



- (51) **International Patent Classification:**
C12N 15/113 (2010.01) *A61K 31/7088* (2006.01)
- (21) **International Application Number:**
PCT/US2015/012060
- (22) **International Filing Date:**
20 January 2015 (20.01.2015)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (71) **Applicant:** OREGON HEALTH & SCIENCE UNIVERSITY [US/US]; 0690 SW Bancroft St, Portland, Oregon 97239 (US).
- (72) **Inventors:** ZHOU, Zhengfeng; 6450 SW Orchid St, Portland, Oregon 97219 (US). GONG, Qiuming; 6450 SW Orchid St, Portland, Oregon 97219 (US). STUMP, Matthew; 3601 SW River Parkway #1600, Portland, Oregon 97239 (US).
- (74) **Agent:** JACKSON, Jeffrey; 0690 SW Bancroft St, Portland, Oregon 97238 (US).
- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM,

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

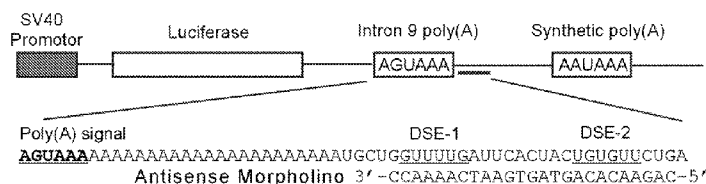
- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR,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

Published:

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

- (54) **Title:** MODULATION OF KCNH2 ISOFORM EXPRESSION BY OLIGONUCLEOTIDES AS A THERAPEUTIC APPROACH FOR LONG QT SYNDROME

Figure 3A



- (57) **Abstract:** Oligonucleotides with activity in preventing poly(A) adenylation at intron 9 of the KCNH2 gene, as well as pharmaceutical compositions comprising the oligonucleotides and methods of using the oligonucleotides to treat long QT syndrome are disclosed. The oligonucleotides include antisense sequences corresponding to sites termed DSE 1 and DSE in intron 9.

TITLE

MODULATION OF KCNH2 ISOFORM EXPRESSION BY OLIGONUCLEOTIDES AS A THERAPEUTIC
APPROACH FOR LONG QT SYNDROME

5

FIELD

Generally, the field is pharmaceutical compositions, more specifically; the field is pharmaceutical compositions comprising oligonucleotides.

ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

10

This invention was made with the support of the United States government under the terms of grant number 2R01 HL68854 awarded by the National Institutes of Health. The United States government has certain rights in this invention.

BACKGROUND

15

20

25

KCNH2 or human ether-a-go-go-related gene 1 (hERG1) encodes the Kv11.1 channel that conducts the rapidly activating delayed rectifier K⁺ current (IKr) in the heart (Warmke JW and Ganetzky B, *Proc Natl Acad Sci U S A* **91**, 3438-3442 (1994); Sanguinetti MC *et al*, *Cell* **81**, 299-307 (1995); Trudeau MC *et al*, *Science* **269**, 92-95 (1995); and Zhou Z *et al*, *Biophys J* **74**, 230-241 (1998); all of which are incorporated by reference herein). Kv11.1 channels are essential for cardiac action potential repolarization and mutations in KCNH2 cause long QT syndrome type 2 (LQT2) (Curran ME *et al*, *Cell* **80**, 795-803 (1995); incorporated by reference herein). Alternative intronic polyadenylation has been shown to direct the expression of two Kv11.1 C-terminal isoforms, the functional Kv11.1a isoform and the non-functional Kv11.1a-USO isoform (Gong Q *et al*, *J Biol Chem* **285**, 32233-32241 (2010); incorporated by reference herein). Kv11.1a is produced by splicing from exon 9 to exon 10 and use of a distal poly(A) site in exon 15, whereas Kv11.1a-USO is generated by the activation of a proximal poly(A) site within intron 9. The last 359 amino acids of Kv11.1a are absent in Kv11.1a-USO and the truncated isoform fails to form functional channels when expressed in mammalian cells

(Kupersmidt S *et al*, J Biol Chem 273, 27231-27235 (1998); incorporated by reference herein). A novel LQT2 mutation that disrupted the alternative processing of KCNH2 intron 9 and resulted in switching the expression of Kv11.1 isoforms from Kv11.1a to Kv11.1a-USO was reported (Gong Q *et al*, *Circ Cardiovasc Genet* **7**, 482-490 (2014); incorporated by reference herein. Thus, the relative expression of Kv11.1a and Kv11.1a-USO isoforms plays an important role in the regulation of Kv11.1 channel function and the pathogenesis of LQT2.

The alternative processing of KCNH2 pre-mRNA is regulated by the relative efficiencies of RNA splicing and polyadenylation events. These events depend on interactions between trans-acting splicing and polyadenylation factors and cis-acting elements present in KCNH2. The poly(A) signal within KCNH2 intron 9 consists of a weak, noncanonical hexamer, AGUAAA (Gong *et al*, 2010 *supra*). When this poly(A) signal is changed to the strong, canonical poly(A) signal, AAUAAA, polyadenylation becomes the dominant reaction, resulting in the predominant expression of Kv11.1a-USO. The elimination of the intron 9 poly(A) signal by the AGUAAA to CGCAAA mutations results in predominant expression of Kv11.1a and an increase in channel current.

SUMMARY

A pharmaceutical composition that acts directly on the poly(A) intron 9 signal of KCNH2 is necessary. One example of such a pharmaceutical composition is an oligonucleotide in an antisense configuration relative to downstream elements of the poly(A) intron 9 signal that facilitate formation of the poly(A) tail.

Disclosed herein are oligonucleotides that include a first sequence of SEQ ID NO: 1 (5'-CAAAAC-3') and a second sequence of SEQ ID NO: 2 (5'-AACACA-3'). The first sequence is the antisense of a site termed DSE-1 herein. The second sequence is the antisense of a site termed DSE-2 herein. In some examples, the oligonucleotide includes both SEQ ID NO: 1 and SEQ ID NO: 2. In still further examples, the oligonucleotide is at least 19 nucleotides in length. In still further examples, the oligonucleotide includes a sequence of SEQ ID NO: 3 (5'-AACACAXXXXXXXXXXCAAAAC-3') wherein X is any nucleic acid. In additional examples, the oligonucleotide includes a sequence of SEQ ID NO: 4 (5'-AACACAGTAGTGAATCAAAAC-3'). In

other examples, the oligonucleotide includes a sequence of SEQ ID NO: 5 (5'-CAGAACACAGTAGTGAATCAAAACC-3'). In more examples, the oligonucleotide consists of a sequence that is exactly SEQ ID NO: 5 with no additional sequence.

Additional modifications can be made to any of the disclosed oligonucleotides including the addition of a locked nucleotide, a G-clamp nucleotide, a nucleotide base analog, a 3'-terminal cap moiety, or a phosphate backbone modification. One example of such a modification is the addition of a morpholino oligonucleotide. In still further examples, all the nucleic acids in the oligonucleotide are morpholino oligonucleotides.

Also disclosed are pharmaceutical compositions comprising an effective amount of the morpholino oligonucleotide of any of claims 1-9 and a pharmaceutically acceptable carrier. These pharmaceutical compositions can be for use in treating long-QT syndrome caused by mutations in the KCNH2 gene.

Also disclosed are methods of treating long-QT syndrome caused by mutations in the KCNH2 gene. These methods involve administering to the subject the disclosed pharmaceutical compositions, thereby treating the long-QT syndrome.

A fully enabling disclosure was made by the inventors in Gong *et al*, *J Mol Cell Cardiol* **76**, 26-32 (14 August 2014) which is incorporated by reference herein.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1A is a diagram of the KCNH2 minigene luciferase reporter construct described herein and includes the sequence of downstream elements of the KCNH2 intron 9 poly(A) signal. Downstream element deletions and mutations are indicated.

Figure 1B is a histogram showing the effect of downstream element deletion and mutations on luciferase activity (n= 3–6).

Figure 2A is a diagram of the structure of the short KCNH2 gene construct described herein and includes the sequence of downstream elements of the KCNH2 intron 9 poly(A) signal. DSE-1 and DSE-2 mutations are indicated.

Figure 2B is an image of a gel resulting from an RNase protection assay analysis of mRNA in Flp-In HEK293 cells stably expressing WT and DSE mutant short KCNH2 genes. Signals

of Kv11.1 isoforms were quantified and shown as a percentage of the total signal (1a + 1a-USO) (mean $\pm$ SD, n= 3).

Figure 2C is an image of an immunoblot analysis of Kv11.1 protein expressed in cells expressing WT and DSE mutant short KCNH2 genes. The expression level of hygromycin B phosphotransferase (HPH) encoded by hygromycin B resistant gene served as a loading control. Signals were quantified and shown as isoform percentage of total (1a + 1a-USO) Kv11.1 protein (mean $\pm$ SD, n = 3).

Figure 2D is a set of two plots showing representative currents recorded from cells stably expressing WT and Mut1 + 2 short KCNH2 genes.

Figure 3A is a diagram of the tandem poly(A) signal construct and the sequence of a morpholino oligonucleotide that includes SEQ ID NO: 1.

Figure 3B is an image of a gel resulting from RNase protection assay analysis of relative usage of intron 9 poly(A) signal and synthetic poly(A) signal following the treatment with invert or antisense MO.

Figure 3C is a histogram quantifying results of RNase protection assays as described in Figure 3B (mean $\pm$ SD, n = 3).

Figure 4A is an image of a gel resulting from an RNase protection assay showing the effect of the antisense morpholino oligonucleotide comprising SEQ ID NO: 1 on Kv11.1 isoform expression. Signals were quantified and shown as an isoform percentage of the total signal (1a + 1a-USO, mean $\pm$ SD, n = 3).

Figure 4B is an image of an immunoblot analysis showing the concentration dependence of antisense morpholino oligonucleotide comprising SEQ ID NO: 1 on protein expression of Kv11.1 isoforms. Signals were quantified and shown as an isoform percentage of total (1a + 1a-USO) Kv11.1 protein (mean $\pm$ SD, n = 3).

Figure 4C is an image of an immunoblot showing the effect of treatment with antisense morpholino oligonucleotide comprising SEQ ID NO: 1 on expression of Kv11.1 isoforms for the indicated amounts of time. Signals were quantified and shown as an isoform percentage of total (1a + 1a-USO) Kv11.1 protein (mean $\pm$ SD, n= 3).

Figure 5A is a set of two plots showing representative currents recorded from Flp-In HEK293 cells stably expressing short KCNH2 gene following treatment with 10 μ M negative control or antisense morpholino oligonucleotide comprising SEQ ID NO: 1 for 48 h.

Figure 5B is an I-V plot of tail current density measured at -50 mV following test voltages from -70 to +50 mV for negative control (■) and antisense morpholino oligonucleotide comprising SEQ ID NO: 1 (●).

Figure 5C is a plot of the activation curves for negative control (■) and antisense morpholino oligonucleotide comprising SEQ ID NO: 1 (●).

Figure 6A is an image of a gel resulting from an RNase protection assay using mRNA from cells stably expressing the short KCNH2 gene containing the canonical poly(A) signal following treatment with 10 μ M negative control or antisense morpholino oligonucleotide comprising SEQ ID NO: 1. Signals were quantified and shown as an isoform percentage of the total signal (1a+ 1a-USO, n=3).

Figure 6B is an image of an immunoblot analysis showing treatment with antisense morpholino oligonucleotide comprising SEQ ID NO: 1 at the indicated concentrations. Signals were quantified and shown as an isoform percentage of total (1a + 1a-USO) Kv11.1 protein (mean $\pm$ SD, n = 3).

Figure 6C is a set of two plots showing representative currents recorded from Flp-In HEK293 cells stably expressing short KCNH2 gene containing the canonical poly(A) signal following treatment with 10 μ M negative control or antisense morpholino oligonucleotide comprising SEQ ID NO: 1 for 48 h.

SEQUENCE LISTING

SEQ ID NO: 1 is a sequence that blocks the DSE-1 site of intron 9 of human KCNH2
5'-CAAAAC-3'

SEQ ID NO: 2 is a sequence that blocks the DSE-2 site of intron 9 of human KCNH2
5'-AACACA-3'

SEQ ID NO: 3 is the sequence of an oligonucleotide that blocks both DSE-1 and DSE-2
5'-AACACAXXXXXXXXXXCAAAAC-3'

SEQ ID NO: 4 is the sequence of an oligonucleotide that blocks both DSE-1 and DSE-2
5'-AACACAGTAGTGAATCAAAAC-3'

SEQ ID NO: 5 is the sequence of an oligonucleotide that blocks DSE-1 and DSE-2 of the
KCNH2 gene and includes intervening sequence. 5'-CAGAACACAGTAGTGAATCAAAACC-3'. It is
5 also called Antisense MO or Anti MO herein.

SEQ ID NO: 6 is the sequence of an inverted morpholino oligonucleotide that acts as a
negative control. 5'-CCAAACTAAGTGATGACACCAAGAC-3'. It is also called Invert MO or Inv MO
herein.

DETAILED DESCRIPTION

10 Terms

Unless otherwise explained, all technical and scientific terms used herein have the same
meaning as commonly understood by one of ordinary skill in the art to which this disclosure
belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly
indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context
15 clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes,
and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are
approximate, and are provided for description. Although methods and materials similar or
equivalent to those described herein can be used in the practice or testing of this disclosure,
suitable methods and materials are described below. The term "comprises" means "includes."
20 In addition, the materials, methods, and examples are illustrative only and not intended to be
limiting.

Administration: To provide or give a subject an agent, such as a composition comprising
therapeutic oligonucleotides by any effective route. Exemplary routes of administration
include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal,
25 intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and
inhalation routes. **Binding or stable binding:** An association between two substances or
molecules, such as the association between an antisense nucleic acid to a sense nucleic acid.
Binding can be detected by any procedure known to one skilled in the art, such as by physical or

functional properties. For example, binding can be detected functionally by determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription, translation, protein activity, and the like.

Contacting: Placement in direct physical association, including contacting of a solid with
5 a solid, a liquid with a liquid, a liquid with a solid, or either a liquid or a solid with a cell or tissue, whether in vitro or in vivo. Contacting can occur in vitro with isolated cells or tissue or in vivo by administering to a subject.

Effective amount: An amount of agent, such as an antisense oligonucleotide that is sufficient to generate a desired response, such as reducing or eliminating a sign or symptom of
10 a condition or disease, such as long-QT syndrome. In some examples, an “effective amount” is one that treats (including prophylaxis) one or more symptoms and/or underlying causes of any of a disorder or disease, for example long-QT syndrome. An effective amount can be a therapeutically effective amount, including an amount that prevents one or more signs or symptoms of a particular disease or condition from developing, such as one or more signs or
15 symptoms associated with long-QT syndrome.

Inhibiting or treating a disease: Inhibiting the full development of a disease or condition, for example, in a subject who has or who is at risk for a disease such as long-QT syndrome. “Treatment” refers to any therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition. The term “ameliorating,” with reference to a
20 disease or pathological condition, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of metastases, an improvement in the overall health or well-being of the subject, or by other clinical or
25 physiological parameters associated with a particular disease. A “prophylactic” treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology. A “therapeutic”

treatment is a treatment administered after the development of significant signs or symptoms of the disease.

Long QT Syndrome: Long QT Syndrome is a heart rhythm disorder that can potentially cause fast, chaotic heartbeats that can trigger fainting or seizures. Long QT can arise as the
5 result of one or more genomic mutations including mutations in the KCNH2 gene.

Mutation: A mutation is any difference in a nucleic acid or polypeptide sequence from a normal, consensus or "wild type" sequence. A mutant is any protein or nucleic acid sequence comprising a mutation. In addition a cell or an organism with a mutation may also be referred to as a mutant.

10 Some types of mutations include point mutations (differences in individual nucleotides or amino acids); silent mutations (differences in nucleotides that do not result in an amino acid changes); deletions (differences in which one or more nucleotides or amino acids are missing); frameshift mutations (differences in which deletion of a number of nucleotides indivisible by 3 results in an alteration of the amino acid sequence.

15 **Sequence identity/similarity:** The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative
20 amino acid substitutions); the higher the percentage, the more similar the sequences are.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, Adv. Appl. Math. 2:482, 1981; Needleman & Wunsch, J. Mol. Biol. 48:443, 1970; Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444, 1988; Higgins & Sharp, Gene, 73:237-44, 1988; Higgins & Sharp, CABIOS
25 5:151-3, 1989; Corpet et al., Nuc. Acids Res. 16:10881-90, 1988; Huang et al. Computer Appls. In the Biosciences 8, 155-65, 1992; and Pearson et al., Meth. Mol. Bio. 24:307-31, 1994. Altschul et al., J. Mol. Biol. 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., J. Mol. Biol. 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1154 nucleotides is 75.0 percent identical to the test sequence ($1166 \div 1554 * 100 = 75.0$). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (that is, $15 \div 20 * 100 = 75$).

Subject: A living multicellular vertebrate organism, a category that includes, for example, mammals and birds. A "mammal" includes both human and non-human mammals, such as mice. In some examples, a subject is a patient, such as a patient diagnosed with long-QT syndrome.

Oligonucleotides

An oligonucleotide may be chemically synthesized. Synthesis of a single stranded nucleic acid makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale
5 syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 micromolar scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides. Alternatively, syntheses at the 0.2 micromolar scale can be performed on a 96-well plate synthesizer from Protogene. However, a larger or smaller scale of synthesis is encompassed by the invention, including any method of synthesis now known or yet to be disclosed. Suitable reagents for synthesis of the
10 oligonucleotides disclosed herein are known to those of skill in the art.

A single stranded oligonucleotide can comprise a modified nucleotide. Examples of modified nucleotides include, but are not limited to, nucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a conformation such as those described in, for
15 example in Sanger, *Principles of Nucleic Acid Structure*, Springer- Verlag Ed. (1984), are also suitable for use in oligonucleotides. Other modified nucleotides include, without limitation: locked nucleic acid (LNA) nucleotides, G-clamp nucleotides, or nucleotide base analogs. LNA nucleotides include but need not be limited to 2'-O, 4'-C-methylene-(D-ribofuranosyl)nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl
20 nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2Cl) nucleotides, and 2'-azido nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (Lin *et al*, *J Am Chem Soc*, **120**, 8531-8532 (1998)). Nucleotide base analogs include for example, C-phenyl, C-naphthyl, other aromatic
25 derivatives, inosine,azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (Loakes, *Nucl Acids Res*, **29**, 2437-2447 (2001)).

An oligonucleotide may comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of classes of terminal

cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β -D-erythrofuransyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α -nucleotides, modified base nucleotides, threo pentofuransyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminoethyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5' phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or nonbridging methylphosphonate or 5'-mercapto moieties (see, e.g., U.S. Pat. No. 5,998,203; Beaucage *et al*, *Tetrahedron* **49**, 1925 (1993)).

Non-limiting examples of phosphate backbone modifications (i.e., resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, peptide nucleic acid, and alkylsilyl substitutions (see, e.g., Hunziker *et al*, *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al*, *Antisense Research*, ACS, 24-39 (1994); Bennett CF and Swayze EE, *Ann Rev Pharmacol Toxicol* **50**, 259-293 (2010); incorporated by reference herein). Additional examples of modified nucleotides and types of chemical modifications that can be introduced into the modified oligonucleotides of the present invention are described, e.g., in UK Patent No. GB 2,397,818 B and U.S. Patent Publication Nos. 20040192626 and 20050282188.

An oligonucleotide can comprise one or more non-nucleotides. A non-nucleotide may be any subunit, functional group, or other molecular entity capable of being incorporated into a nucleic acid chain in the place of one or more nucleotide units that is not or does not comprise a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine, such as a sugar or phosphate.

Chemical modification of the oligonucleotide may also comprise attaching a conjugate to the oligonucleotide molecule. The conjugate can be attached at the 5'-and/or the 3'-end of an oligonucleotide via a covalent attachment such as a nucleic acid or non-nucleic acid linker. The conjugate can also be attached to the oligonucleotide through a carbamate group or other
5 linking group (see, e.g., U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). A conjugate may be added to the oligonucleotide for any of a number of purposes. For example, the conjugate may be a molecular entity that facilitates the delivery of the oligonucleotide into a cell or the conjugate a molecule that comprises a drug or label.

Examples of conjugate molecules suitable for attachment to the disclosed
10 oligonucleotides include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (e.g., folic acid, folate analogs and derivatives thereof), sugars (e.g., galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, etc.), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and
15 combinations thereof (see, e.g., U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Pat. No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Other examples
20 include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidinium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples of conjugate molecules include a hydrophobic group, a membrane active compound, a cell penetrating compound, a cell targeting signal, an interaction modifier, or a steric stabilizer
25 as described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739.

The type of conjugate used and the extent of conjugation to the oligonucleotide can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the

oligonucleotide while retaining activity. As such, one skilled in the art in light of this disclosure can screen oligonucleotides having various conjugates attached thereto to identify oligonucleotide conjugates having improved properties using any of a variety of well-known in vitro cell culture or in vivo animal models, such as for long-QT syndrome caused by mutations in the KCNH2 gene.

Morpholino oligonucleotides

A morpholino oligonucleotide (MO) is a polymeric molecule having a backbone which supports bases capable of hydrogen bonding to typical polynucleotides, wherein the polymer lacks a pentose sugar backbone moiety, and more specifically a ribose backbone linked by phosphodiester bonds which is typical of nucleotides and nucleosides. The morpholino oligonucleotide instead contains a ring nitrogen with coupling through the ring nitrogen.

Morpholino oligonucleotides are structures from 12 to 25 nucleotides, including a targeting base sequence that is complementary to a target region of a selected preprocessed mRNA such as preprocessed intron 9 of KCNH2. The morpholino antisense oligonucleotide inhibits formation of a poly(A) signal at a site within intron 9 and thereby results in the proper processing of the mRNA to form KCNH2 protein.

The antisense compound employed in the present invention is one that does not activate RNase H. RNase-H active oligomers, of which phosphorothioate oligonucleotides are the most prominent example, operate primarily by a mechanism in which the target mRNA is cleaved. RNase-incompetent oligomers, on the other hand, are believed to act by a steric blocking mechanism. Such compounds include morpholino oligonucleotides, PNA's (peptide nucleic acids), methylphosphonates, and 2'-O-alkyl or -allyl modified oligonucleotides, all of which are known in the art. The morpholino oligonucleotides are composed of morpholino subunits of the form shown in U.S. Pat. Nos. 5,698,685, 5,217,866, 5,142,047, 5,034,506, 5,166,315, 5,521,063, and 5,506,337. The synthesis, structures, and binding characteristics of morpholino oligonucleotides are detailed in these patents so that one of skill in the art in light of this disclosure can create the morpholino oligonucleotides with the sequences disclosed herein.

In a morpholino oligonucleotide, (i) the morpholino groups are linked together by uncharged phosphorus-containing linkages, one to three atoms long, joining the morpholino nitrogen of one subunit to the 5' exocyclic carbon of an adjacent subunit, and (ii) the base attached to the morpholino group is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. The purine or pyrimidine base-pairing moiety is typically adenine, cytosine, guanine, uracil or thymine. Preparation of such oligomers is described in detail in U.S. Pat. No. 5,185,444 (Summerton and Weller, 1993), which is hereby incorporated by reference in its entirety. As shown in the reference, several types of nonionic linkages may be used to construct a morpholino backbone.

Such morpholino oligonucleotides have shown high binding affinity for RNA targets, and the uncharged backbone favors uptake into cells and reduces non-specific binding interactions, relative to charged analogs such as phosphorothioates. They have been shown to provide significantly improved activity and selectivity in inhibiting translation of targeted sequences in comparison to phosphorothioate oligonucleotides. See, for example, Summerton et al., *Antisense & Nucleic Acid Drug Dev.* 7, 63-70, (1997). The morpholino oligonucleotides have very high nuclease resistance and good water solubility, making them good candidates for in vivo use.

The solubility of the morpholino oligonucleotides, and the ability of the compound to resist precipitation on storage in solution, can be further enhanced by derivatizing the oligomer with a solubilizing moiety, such as a hydrophilic oligomer, or a charged moiety, such as a charged amino acid or organic acid. The moiety may be any biocompatible hydrophilic or charged moiety that can be coupled to the antisense compound and that does not interfere with compound binding to the target sequence. The moiety can be chemically attached to the antisense compound, e.g., at its 5' end, by well-known derivatization methods. One preferred moiety is a defined length oligo ethylene glycol moiety, such as triethyleneglycol, coupled covalently to the 5' end of the antisense compound through a carbonate linkage, via a piperazine linking group forming a carbamate linkage with triethyleneglycol, where the second piperazine nitrogen is coupled to the 5'-end phosphorodiamidate linkage of the antisense.

Alternatively, or in addition, the compound may be designed to include one a small number of charged backbone linkages, such as a phosphodiester linkage.

The compound is designed to hybridize to the target sequence under physiological conditions with a T_m substantially greater than 37° C., *e.g.*, at least 50° C. and preferably 60° C-
5 80° C. Although the compound may not be necessarily 100% complementary to the target sequence, so long as it is effective to stably and specifically bind to the target sequence such that formation of a poly(A) at intron 9 is inhibited. The appropriate length of the oligomer to allow stable, effective binding combined with good specificity is about 8 to 40 nucleotide base units, and preferably about 12-25 base units. Mismatches, if present, are less destabilizing
10 toward the end regions of the hybrid duplex than in the middle. Oligomer bases that allow degenerate base pairing with target bases are also contemplated, assuming base-pair specificity with the target and inhibition of poly(A) formation at intron 9 is maintained.

Because morpholino oligonucleotides have bases that are analogs of those of nucleic acids, a description of a morpholino oligonucleotide as having a particular sequence (or a
15 homolog thereof) is a recitation of the structure of the morpholino oligonucleotide.

Chemical modification of the morpholino oligonucleotide can involve attaching a conjugate to the morpholino oligonucleotide. The conjugate can be attached at the 5'- and/or the 3'-end of the sense and/or the antisense strand of the oligonucleotide via a covalent attachment such as a nucleic acid or non-nucleic acid linker. The conjugate can also be attached
20 to the morpholino oligonucleotide through a carbamate group or other linking group (see, *e.g.*, U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). A conjugate can be added to the morpholino oligonucleotide for any of a number of purposes. For example, the conjugate may be a molecular entity that facilitates the delivery of the morpholino oligonucleotide into a cell or the conjugate a molecule that comprises a drug or label.

25 Examples of conjugate molecules suitable for attachment to a morpholino oligonucleotide include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.*, folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose,

galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, etc.), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (see, e.g., U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Pat. No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidinium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples of conjugate molecules include a hydrophobic group, a membrane active compound, a cell penetrating compound, a cell targeting signal, an interaction modifier, or a steric stabilizer as described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739.

The type of conjugate used and the extent of conjugation to the morpholino oligonucleotide can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the morpholino oligonucleotide while retaining activity. As such, one skilled in the art can screen oligonucleotides having various conjugates attached thereto to identify oligonucleotide conjugates having improved properties using any of a variety of well-known in vitro cell culture or in vivo animal models.

Pharmaceutical Compositions

A pharmaceutical composition may be any chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. A pharmaceutical composition can include a therapeutic agent, a diagnostic agent or a pharmaceutical agent. A therapeutic agent is one that alone or together with one or more additional compounds induces the desired response (such as inducing a therapeutic effect when administered to a subject). In a particular example, a pharmaceutical agent is an agent that significantly reduces one or more symptoms associated with long-QT syndrome. One

example is a pharmaceutical composition comprising an oligonucleotide comprising a sequence of SEQ ID NO: 1 and/or a sequence of SEQ ID NO: 2 that is sufficient to block formation of a poly(A) signal in intron 9 of KCNH2.

5 A pharmaceutically acceptable carrier (interchangeably termed a vehicle) can be any material or molecular entity that facilitates the administration or other delivery of the morpholino oligonucleotides described herein. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, 10 aqueous dextrose, glycerol or the like as a vehicle. In a particular embodiment the carrier is one that allows trafficking of the morpholino oligonucleotide to the heart or one that allows the morpholino oligonucleotide to be taken up by the heart.

An effective amount or concentration of a compound may be any amount of a composition that alone, or together with one or more additional therapeutic agents is sufficient 15 to achieve a desired effect in a subject, or in a cell being treated with the agent. The effective amount of the agent will be dependent on several factors, including, but not limited to, the subject or cells being treated and the manner of administration of the therapeutic composition. In one example, an effective amount or concentration is one that is sufficient to prevent advancement, delay progression, or to cause regression of a disease, or which is capable of 20 reducing symptoms caused by any disease, including long-QT syndrome. In one example, a desired effect is to reduce or inhibit one or more symptoms associated with long-QT syndrome. The one or more symptoms do not have to be completely eliminated for the composition to be effective. For example, a composition can decrease the sign or symptom by a desired amount, for example by at least 20%, at least 50%, at least 80%, at least 90%, at least 95%, at least 98%, 25 or even at least 100%, as compared to the sign or symptom in the absence of the composition. A therapeutically effective amount of a pharmaceutical composition can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the therapeutically effective amount can depend on the subject being treated, the severity and

type of the condition being treated, and the manner of administration. For example, an effective amount of such agent can vary from about 100 µg -10 mg per kg body weight if administered intravenously.

The actual dosages will vary according to factors such as the particular status of the subject (for example, the subject's age, size, fitness, extent of symptoms, susceptibility factors, and the like) time and route of administration, other drugs or treatments being administered concurrently, as well as the specific pharmacology of treatments for long-QT syndrome for eliciting the desired activity or biological response in the subject. Dosage regimens can be adjusted to provide an optimum prophylactic or therapeutic response.

An effective amount is also one in which any toxic or detrimental side effects of the compound and/or other biologically active agent is outweighed in clinical terms by therapeutically beneficial effects. A non-limiting range for a therapeutically effective amount of treatments for long-QT syndrome within the methods and formulations of the disclosure is about 0.0001 µg/kg body weight to about 10 mg/kg body weight per dose, such as about 0.0001 µg/kg body weight to about 0.001 µg/kg body weight per dose, about 0.001 µg/kg body weight to about 0.01 µg/kg body weight per dose, about 0.01 µg/kg body weight to about 0.1 µg/kg body weight per dose, about 0.1 µg/kg body weight to about 10 µg/kg body weight per dose, about 1 µg/kg body weight to about 100 µg/kg body weight per dose, about 100 µg/kg body weight to about 500 µg/kg body weight per dose, about 500 µg/kg body weight per dose to about 1000 µg/kg body weight per dose, or about 1.0 mg/kg body weight to about 10 mg/kg body weight per dose.

The effective amount can be selected based on the mode of delivery, for example, trans-epidermal, rectal, oral, pulmonary, intranasal delivery, intravenous or subcutaneous delivery.

Determination of effective amount is typically based on animal model studies followed up by human clinical trials and is guided by administration protocols that significantly reduce the occurrence or severity of targeted disease symptoms or conditions in the subject. Suitable models in this regard include, for example, murine, rat, porcine, feline, non-human primate,

and other accepted animal model subjects known in the art. Alternatively, effective dosages can be determined using in vitro models (for example, mouse models of long-QT syndrome). Using such models, only ordinary calculations and adjustments are required to determine an appropriate concentration and dose to administer a therapeutically effective amount of the treatments for long-QT syndrome (for example, amounts that are effective to alleviate one or more symptoms of long-QT syndrome).

Treatment of long-QT Syndrome

Disclosed herein include methods of treating a subject that has or may have long QT syndrome comprising administering a pharmaceutical composition comprising a morpholino oligonucleotide of SEQ ID NO: 1 or a homolog thereof to the subject. The subject may be treated therapeutically or prophylactically.

The administration of pharmaceutical compositions for treatment of long-QT syndrome can be for either prophylactic or therapeutic purposes. When provided prophylactically, the pharmaceutical composition is provided in advance of any clinical symptom of long-QT syndrome. Prophylactic administration serves to prevent or ameliorate any subsequent disease process. When provided therapeutically, the compounds are provided in response to symptoms of the disease. For prophylactic and therapeutic purposes, the pharmaceutical compositions described herein can be administered to the subject in a single bolus delivery, via continuous delivery (for example, continuous transdermal, mucosal or intravenous delivery) over an extended time period, or in a repeated administration protocol (for example, by an hourly, daily or weekly, repeated administration protocol) or as repeated doses within a prolonged prophylaxis or treatment regimen that will yield clinically significant results to alleviate one or more symptoms or detectable conditions associated with long-QT syndrome in the subject.

A subject can be any multi-cellular vertebrate organism, a category that includes human and non-human mammals, such as mice. In some examples a subject is a male. In some examples a subject is a female. Further types of subjects to which the pharmaceutical composition may be properly administered include subjects known to have long-QT syndrome

(through, for example, a molecular diagnostic test or clinical diagnosis,) subjects having a predisposition to long-QT syndrome or subjects displaying one or more symptoms of long-QT syndrome.

Administration of the pharmaceutical composition can be by any method of providing or giving a subject a pharmaceutical composition, by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Treating a subject encompasses any therapeutic intervention that can ameliorate a sign or symptom of a disease or pathological condition after it has begun to develop, whether or not the subject has developed symptoms of the disease. Ameliorating, with reference to a disease, pathological condition or symptom refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of relapses of the disease, an improvement in the memory and/or cognitive function of the subject, a qualitative improvement in symptoms observed by a clinician or reported by a patient, or by other parameters well known in the art that are specific to long-QT syndrome.

A symptom may be any subjective evidence of disease or of a subject's condition, for example, such evidence as perceived by the subject; a noticeable change in a subject's condition indicative of some bodily or mental state. A sign may be any abnormality indicative of disease, discoverable on examination or assessment of a subject. A sign is generally an objective indication of disease.

EXAMPLES

The following examples are illustrative of disclosed methods. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other examples of the disclosed method would be possible without undue experimentation.

Example 1 – Inhibition of Intronic Polyadenylation with Antisense Morpholino Oligonucleotides

Inhibition of cis acting elements in intron 9 of human KCNH2 using antisense morpholino oligonucleotides (MO) results in the further inhibition of Kv11.1a-USO expression, allowing the transcript to be processed to the functional Kv11.1a isoform. Surprisingly, expression of the functional Kv11.1a isoform is upregulated by this antisense oligonucleotide inhibition of KCNH2 intronic polyadenylation.

Generation of a minigene luciferase reporter construct

A minigene luciferase reporter construct was generated by subcloning the Renilla luciferase gene downstream of a splicing competent KCNH2 minigene comprising KCNH2 genomic DNA from exon 8 to exon 11. The construction of the KCNH2 minigene has been previously described in Gong Q *et al*, *J Mol Cell Cardiol* **44**, 502-509 (2008) which is incorporated by reference herein. The N-terminus of the minigene was Myc tagged. The Myc tag was inserted in-frame with the KCNH2 and luciferase translation sequence. Expression of the minigene luciferase reporter is driven by a CMV promoter. The vector also contains the firefly luciferase gene driven by the SV40 promoter, which was used as a control for transfection efficiency. The deletion and mutations of U/GU-rich elements downstream of the KCNH2 intron 9 poly(A) signal were performed using the pAlter® in vitro mutagenesis system (Promega, Madison, WI). HEK293 cells were transiently transfected with the minigene luciferase reporter construct using the Effectene® method (Qiagen, Valencia, CA). After 24 hours, cells were harvested and assayed for both firefly and Renilla luciferase activity using the Dual-Luciferase assay kit (Promega). Data were analyzed by normalizing Renilla luciferase activity to firefly luciferase activity and presented as mean ± SEM.

Generation of a short KCNH2 gene and stable transfection in Flp-In HEK293 cells

The generation of a short KCNH2 gene construct, in which the two longest introns, intron 2 (14.9 kb) and intron 5 (4.4 kb), were shortened to 600 bp has been previously described (Gong Q *et al*, *Circ Cardiovasc Genet* **7**, 482-490 (2014); incorporated by reference herein). Mutations were introduced into the short KCNH2 gene by the pAlter® in vitro

mutagenesis system. Stably transfected Flp-In HEK293 cells were generated by the co-transfection of the KCNH2 gene constructs (0.1 µg) with the Flp recombinase expression vector pOG44 (0.9 µg) using the Effectene® method and selected with 100 µg/ml hygromycin.

Generation of a tandem KCNH2 poly(A) signal construct

5 The generation of a tandem poly(A) signal construct was previously described in Gong Q *et al*, *J Biol Chem* **285**, 32233-32241 (2010); which incorporated by reference herein. The construct contained the SV40 promoter, the firefly luciferase gene, and 308 bp of KCNH2 intron 9 followed by a synthetic poly(A) signal. HEK293 cells were transiently transfected with the tandem poly(A) construct as described in Gong *et al* 2010 *supra*.

Antisense morpholino oligonucleotide generation and use

10 Morpholino oligonucleotides were synthesized by Gene Tools (Philomath, OR). The KCNH2 antisense morpholino oligonucleotide was designed to target a 25 nt sequence within KCNH2 intron 9 containing U/GU-rich elements (underlined) essential for the activation of the intron 9 poly(A) signal, 5'-CAGAACACAGTAGTGAATCAAAACC-3'. An inverted morpholino
15 oligonucleotide with the same sequence but in a reverse orientation was used as a control 5'-CCAAACTAAGTGATGACACAAGAC-3'. The Endo-Porter® delivery system (Gene Tools) was used to deliver antisense and control morpholino oligonucleotides into the cells.

RNAse Protection Assay

20 RNA isolation and an RNase protection assay (RPA) were performed as previously described in Gong *et al*, 2006 *supra*. Briefly, antisense RNA riboprobes were transcribed *in vitro* in the presence of biotin-14-CTP. Yeast RNA was used as a control for the complete digestion of the probes by RNase. The relative intensity of each band was quantified using ImageJ® software and adjusted for the number of biotin-labeled cytidines in each protected fragment. The
25 expression level of the hygromycin B resistance gene from the KCNH2 gene constructs was used to normalize the relative expression of Kv11.1 isoforms.

Immunoblot analysis

 Immunoblot analysis was performed as previously described in Gong *et al*, 2014 *supra*. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then

electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated with an anti-Kv11.1 antibody against the N-terminus of Kv11.1a and Kv11.1a-USO proteins (H-175, Santa Cruz, Santa Cruz, CA) at a 1:600 dilution and visualized with the ECL detection kit (Amersham, Piscataway, NJ). The expression level of hygromycin B phosphotransferase (HPH) encoded by hygromycin B resistance gene was used as loading control (Gong et al 2010 *supra*).

Patch-clamp recordings

Membrane currents were recorded in whole cell configuration using suction pipettes as previously described in Zhou *et al*, 1998 *supra*. The bath solution contained 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). The pipette solution contained 130 mM KCl, 1 mM MgCl₂, 5 mM EGTA, 5 mM MgATP, and 10 mM HEPES (pH 7.2 with KOH). All patch-clamp experiments were performed at 22–23 °C. Kv11.1 current was activated by depolarizing steps between –70 and +50 mV from a holding potential of –80 mV and Kv11.1 tail current was recorded following repolarization to –50 mV. The patch-clamp data are presented as mean ± SEM and analyzed by Student's t-test. P < 0.05 is considered statistically significant.

Intron 9 poly(A) signal downstream elements are required for Kv11.1 alternative polyadenylation

Polyadenylation sites are primarily defined by a hexameric poly(A) signal AAUAAA or other close variants (Tian B and Manley JL, *Trends Biochem Sci* **38**, 312-320 (2013); incorporated by reference herein). In addition to the poly(A) signal, a cis-acting, U/GU-rich downstream element (DSE) is also required for the formation of the poly(A) tail. We have reported that alternative polyadenylation within KCNH2 intron 9 is directed by a noncanonical poly(A) signal AGUAAA (Gong et al 2010 *supra*). A reporter construct was designed to identify cis-acting elements required for intron 9 polyadenylation by subcloning the Renilla luciferase gene downstream of a splicing competent minigene composed of KCNH2 genomic DNA from exon 8 to exon 11. In this reporter construct, the removal of intron 9 generated active luciferase and polyadenylation of intron 9 resulted in no luciferase activity (Figure 1A). When 107 nucleotides

downstream of the poly(A) signal in the minigene luciferase reporter construct were deleted, the deletion significantly increased the luciferase activity (Figure 1B, d-DS). This indicates that the downstream region contains elements that are required for polyadenylation. Analysis of the intron 9 sequence revealed several putative U/GU-rich elements referred to herein as DSE-1, DSE-2 and DSE-3 (Figure 1A). DSE-1 was mutated from GUUUUG to CCACAA (Mut1), DSE-2 from UGUGUU to CAACCA (Mut2) and DSE-3 from UCUUU to CCAAC (Mut3) in the minigene luciferase reporter construct. Mut1 and Mut2, but not Mut3, resulted in higher luciferase expression relative to unmutated (Figure 1B). When both DSE-1 and DSE-2 were mutated (Mut1 + 2), luciferase expression was higher when compared to Mut1 and Mut2 alone. When both DSE-2 and DSE-3 were mutated (Mut2 + 3), the luciferase activity was increased, but similar to Mut2 alone. As a result, DSE-1 and DSE-2 are important in KCNH2 intron 9 polyadenylation.

Regulation of Kv11.1 isoform expression by intron 9 poly(A) signal downstream elements

To test the effects of the downstream elements in the regulation of Kv11.1 isoform expression, we introduced the DSE-1 and DSE-2 mutations into the short KCNH2 gene construct (Figure 2A). Wild-type (WT) and mutant short gene constructs were stably transfected into Flp-In HEK293 cells. The Mut1 and Mut2 mutations increased Kv11.1a expression and decreased Kv11.1a-USO expression in RPA and immunoblot analyses (Figures 2B and 2C). When both DSE-1 and DSE-2 were mutated (Mut1 + 2) Kv11.1a was predominantly expressed.

Patch-clamp studies showed that Kv11.1 channel current was significantly increased in Mut1 + 2 (Figure 2D). The maximum tail current densities of WT and Mut1 + 2 were 7.0 ± 0.6 pA/pF ($n = 9$) and 17.8 ± 1.7 pA/pF ($n = 10$, $P < 0.001$), respectively. These results suggest that DSE-1 and DSE-2 are important downstream elements of the intron 9 poly(A) signal, and that disruption of these elements leads to a shift in KCNH2 pre-mRNA processing toward the production of the Kv11.1a isoform and an increase in Kv11.1 channel function.

Antisense morpholino oligonucleotide inhibition of the KCNH2 intron 9 polyadenylation

Because DSE-1 and DSE-2 are important for KCNH2 intron 9 polyadenylation, it was hypothesized that blocking these elements using an antisense oligonucleotide would inhibit KCNH2 intron 9 polyadenylation and lead to the upregulation of the functional Kv11.1a isoform.

A 25-mer antisense morpholino oligonucleotide complementary to a sequence comprising the KCNH2 intron 9 downstream elements DSE-1 and DSE-2 (Fig. 3A) was generated. To determine whether the antisense morpholino oligonucleotide could inhibit the poly(A) signal in intron 9, a competition assay using a tandem poly(A) signal construct was performed as described in Gong et al 2010 *supra*. The KCNH2 intron 9 poly(A) signal AGUAAA and flanking sequences (–130/+172 nt) were positioned upstream of a relatively strong synthetic poly(A) signal (Figure 3A). Cells expressing the tandem poly(A) construct were treated the with 5 μ M negative control or antisense morpholino oligonucleotide of SEQ ID NO: 5. Then an RNase protection assay was performed using a probe specific to 249 nt of KCNH2 intron 9 (Gong, 2010 *supra*). Use of the probe results in termination of transcription after generation of a 158 nt fragment, indicating polyadenylation at the intron 9 poly(A) signal and termination of transcription after generation of a 249 nt fragment indicating polyadenylation at the downstream synthetic poly(A) signal.

In the presence of the negative control morpholino oligonucleotide of SEQ ID NO: 6, transcription terminated predominantly at the KCNH2 intron 9 poly(A) signal (Figure 3B). In the presence of the antisense morpholino oligonucleotide of SEQ ID NO: 1 the transcription was predominantly terminated predominantly at the synthetic poly(A) signal. Treatment with the morpholino oligonucleotide of SEQ ID NO: 5 resulted in a 41% usage of the intron 9 poly(A) site relative to 83% usage of the intron 9 poly(A) site in controls (n = 3, Figure 3C). These results show that treatment with the morpholino oligonucleotide of SEQ ID NO: 5 inhibits the poly(A) signal in KCNH2 intron 9.

Modulation of Kv11.1 isoform expression by the antisense morpholino oligonucleotide of SEQ ID NO: 1

Flp-In HEK293 stably expressing a short KCNH2 gene were treated with the antisense morpholino oligonucleotide of SEQ ID NO: 1. RNase protection assays showed that treatment with 5 μ M of the antisense morpholino oligonucleotide of SEQ ID NO: 1 for 48 h resulted in greater expression of the Kv11.1a transcript and less expression of the Kv11.1a-USO transcript than the negative control morpholino oligonucleotide (Figure 4A).

The effects of the antisense morpholino oligonucleotide of SEQ ID NO: 1 on Kv11.1 protein expression was analyzed by immunoblot. Flp-In HEK293 cells stably expressing the short KCNH2 gene were treated with the indicated concentrations of the antisense morpholino oligonucleotide of SEQ ID NO: 5 for 48 hours or with 5 μ M for the indicated amount of time (Figures 4B and 4C). Treatment with the antisense morpholino oligonucleotide of SEQ ID NO: 5 resulted in significantly higher expression of Kv11.1a protein and lower expression of Kv11.1a-USO protein relative to controls.

Treatment with the antisense morpholino oligonucleotide exhibited concentration and time dependence. The expression of Kv11.1a protein was higher than the control at a concentration of morpholino oligonucleotide SEQ ID NO: 5 as low as 2 μ M with maximal expression at 10 μ M (Figure 4B). The increase in Kv11.1a protein level in cells treated with the morpholino oligonucleotide of SEQ ID NO: 5 relative to controls was observed by 24 hours and reached a maximum by 72 h following antisense MO treatment (Figure 4C).

Treatment with the antisense morpholino oligonucleotide of SEQ ID NO: 1 resulted in higher Kv11.1 channel current relative to controls

Patch-clamp recordings of Kv11.1 channel current in cells treated with the antisense morpholino oligonucleotide of SEQ ID NO: 5 were performed. Cells stably expressing the short KCNH2 gene were treated with 10 μ M antisense or a negative control morpholino oligonucleotide for 48 hours. Treatment with the antisense morpholino oligonucleotide of SEQ ID NO: 5 significantly increased Kv11.1 channel current compared to the negative control (Figure 5A). The maximum tail current density in cells treated with the negative control was 7.6 ± 1.0 pA/pF ($n = 8$). The maximum tail current density in cells treated with the antisense morpholino oligonucleotide of SEQ ID NO: 1 was 16.8 ± 2.1 pA/pF ($n = 9$, $P < 0.001$, Figure 5B).

The voltage dependence of Kv11.1 channel activation was determined by fitting the normalized tail currents with a Boltzmann function (Figure 5C). The half maximal activation voltages ($V_{1/2}$) in cells treated with the negative control was -4.2 ± 2.9 mV. The half maximal activation voltage in cells treated with antisense morpholino oligonucleotide of SEQ ID NO: 5 was -5.6 ± 2.0 mV. The slope factor (k) in cells treated with the negative control (SEQ ID NO: 6)

was 8.7 ± 0.2 . The slope factor in cells treated with antisense morpholino oligonucleotide of SEQ ID NO: 5 was 7.9 ± 0.3 . These patch-clamp experiments demonstrated that inhibition of polyadenylation of intron 9 using antisense morpholino oligonucleotide of SEQ ID NO: 5 results in higher Kv11.1 channel current.

5 *Effect of antisense MO on Kv11.1 isoform expression in canonical poly(A) signal construct*

It has been previously shown that the poly(A) signal in KCNH2 intron 9 is intrinsically weak due to the presence of the noncanonical hexamer AGUAAA. When the intron 9 poly(A) signal AGUAAA was changed to the canonical poly(A) signal AAUAAA, the Kv11.1a-USO isoform was predominantly expressed (Gong et al, 2010 *supra*). To test whether the antisense morpholino oligonucleotide of SEQ ID NO: 5 can mediate the switch of the expression from Kv11.1a-USO to the Kv11.1a isoform in the presence of a strong poly(A) signal, HEK293 cells stably expressing the short KCNH2 gene construct containing a canonical poly(A) signal were treated with negative control (SEQ ID NO: 6) or antisense morpholino oligonucleotide of SEQ ID NO: 5. RNAse protection assay (Figure 6A), shows that in the presence of the negative control, Kv11.1a-USO was predominantly expressed with no detectable expression of Kv11.1a mRNA. When treated with 10 μ M antisense oligonucleotide of SEQ ID NO: 5, the expression of the Kv11.1a isoform was significantly higher.

Immunoblot analysis revealed that the expression of Kv11.1a protein correlated with treatment with higher concentrations of antisense morpholino oligonucleotide of SEQ ID NO: 5 (Figure 6B). Patch clamp studies showed that treatment with 10 μ M antisense morpholino oligonucleotide of SEQ ID NO: 5 resulted in significantly higher Kv11.1 current than the negative control (Figure 6C). The maximum tail current densities in cells treated with the negative control (SEQ ID NO: 6) was 1.3 ± 0.3 pA/pF ($n = 8$). The maximum tail current densities in cells treated with the antisense morpholino oligonucleotide of SEQ ID NO: 5 was 11.5 ± 1.4 pA/pF ($n = 9$, $P < 0.001$). This indicates that the antisense morpholino oligonucleotide of SEQ ID NO: 1 can cause a Kv11.1 isoform switch in the presence of a strong intron 9 poly(A) signal.

CLAIMS

1. An oligonucleotide comprising a first sequence of SEQ ID NO: 1 or a second sequence of SEQ ID NO: 2 that inhibits formation of a poly(A) signal in intron 9 of KCNH2.
2. The oligonucleotide of claim 1 wherein the oligonucleotide is at least 19 nucleotides in length.
3. The oligonucleotide of claim 1 comprising a first sequence of SEQ ID NO: 1 and a second sequence of SEQ ID NO: 2.
4. The oligonucleotide of claim 3 comprising SEQ ID NO: 3.
5. The oligonucleotide of claim 4 comprising SEQ ID NO: 4.
6. The oligonucleotide of claim 5 comprising SEQ ID NO: 5.
7. The oligonucleotide of claim 6 consisting of a sequence of SEQ ID NO: 5.
8. The oligonucleotide of any of claims 1-7 wherein the oligonucleotide is no more than 30 nucleotides in length.
9. The oligonucleotide of any of claims 1-8 comprising a modified nucleotide, locked nucleotide, G-clamp nucleotide, nucleotide base analog, 3'-terminal cap moiety, or phosphate backbone modification.
10. The oligonucleotide of claim 9 comprising a morpholino nucleotide.

11. The oligonucleotide of claim 10 wherein all of the nucleic acids in the oligonucleotide are morpholino nucleotides.
12. A pharmaceutical composition comprising an effective amount of the morpholino oligonucleotide of any of claims 1-11 and a pharmaceutically acceptable carrier.
13. The pharmaceutical composition of claim 12 for use in treating long-QT syndrome caused by mutations in the KCNH2 gene.

Figure 1A

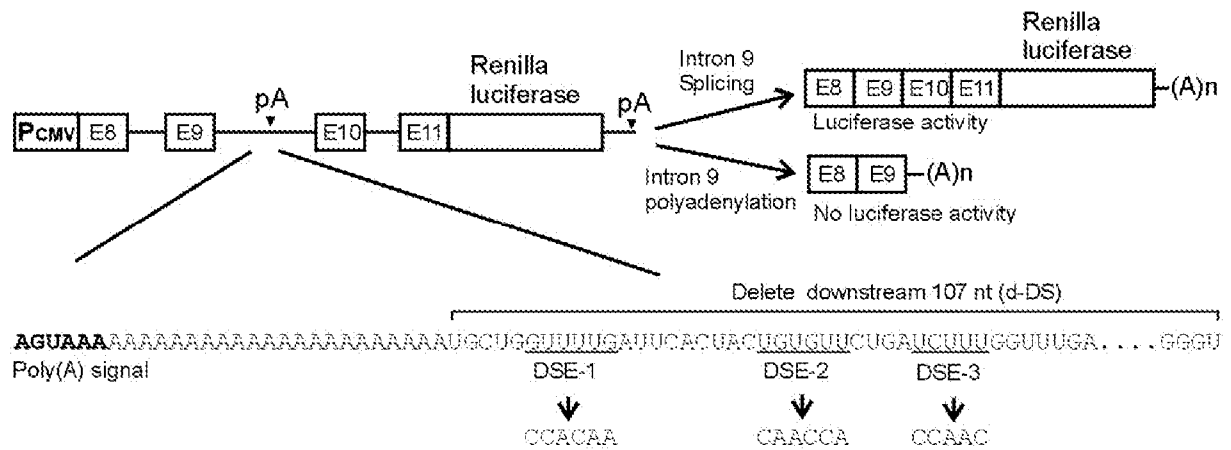


Figure 1B

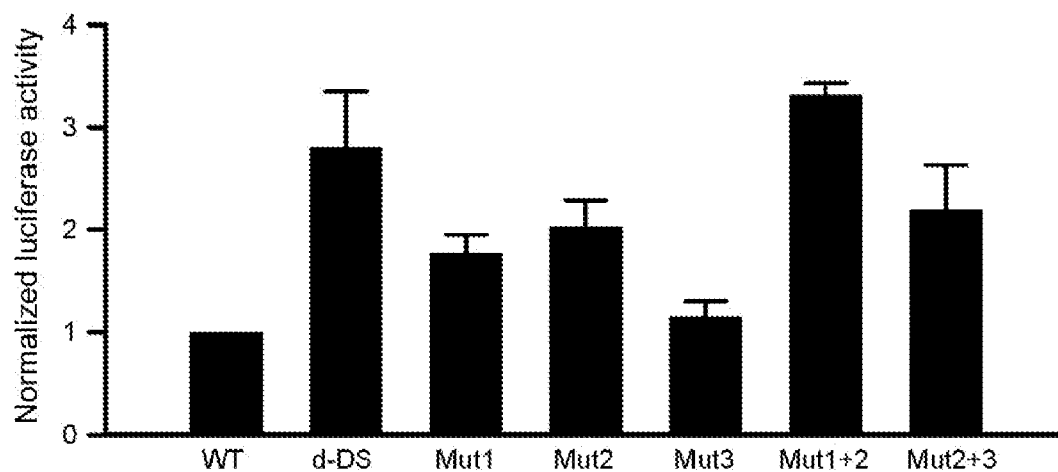


Figure 2A



Figure 2B

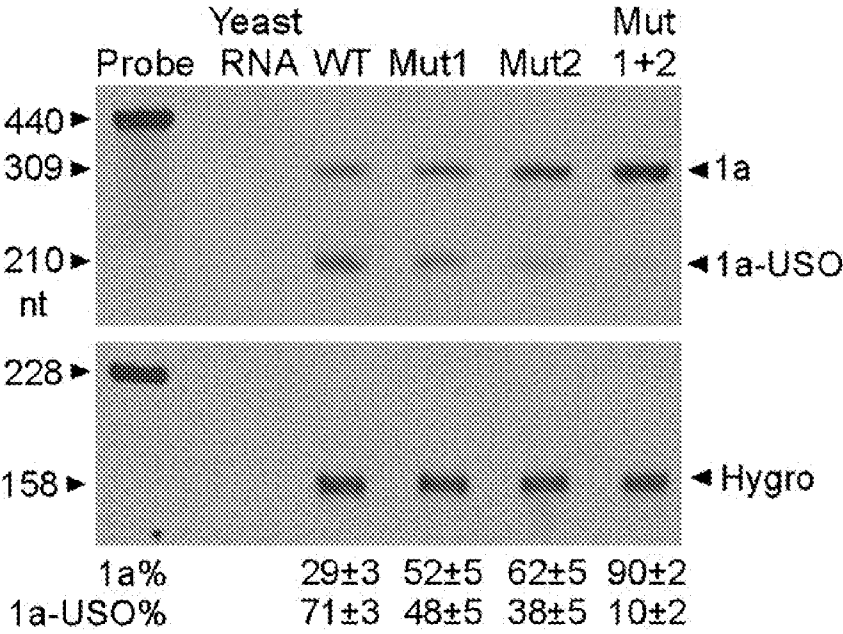


Figure 2C

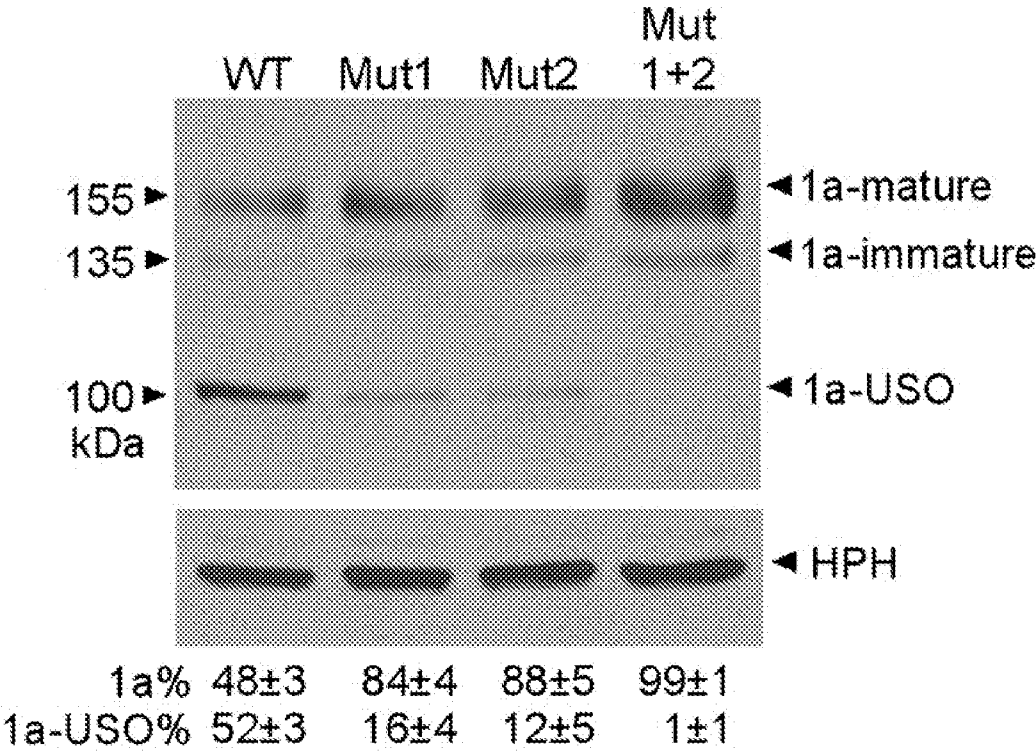


Figure 2D

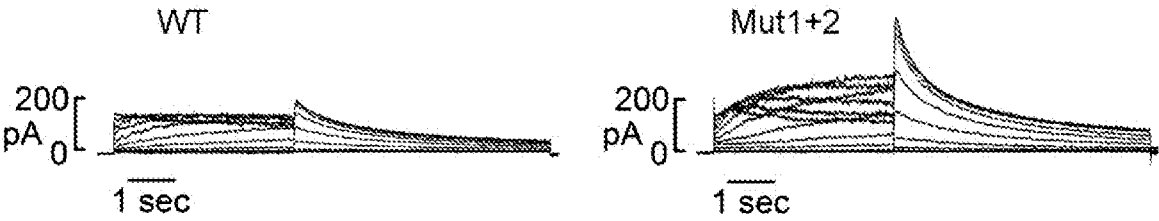


Figure 3A

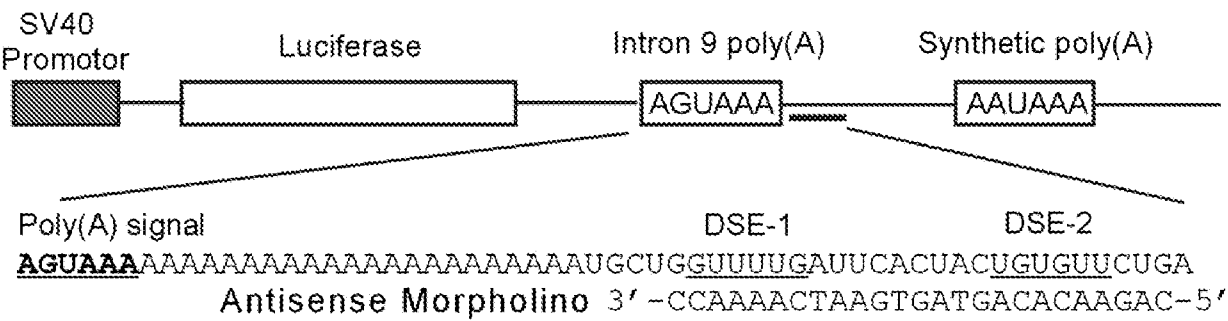


Figure 3B

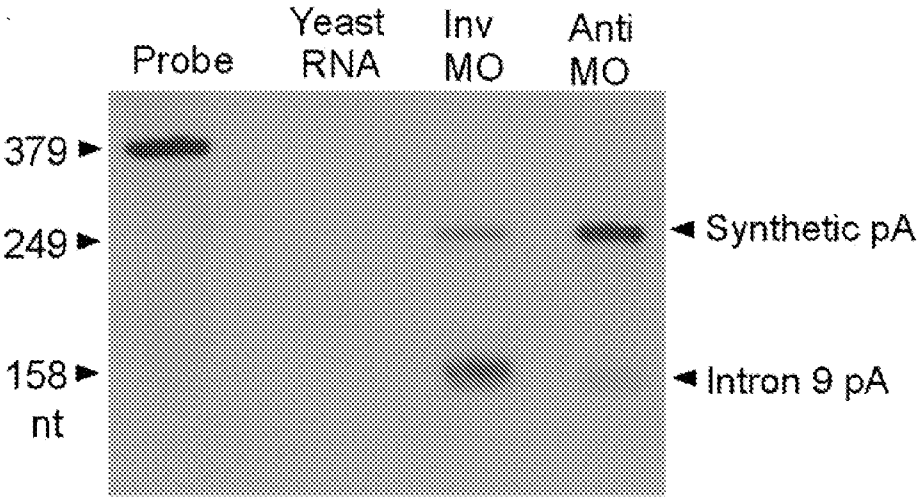


Figure 3C

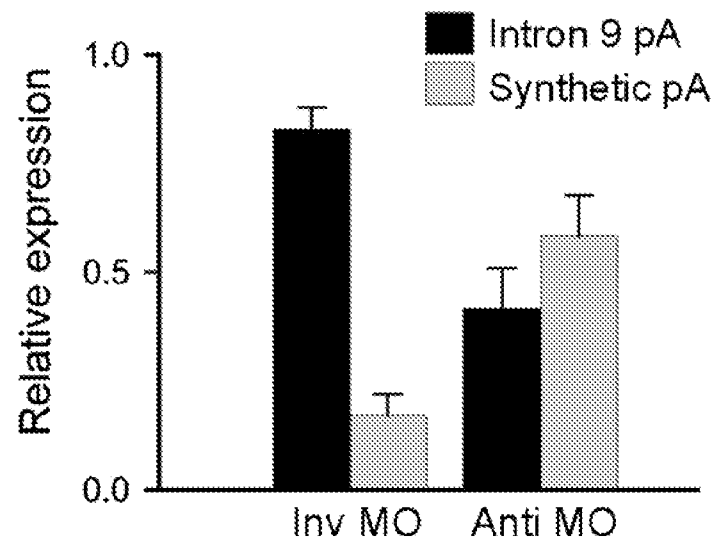


Figure 4A

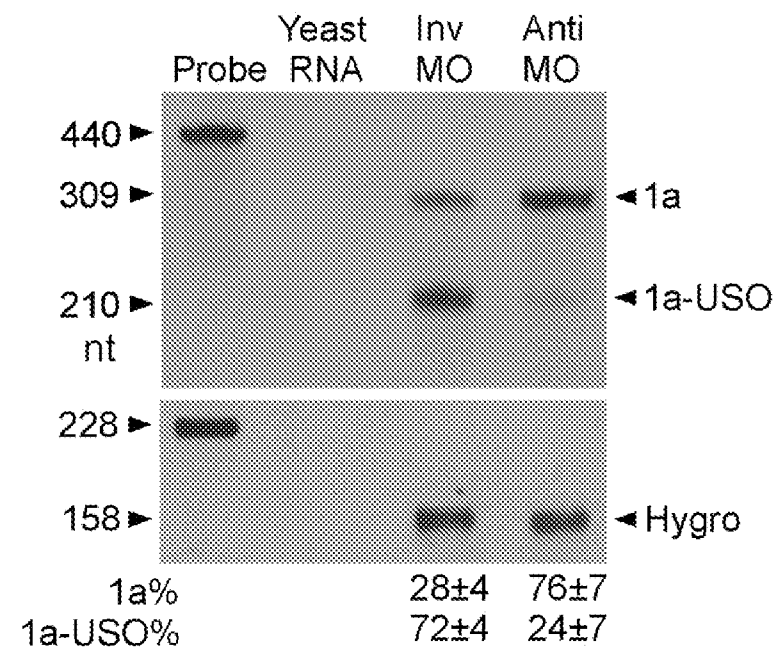


Figure 4B

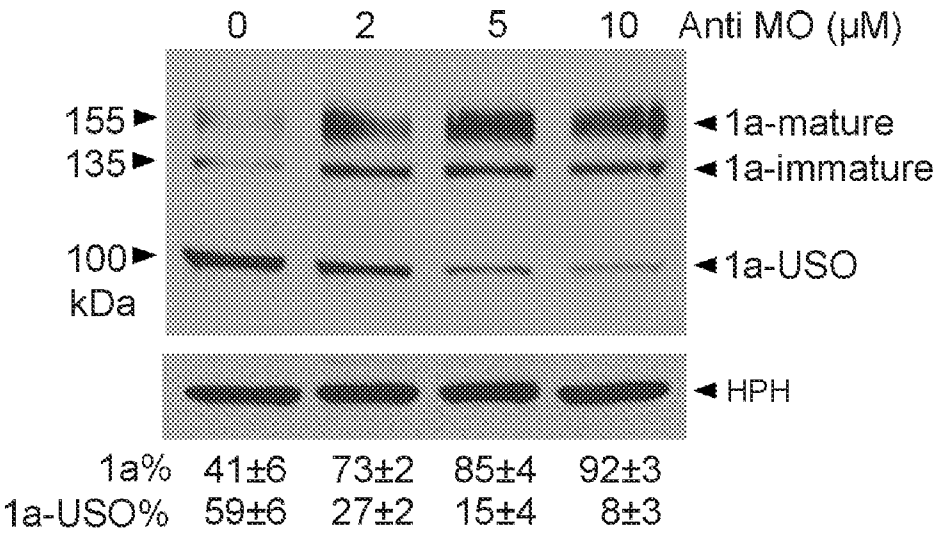


Figure 4C

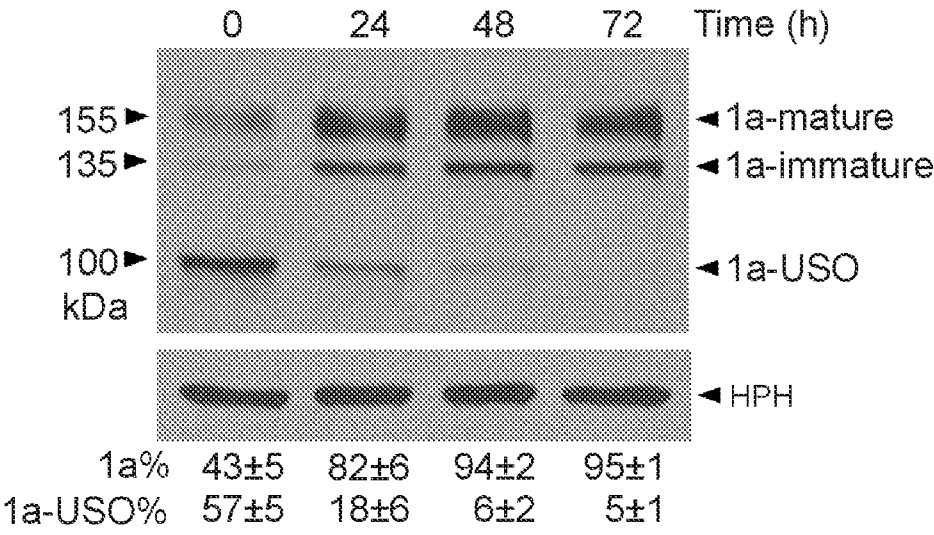


Figure 5A

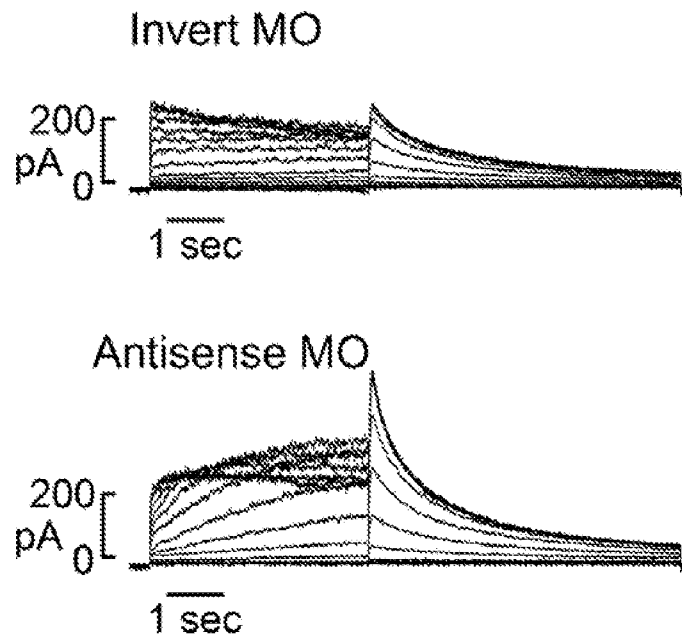


Figure 5B

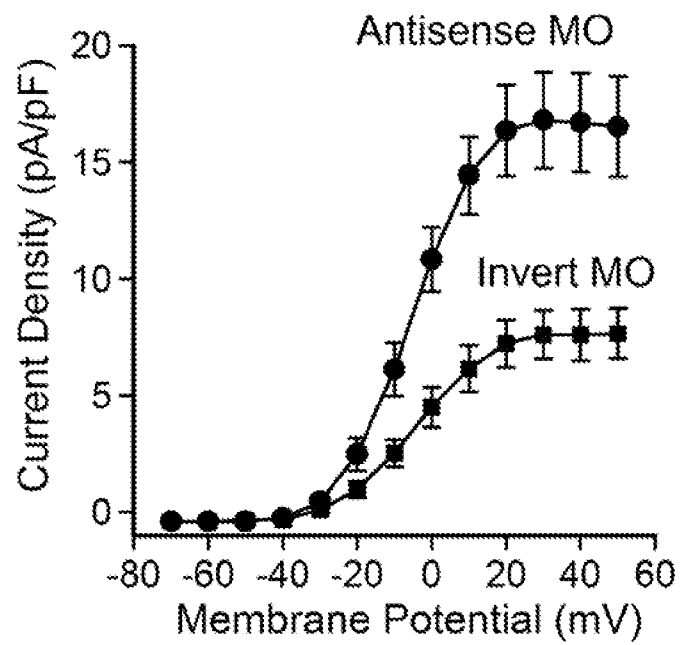


Figure 5C

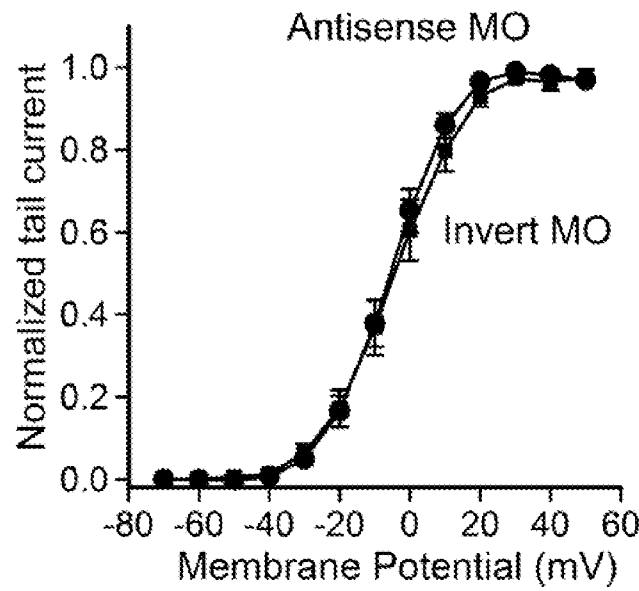


Figure 6A

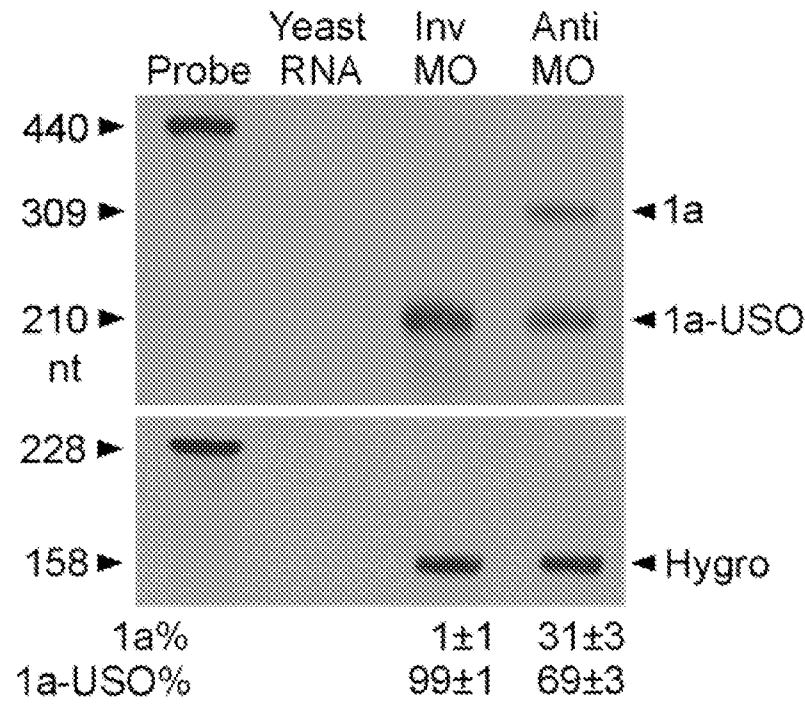


Figure 6B

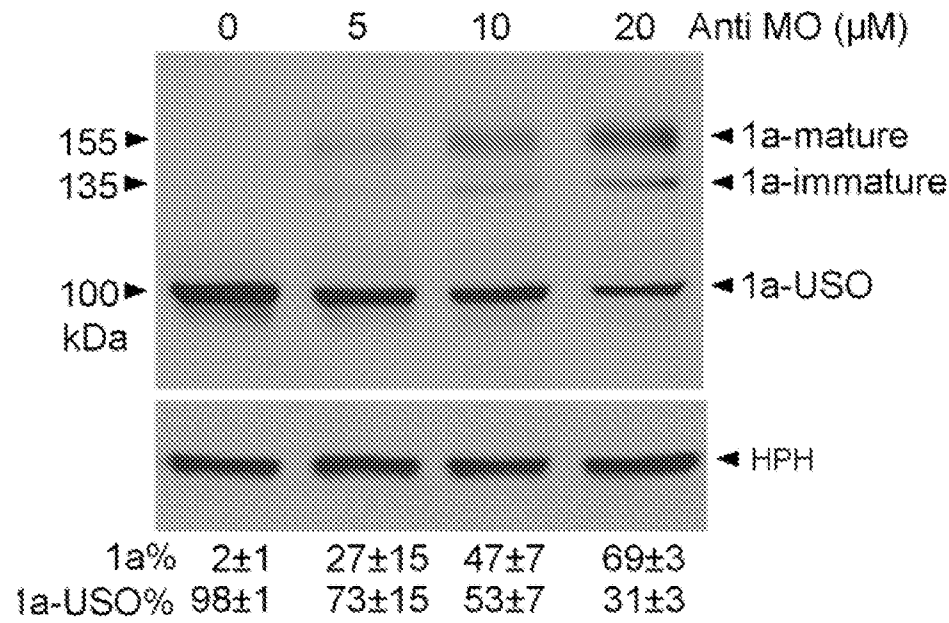
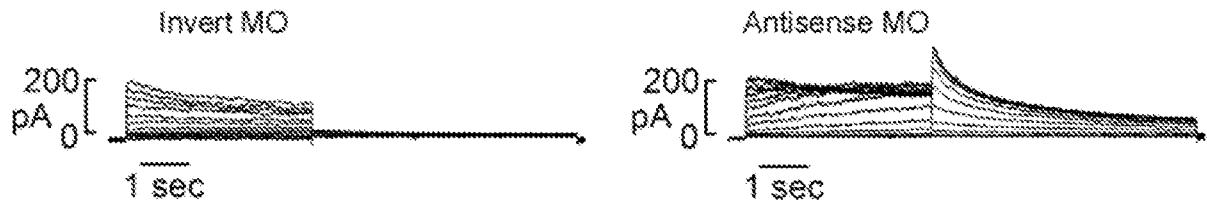


Figure 6C



A. CLASSIFICATION OF SUBJECT MATTER**C12N 15/113(2010.01)i, A61K 31/7088(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/113; C07K 14/705; B01D 57/02; C12Q 1/68; A61K 31/7088

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: KCNH2, Kv11.1, intron 9, long QT syndrome, antisense inhibition

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONG, QIUMING et al., 'Upregulation of functional Kv11.1 isoform expression by inhibition of intronic polyadenylation with antisense morpholino oligonucleotides', Journal of Molecular and Cellular Cardiology, November 2014, Vol. 76, pp. 26-32. See abstract; page 27, left column, paragraph 4; page 29, left column, paragraph 2.	1-8
A	GONG, QIUMING et al., 'Identification of Kv11.1 isoform switch as a novel pathogenic mechanism of long-QT syndrome', Circulation. Cardiovascular Genetics, August 2014, Vol. 7, No. 4, pp. 482-490. See the whole document.	1-8
A	DONOVAN, ALEXANDER J. et al., 'Long QT2 mutation on the Kv11.1 ion channel inhibits current activity by ablating a protein kinase Ca consensus site', Molecular Pharmacology, September 2012, Vol. 82, No. 3, pp. 428-437. See the whole document.	1-8
A	US 2009-0220949 A1 (PRIORI, SILVIA GIULIANA) 3 September 2009 See abstract; claims 58-59.	1-8
A	US 2005-0130190 A1 (ANTZELEVITCH, CHARLES et al.) 16 June 2005 See abstract; claims 1-8.	1-8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 September 2015 (24.09.2015)

Date of mailing of the international search report

24 September 2015 (24.09.2015)

Name and mailing address of the ISA/KR

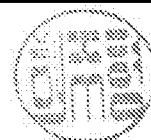
International Application Division
Korean Intellectual Property Office
189 Cheongsu-ro, Seo-gu, Daejeon Metropolitan City, 35208,
Republic of Korea

Facsimile No. +82-42-472-7140

Authorized officer

HEO, Joo Hyung

Telephone No. +82-42-481-8150



INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2015/012060**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 10-11,13
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 10-11 and 13 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 9,12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/012060

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009-0220949 A1	03/09/2009	AT 486963 T DE 602006018003 D1 EP 1891235 A2 EP 1891235 B1 IT MI20051047 A1 WO 2006-131528 A2 WO 2006-131528 A3	15/11/2010 16/12/2010 27/02/2008 03/11/2010 08/12/2006 14/12/2006 29/03/2007
US 2005-0130190 A1	16/06/2005	US 2009-0317905 A1 US 7537928 B2 US 7745213 B2	24/12/2009 26/05/2009 29/06/2010