial Length (mm)
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0.1
0
-0.1
-0.2
NS
DNA 16mer
17mer
LNA403
404
405

Figure 3

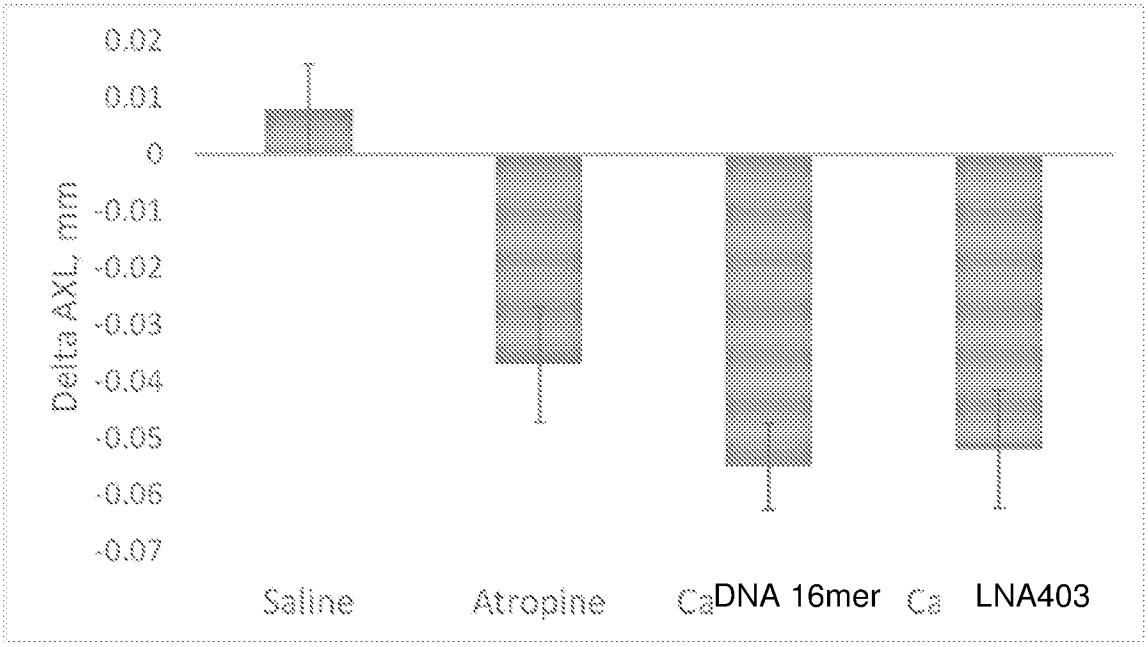

Figure 4

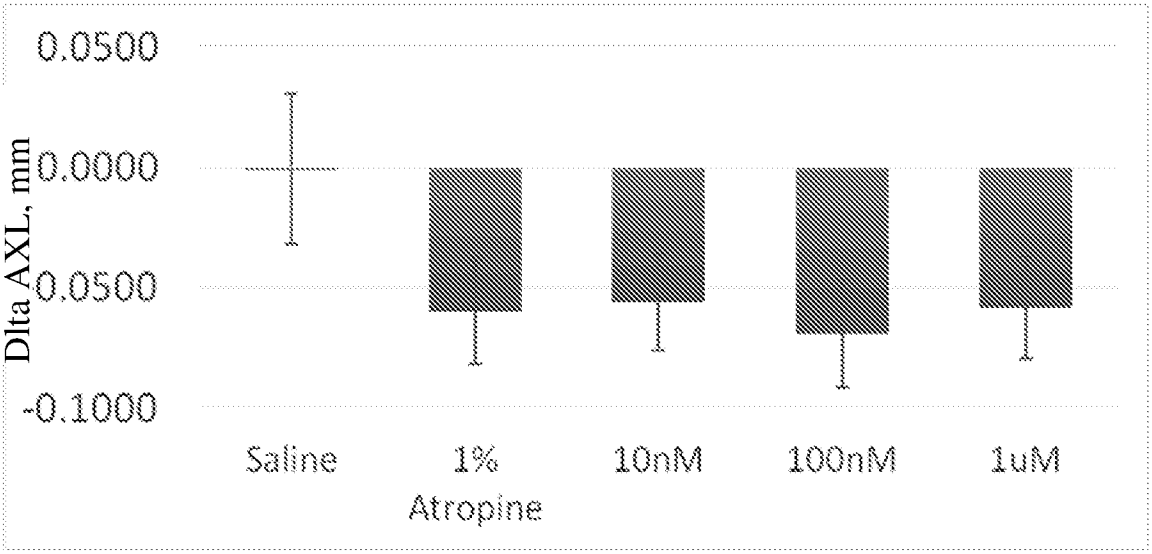

Figure 5

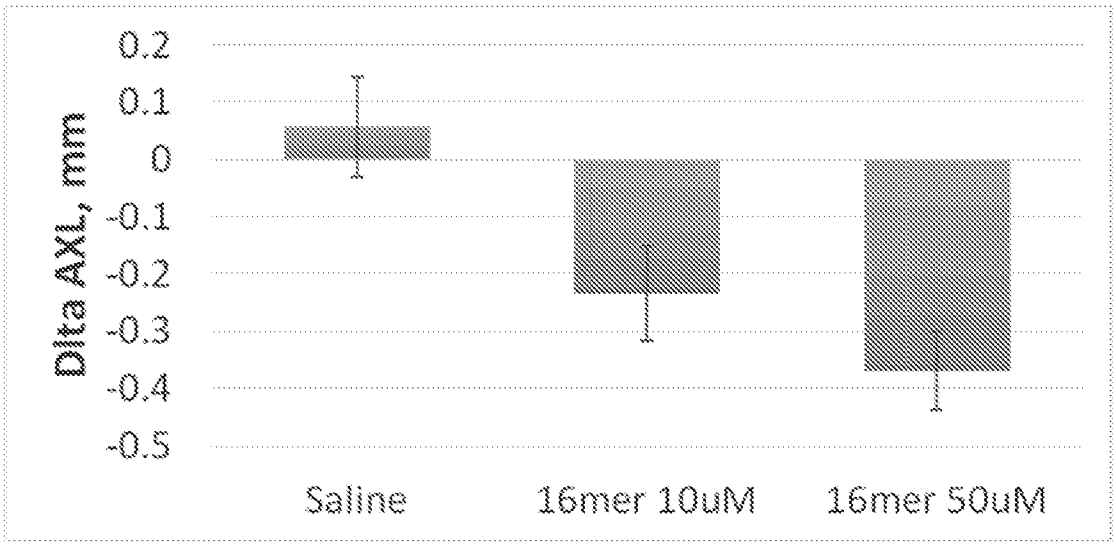

Figure 6