

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization

International Bureau

(43) International Publication Date  
20 June 2019 (20.06.2019)



(10) International Publication Number  
**WO 2019/115417 A2**

(51) International Patent Classification:

Not classified

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(21) International Application Number:

PCT/EP20 18/084077

(22) International Filing Date:

10 December 2018 (10.12.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

17206565.8 12 December 2017 (12.12.2017) EP

(71) Applicant: ROCHE INNOVATION CENTER COPENHAGEN A/S [DK/DK]; Fremtidsvej 3, 2970 Horsholm (DK).

(72) Inventors: HAGEDORN, Peter; c/o Roche Innovation Center Copenhagen AS Fremtidsvej 3, 2970 Florsholm (DK). PEDERSEN, Lykke; c/o Roche Innovation Center Copenhagen AS Fremtidsvej 3, 2970 Florsholm (DK).

(74) Agent: RUDOLPH HANSEN, Marianne; Grenzacherstrasse 124, 4070 Basel (CH).

(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, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, 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).

Declarations under Rule 4.17:

- of inventorship (Rule 4.17(iv))

(54) Title: OLIGONUCLEOTIDES FOR MODULATING RB1 EXPRESSION

(57) Abstract: The present invention relates to antisense oligonucleotides that are capable of modulating expression of RB 1 in a target cell. The oligonucleotides hybridize to RB 1 pre-mRNA. The present invention further relates to conjugates of the oligonucleotide and pharmaceutical compositions and methods for treatment neurological injury or neuronal degeneration using the oligonucleotide.



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## OLIGONUCLEOTIDES FOR MODULATING RB1 EXPRESSION

### FIELD OF INVENTION

The present invention relates to oligonucleotides (oligomers) that are complementary to retinoblastoma transcriptional corepressor 1 (RB1) transcript. Such oligonucleotides may be used for reducing RB1 transcript in the cell, leading to modulation of the expression of RB1 protein. Modulation of RB1 expression is beneficial for a range of medical disorders related to neurological injury or neuronal degeneration.

### BACKGROUND

The protein encoded by the retinoblastoma transcriptional corepressor 1 (RB1) gene is a negative regulator of the cell cycle and was the first tumor suppressor gene found. The protein encoded by RB1 stabilizes constitutive heterochromatin to maintain the overall chromatin structure. The active, hypophosphorylated form of the protein binds transcription factor E2F1. Defects in the RB1 gene is a cause of among others, childhood cancer, retinoblastoma (RB), urinary bladder cancer, and osteogenic sarcoma (see for example Wang et al 2017 Int J One vol 50 P1221).

WO9205272 discloses a method and composition for reversibly increasing the proliferation of cells *in vitro*. Cell populations are treated *in vitro* with antisense oligonucleotides targeting a tumor suppressor gene, such as the retinoblastoma gene. Such treatment inhibits expression of the tumor suppressor gene product and results in a reversible increase in the proliferation of the *in vitro* cell population, which can then be introduced back into the patient.

US 2005/260620 suggests a use of short interfering nucleic acid (siRNA) for inhibition of retinoblastoma gene expression in cancers, in particular for inhibiting particular retinoblastoma genes that are associated with hearing loss, deafness, tinnitus, motion and balance disorders. However, the reference does not provide any experimental data supporting the intended use of siRNA. Moreover, the whole document does not contain any reference to selective targeting of introns of RB1 pre-mRNA.

Christie et al 2016 Nat Commun., 5: 3670; discloses that siRNA knockdown or RB1 deletion *in vitro* enhances neurite outgrowth and branching. Also, *in vivo* RB1 siRNA delivery following a nerve crush improves the number of outgrowing axons.

The siRNA approach is however significantly different from the single stranded antisense oligonucleotide approach since the mode of actions is quite different. As described in Xu et al 2003 Biochem. Biophys. Res. Comm. Vol 306 page 712-717 antisense oligonucleotides and siRNAs have different preferences for target sites in the mRNA.

None of the references above disclose a concept of targeting intron sequences or repeated sequences in the RB1 gene.

Antisense oligonucleotides targeting repeated sites in the same RNA has been shown to have enhanced potency for downregulation of target mRNA in some cases of in vitro transfection experiments. This has been the case for GCGR, STST3, MAPT, OGFR, and BOK RNA (Vickers at al. PLOS one, October 2014, Volume 9, Issue 10). WO 2013/120003 also refers to modulation of RNA by repeat targeting.

### OBJECTIVE OF THE INVENTION

RB1 is a known tumor suppressor and defectes in the RB1 gene may result in the progression of certain cancers as well as hearing loss, deafness, tinnitus, and motion and balance disorders. Systemic removal of RB1 is therefore undesired, but may have a positive effect when administred locally in patients to tissues where cell proliferation is desired, e.g. in connection with neurological injury or neuronal degeneration.

The present invention provides antisense oligonucleotides capable of modulating RB1 mRNA and protein expression both *in vivo* and *in vitro*. The expression of RB1 is targeted at the pre-mRNA level which prevents formation of mature RB1 mRNA. The antisense oligonucleotides targeting repeated sites have higher potency compared to antisense oligonucleotides targeting single regions within the same target sequence. Accordingly the present invention provide antisense oligonucleotides targeting repeated sequences in the RB1 gene which can be used for local administration in to treat nurological disorders or nuerological injuries.

### SUMMARY OF INVENTION

The present invention provides antisense oligonucleotides, which are complementary to and target introns of mammalian RB1 nucleic acids, such as SEQ ID NO: 1 and/or SEQ ID NO: 3, and uses thereof.

The present invention provides antisense oligonucleotides which comprise a contiguous nucleotide sequence which is complementary to certain regions or sequences present in the introns of target mammalian RB1 nucleic acids.

The oligonucleotides of the invention are capable of reducing mammalian RB1 nucleic acids in a cell which is expressing the mammalian RB1 nucleic acid.

Accordingly, in a first aspect the invention provides antisense oligonucleotides which comprise a contiguous nucleotide sequence of 10 to 30 nucleotides in length with at least 95%

In a further aspect the invention provides the oligonucleotide according to the previous aspects, wherein the contiguous nucleotide sequence is complementary to a target region of the target nucleic acid, selected from the group consisting of position 123370-123389, 123473-123492,

123526-123545, 123579-123598, 123632-123651, 123685-123704, 123738-123757, 123791-123810, 123897-123916, 123950-123969, 124003-124022, 124056-124075, 124109-124128, 124162-124181, 124215-124234, 124268-124287, 124321-124340, 124374-124393, 124427-124446, 124480-124499, 124533-124552, 124586-124605, 124639-124658, 124692-124711; 5 123370-123387, 123473-123490, 123526-123543, 123579-123596, 123632-123649, 123685-123702, 123738-123755, 123791-123808, 123897-123914, 123950-123967, 124003-124020, 124056-124073, 124109-124126, 124162-124179, 124215-124232, 124268-124285, 124321-124338, 124374-124391, 124427-124444, 124480-124497, 124533-124550, 124586-124603, 124639-124656, 124692-124709; 123372-123387, 123422-123437, 123475-123490, 123528-10 123543, 123581-123596, 123634-123649, 123687-123702, 123740-123755, 123793-123808, 123899-123914, 123952-123967, 124005-124020, 124058-124073, 124111-124126, 124164-124179, 124217-124232, 124270-124285, 124323-124338, 124376-124391, 124429-124444, 124482-124497, 124535-124550, 124588-124603, 124641-124656, 124694-124709; 123375-123388, 123425-123438, 123478-123491, 123531-123544, 123584-123597, 123637-123650, 15 123690-123703, 123743-123756, 123796-123809, 123902-123915, 123955-123968, 124008-124021, 124061-124074, 124114-124127, 124167-124180, 124220-124233, 124273-124286, 124326-124339, 124379-124392, 124432-124445, 124485-124498, 124538-124551, 124591-124604, 124644-124657, 124697-124710 of SEQ ID NO:1.

In a further aspect the invention provides antisense oligonucleotides which comprises a 20 contiguous nucleotide sequence of 10 to 30 nucleotides in length with at least 95% complementarity to at least two repeated target regions present in the target nucleic acids of SEQ ID NO:1 and SEQ ID NO: 3.

In a further aspect, the invention provides antisense oligonucleotides which comprises a 25 wherein the contiguous nucleotide sequence is 95% complementary, such as fully complementary, to a target region of 10-22, such as 14-20, nucleotides in length of the target nucleic acid of SEQ ID NO: 1, wherein the target region is repeated at least 6 or more times across the introns of the target nucleic acid.

In a further aspect the invention provides the antisense oligonucleotide, wherein the antisense oligonucleotide, or contiguous nucleotide sequence thereof is selected from the group 30 consisting of GAggtgaggtttgtagaAG (Compound ID 9\_1); GGTgaggtttgtagaAG (Compound ID 10\_1); GGTgaggtttgtaGA (Compound ID 11\_1) and AGGTgaggtttgGT /Compound ID 12\_1); wherein capital letters represent LNA nucleosides, such as beta-D-oxy LNA nucleosides, lower case letters represent DNA nucleosides, optionally all LNA C are 5-methyl cytosine, wherein the compounds comprise phosphorothioate internucleoside linkages.

In a further aspect the invention provides a conjugate comprising the antisense oligonucleotide according to the invention, and at least one conjugate moiety covalently attached to said oligonucleotide.

5 In a further aspect the invention provides a pharmaceutical composition comprising the oligonucleotide according to the invention or the conjugate according to the invention, and a pharmaceutically acceptable diluent, solvent, carrier, salt and/or adjuvant.

In a further aspect, the invention provides a pharmaceutically acceptable salt of the antisense oligonucleotide or conjugate according to the invention the invention.

10 In a further aspect, the invention provides *in vivo* or *in vitro* methods for modulation of RB1 expression in a target cell which is expressing RB1 , by administering an oligonucleotide or composition of the invention in an effective amount to said cell.

In a further aspect the invention provides a method for treating or preventing a disease comprising administering a therapeutically or prophylactically effective amount of an oligonucleotide, the conjugate, the pharmaceutically acceptable salt, or the pharmaceutical  
15 composition according to the invention to a subject suffering from or susceptible to the disease

In a further aspect the oligonucleotide or composition of the invention is used for the treatment or prevention of disorders related to neurological injury or neuronal degeneration.

## DEFINITIONS

### ***Oligonucleotide***

20 The term “oligonucleotide” as used herein is defined as it is generally understood by the skilled person as a molecule comprising two or more covalently linked nucleosides. Such covalently bound nucleosides may also be referred to as nucleic acid molecules or oligomers.

Oligonucleotides are commonly made in the laboratory by solid-phase chemical synthesis followed by purification and isolation. When referring to a sequence of the oligonucleotide,  
25 reference is made to the sequence or order of nucleobase moieties, or modifications thereof, of the covalently linked nucleotides or nucleosides. The oligonucleotide of the invention is man-made, and is chemically synthesized, and is typically purified or isolated. The oligonucleotide of the invention may comprise one or more modified nucleosides or nucleotides.

### ***Antisense oligonucleotides***

30 The term “Antisense oligonucleotide” as used herein is defined as oligonucleotides capable of modulating expression of a target gene by hybridizing to a target nucleic acid, in particular to a contiguous sequence on a target nucleic acid. The antisense oligonucleotides are not essentially double stranded and are therefore not siRNAs or shRNAs. Preferably, the antisense oligonucleotides of the present invention are single stranded. . It is understood that single

stranded oligonucleotides of the present invention can form hairpins or intermolecular duplex structures (duplex between two molecules of the same oligonucleotide), as long as the degree of intra or inter self complementarity is less than 50% across of the full length of the oligonucleotide

- 5 Advantageously, the antisense oligonucleotide of the invention comprise one or more modified nucleosides or nucleotides.

### ***Contiguous Nucleotide Sequence***

The term “contiguous nucleotide sequence” refers to the region of the oligonucleotide which is complementary to the target nucleic acid. The term is used interchangeably herein with the  
10 term “contiguous nucleobase sequence” and the term “oligonucleotide motif sequence”. In some embodiments all the nucleotides of the oligonucleotide constitute the contiguous nucleotide sequence. In some embodiments the oligonucleotide comprises the contiguous nucleotide sequence, such as a F-G-F’ gapmer region, and may optionally comprise further nucleotide(s), for example a nucleotide linker region which may be used to attach a functional group to the  
15 contiguous nucleotide sequence. The nucleotide linker region may or may not be complementary to the target nucleic acid.

### ***Nucleotides***

Nucleotides are the building blocks of oligonucleotides and polynucleotides, and for the purposes of the present invention include both naturally occurring and non-naturally occurring  
20 nucleotides. In nature, nucleotides, such as DNA and RNA nucleotides comprise a ribose sugar moiety, a nucleobase moiety and one or more phosphate groups (which is absent in nucleosides). Nucleosides and nucleotides may also interchangeably be referred to as “units” or “monomers”.

### ***Modified nucleoside***

25 The term “modified nucleoside” or “nucleoside modification” as used herein refers to nucleosides modified as compared to the equivalent DNA or RNA nucleoside by the introduction of one or more modifications of the sugar moiety or the (nucleo)base moiety. In a preferred embodiment the modified nucleoside comprise a modified sugar moiety. The term modified nucleoside may also be used herein interchangeably with the term “nucleoside analogue” or  
30 modified “units” or modified “monomers”. Nucleosides with an unmodified DNA or RNA sugar moiety are termed DNA or RNA nucleosides herein. Nucleosides with modifications in the base region of the DNA or RNA nucleoside are still generally termed DNA or RNA if they allow Watson Crick base pairing.

***Modified internucleoside linkage***

The term "modified internucleoside linkage" is defined as generally understood by the skilled person as linkages other than phosphodiester (PO) linkages, that covalently couples two nucleosides together. The oligonucleotides of the invention may therefore comprise modified internucleoside linkages. In some embodiments, the modified internucleoside linkage increases the nuclease resistance of the oligonucleotide compared to a phosphodiester linkage. For naturally occurring oligonucleotides, the internucleoside linkage includes phosphate groups creating a phosphodiester bond between adjacent nucleosides. Modified internucleoside linkages are particularly useful in stabilizing oligonucleotides for in vivo use, and may serve to protect against nuclease cleavage at regions of DNA or RNA nucleosides in the oligonucleotide of the invention, for example within the gap region of a gapmer oligonucleotide, as well as in regions of modified nucleosides, such as region F and F'.

In an embodiment, the oligonucleotide comprises one or more internucleoside linkages modified from the natural phosphodiester, such one or more modified internucleoside linkages that is for example more resistant to nuclease attack. Nuclease resistance may be determined by incubating the oligonucleotide in blood serum or by using a nuclease resistance assay (e.g. snake venom phosphodiesterase (SVPD)), both are well known in the art. Internucleoside linkages which are capable of enhancing the nuclease resistance of an oligonucleotide are referred to as nuclease resistant internucleoside linkages. In some embodiments at least 50% of the internucleoside linkages in the oligonucleotide, or contiguous nucleotide sequence thereof, are modified, such as at least 60%, such as at least 70%, such as at least 75% such as at least 80% or such as at least 90% of the internucleoside linkages in the oligonucleotide, or contiguous nucleotide sequence thereof, are nuclease resistant internucleoside linkages. In some embodiments all of the internucleoside linkages of the oligonucleotide, or contiguous nucleotide sequence thereof, are nuclease resistant internucleoside linkages. It will be recognized that, in some embodiments the nucleosides which link the oligonucleotide of the invention to a non-nucleotide functional group, such as a conjugate, may be phosphodiester. A preferred modified internucleoside linkage for use in the oligonucleotide of the invention is phosphorothioate.

Phosphorothioate internucleoside linkages are particularly useful due to nuclease resistance, beneficial pharmacokinetics and ease of manufacture. In some embodiments at least 50% of the internucleoside linkages in the oligonucleotide, or contiguous nucleotide sequence thereof, are phosphorothioate, such as at least 60%, such as at least 70%, such as at least 75%, such as at least 80% or such as at least 90% of the internucleoside linkages in the oligonucleotide, or contiguous nucleotide sequence thereof, are phosphorothioate. In some embodiments, all of the internucleoside linkages of the oligonucleotide, or contiguous nucleotide sequence thereof, are phosphorothioate. In some embodiments, the oligonucleotide of the invention comprises both

phosphorothioate internucleoside linkages and at least one phosphodiester linkage, such as 2, 3 or 4 phosphodiester linkages, in addition to the phosphorodithioate linkage(s). In a gapmer oligonucleotide, phosphodiester linkages, when present, are suitably not located between contiguous DNA nucleosides in the gap region G.

- 5 Nuclease resistant linkages, such as phosphorothioate linkages, are particularly useful in oligonucleotide regions capable of recruiting nuclease when forming a duplex with the target nucleic acid, such as region G for gapmers. Phosphorothioate linkages may, however, also be useful in non-nuclease recruiting regions and/or affinity enhancing regions such as regions F and F' for gapmers. Gapmer oligonucleotides may, in some embodiments comprise one or  
10 more phosphodiester linkages in region F or F', or both region F and F', where all the internucleoside linkages in region G may be phosphorothioate.

Advantageously, all the internucleoside linkages of the contiguous nucleotide sequence of the oligonucleotide, or all the internucleoside linkages of the oligonucleotide, are phosphorothioate linkages.

- 15 It is recognized that, as disclosed in EP 2 742 135, antisense oligonucleotides may comprise other internucleoside linkages (other than phosphodiester and phosphorothioate), for example alkyl phosphonate/methyl phosphonate internucleosides, which according to EP 2 742 135 may for example be tolerated in an otherwise DNA phosphorothioate the gap region.

### **Nucleobase**

- 20 The term nucleobase includes the purine (e.g. adenine and guanine) and pyrimidine (e.g. uracil, thymine and cytosine) moiety present in nucleosides and nucleotides which form hydrogen bonds in nucleic acid hybridization. In the context of the present invention the term nucleobase also encompasses modified nucleobases which may differ from naturally occurring nucleobases, but are functional during nucleic acid hybridization. In this context "nucleobase"  
25 refers to both naturally occurring nucleobases such as adenine, guanine, cytosine, thymidine, uracil, xanthine and hypoxanthine, as well as non-naturally occurring variants. Such variants are for example described in Hirao et al (2012) Accounts of Chemical Research vol 45 page 2055 and Bergstrom (2009) Current Protocols in Nucleic Acid Chemistry Suppl. 37 1.4.1 .

- In a some embodiments the nucleobase moiety is modified by changing the purine or pyrimidine  
30 into a modified purine or pyrimidine, such as substituted purine or substituted pyrimidine, such as a nucleobase selected from isocytosine, pseudoisocytosine, 5-methyl cytosine, 5-thiazolo-cytosine, 5-propynyl-cytosine, 5-propynyl-uracil, 5-bromouracil 5-thiazolo-uracil, 2-thio-uracil, 2'thio-thymine, inosine, diaminopurine, 6-aminopurine, 2-aminopurine, 2,6-diaminopurine and 2-chloro-6-aminopurine.

The nucleobase moieties may be indicated by the letter code for each corresponding nucleobase, e.g. A, T, G, C or U, wherein each letter may optionally include modified nucleobases of equivalent function. For example, in the exemplified oligonucleotides, the nucleobase moieties are selected from A, T, G, C, and 5-methyl cytosine. Optionally, for LNA gapmers, 5-methyl cytosine LNA nucleosides may be used.

### ***Modified oligonucleotide***

The term modified oligonucleotide describes an oligonucleotide comprising one or more sugar-modified nucleosides and/or modified internucleoside linkages. The term chimeric oligonucleotide is a term that has been used in the literature to describe oligonucleotides with modified nucleosides.

### ***Complementarity***

The term "complementarity" describes the capacity for Watson-Crick base-pairing of nucleosides/nucleotides. Watson-Crick base pairs are guanine (G)-cytosine (C) and adenine (A) - thymine (T)/uracil (U). It will be understood that oligonucleotides may comprise nucleosides with modified nucleobases, for example 5-methyl cytosine is often used in place of cytosine, and as such the term complementarity encompasses Watson Crick base-pairing between non-modified and modified nucleobases (see for example Hirao et al (2012) Accounts of Chemical Research vol 45 page 2055 and Bergstrom (2009) Current Protocols in Nucleic Acid Chemistry Suppl. 37 1.4.1 ).

The term "% complementary" as used herein, refers to the proportion of nucleotides (in percent) of a contiguous nucleotide sequence in a nucleic acid molecule (e.g. oligonucleotide) which across the contiguous nucleotide sequence, are complementary to a reference sequence (e.g. a target sequence or sequence motif). The percentage of complementarity is thus calculated by counting the number of aligned nucleobases that are complementary (from Watson Crick base pair) between the two sequences (when aligned with the target sequence 5'-3' and the oligonucleotide sequence from 3'-5'), dividing that number by the total number of nucleotides in the oligonucleotide and multiplying by 100. In such a comparison a nucleobase/nucleotide which does not align (form a base pair) is termed a mismatch. Insertions and deletions are not allowed in the calculation of % complementarity of a contiguous nucleotide sequence. It will be understood that in determining complementarity, chemical modifications of the nucleobases are disregarded as long as the functional capacity of the nucleobase to form Watson Crick base pairing is retained (e.g. 5'-methyl cytosine is considered identical to a cytosine for the purpose of calculating % identity).

The term "fully complementary", refers to 100% complementarity.

The following is an example of an oligonucleotide (SEQ ID NO:12) that is fully complementary to the target nucleic acid (SEQ ID NO: 13).

5 'CTTCTACCAAACCTCACCTCCAGCATTGGGGA 3' (SEQ ID NO :4 ) ; and  
 |||||  
 3' TGGTTTGGAGTGGA 5' (SEQ ID NO: 12) ,

### **Identity**

5 The term "Identity" as used herein, refers to the proportion of nucleotides (expressed in percent) of a contiguous nucleotide sequence in a nucleic acid molecule (e.g. oligonucleotide) which across the contiguous nucleotide sequence, are identical to a reference sequence (e.g. a sequence motif). The percentage of identity is thus calculated by counting the number of aligned nucleobases that are identical (a Match) between two sequences (in the contiguous  
 10 nucleotide sequence of the compound of the invention and in the reference sequence), dividing that number by the total number of nucleotides in the oligonucleotide and multiplying by 100. Therefore, Percentage of Identity = (Matches x 100)/Length of aligned region (e.g. the contiguous nucleotide sequence). Insertions and deletions are not allowed in the calculation the percentage of identity of a contiguous nucleotide sequence. It will be understood that in  
 15 determining identity, chemical modifications of the nucleobases are disregarded as long as the functional capacity of the nucleobase to form Watson Crick base pairing is retained (e.g. 5-methyl cytosine is considered identical to a cytosine for the purpose of calculating % identity).

### **Hybridization**

20 The term "hybridizing" or "hybridizes" as used herein is to be understood as two nucleic acid strands (e.g. an oligonucleotide and a target nucleic acid) forming hydrogen bonds between base pairs on opposite strands thereby forming a duplex. The affinity of the binding between two nucleic acid strands is the strength of the hybridization. It is often described in terms of the melting temperature ( $T_m$ ) defined as the temperature at which half of the oligonucleotides are duplexed with the target nucleic acid. At physiological conditions  $T_m$  is not strictly proportional to  
 25 the affinity (Mergny and Lacroix, 2003, *Oligonucleotides* 13:515-537). The standard state Gibbs free energy  $AG^\circ$  is a more accurate representation of binding affinity and is related to the dissociation constant ( $K_d$ ) of the reaction by  $AG^\circ = -RT \ln(K_d)$ , where R is the gas constant and T is the absolute temperature. Therefore, a very low  $AG^\circ$  of the reaction between an oligonucleotide and the target nucleic acid reflects a strong hybridization between the  
 30 oligonucleotide and target nucleic acid.  $AG^\circ$  is the energy associated with a reaction where aqueous concentrations are 1M, the pH is 7, and the temperature is 37°C. The hybridization of oligonucleotides to a target nucleic acid is a spontaneous reaction and for spontaneous reactions  $AG^\circ$  is less than zero.  $AG^\circ$  can be measured experimentally, for example, by use of the isothermal titration calorimetry (ITC) method as described in Hansen et al., 1965, *Chem. Comm.* 36-38 and Holdgate et al., 2005, *Drug Discov Today*. The skilled person will know that commercial equipment is available for  $AG^\circ$  measurements.  $AG^\circ$  can also be estimated numerically by using the nearest neighbor model as described by SantaLucia, 1998, *Proc Natl*

*Acad Sci USA*. 95: 1460-1465 using appropriately derived thermodynamic parameters described by Sugimoto et al., 1995, *Biochemistry* 34:1 121 1-1 1216 and McTigue et al., 2004, *Biochemistry* 43:5388-5405. In order to have the possibility of modulating its intended nucleic acid target by hybridization, oligonucleotides of the present invention hybridize to a target nucleic acid with estimated AG° values below -10 kcal for oligonucleotides that are 10-30 nucleotides in length. In some embodiments the degree or strength of hybridization is measured by the standard state Gibbs free energy AG°. The oligonucleotides may hybridize to a target nucleic acid with estimated AG° values below the range of -10 kcal, such as below -15 kcal, such as below -20 kcal and such as below -25 kcal for oligonucleotides that are 8-30 nucleotides in length. In some embodiments the oligonucleotides hybridize to a target nucleic acid with an estimated AG° value of -10 to -60 kcal, such as -12 to -40, such as from -15 to -30 kcal or -16 to -27 kcal such as -18 to -25 kcal.

**Target nucleic acid**

According to the present invention, the target nucleic acid is a nucleic acid which encodes mammalian RB1 and may for example be a gene, a RNA, a mRNA, and pre-mRNA, a mature mRNA or a cDNA sequence. The target may therefore be referred to as an RB1 target nucleic acid. The oligonucleotide of the invention may, for example, target exon regions of a mammalian RB1, or may, for example, target intron regions in the RB1 pre-mRNA as predicted below in Table 1.

Table 1. Exon and intron regions in the human RB1 pre-mRNA

Exon regions in the human RB1 premRNA (SEQ ID NO: 1)			Intron regions in the human RB1 premRNA (SEQ ID NO: 1)		
ID	start	end	ID	start	end
E1	25	299	I1	300	3529
E2	3530	3656	I2	3657	38848
E3	38849	38964	I3	38965	41329
E4	41330	41449	I4	41450	44074
E5	44075	44113	I5	44114	45205
E6	45206	45273	I6	45274	56266
E7	56267	56377	I7	56378	59064
E8	59065	59207	I8	59208	61143
E9	61144	61221	I9	61221	63743
E10	63744	63853	I10	63854	64776
E11	64777	64854	I11	64855	69654
E12	69655	69742	I12	69743	73167
E13	73168	73284	I13	73285	75843
E14	75844	75900	I14	75901	76302
E15	76303	76334	I15	76335	76414
E16	76415	76491	I16	76492	77496
E17	77497	77693	I17	77694	149242
E18	149243	149361	I18	149362	152453
E19	152454	152599	I19	152600	155937
E20	155938	156083	I20	156084	159980

Exon regions in the human RB1 premRNA (SEQ ID NO: 1)			Intron regions in the human RB1 premRNA (SEQ ID NO: 1)		
E21	159981	160085	I21	160086	161247
E22	161248	161361	I22	161362	161454
E23	161455	161618	I23	161619	169609
E24	169610	169640	I24	169641	172950
E25	172951	173093	I25	173094	173604
E26	173605	173654	I26	173655	176247
E27	176248	178236			

Suitably, the target nucleic acid encodes an RB1 protein, in particular mammalian RB1, such as human RB1 (See for example Table3) which provides the mRNA and pre-mRNA sequences for human and Cynomolgus monkey, RB1).

In some embodiments, the target nucleic acid is selected from the group consisting of SEQ ID NO: 1, 2, and 3 or naturally occurring variants thereof (e.g. sequences encoding a mammalian RB1 protein).

The target nucleic acid may, in some embodiments, be DNA or RNA, such as a messenger RNA, such as a mature mRNA or pre-mRNA which encodes mammalian RB1 protein, such as human RB1, e.g. the human pre-mRNA sequence, such as that disclosed as SEQ ID NO:1 or human mature mRNA as disclosed in SEQ ID NO:2.

If employing the oligonucleotide of the invention in research or diagnostics the target nucleic acid may be a cDNA or a synthetic nucleic acid derived from DNA or RNA.

For *in vivo* or *in vitro* application, the oligonucleotide of the invention is typically capable of inhibiting the expression of the RB1 target SEQ ID NO:2 nucleic acid in a cell which is expressing the RB1 target nucleic acid. The contiguous sequence of nucleobases of the oligonucleotide of the invention is typically complementary to the RB1 target nucleic acid, as measured across the length of the oligonucleotide, optionally with the exception of one or two mismatches, and optionally excluding nucleotide based linker regions which may link the oligonucleotide to an optional functional group such as a conjugate, or other non-complementary terminal nucleotides (e.g. region D' or D'').

Table 2. Genome and assembly information for RB1 across species.

Species	Chr.	Strand	Genomic coordinates		Assembly	Essamble ID
			Start	End		
Human	13	fwd	48303751	48481986	GRCh38.p2 release 107	ENSG00000139687
Cynomolgus monkey	17	fwd	27464762	27642897	Macaca_fascicularis_5.0 release 100 (GCF_000364345.1)	
Mouse (mmusculus)	14	rev	73183673	73325822	GRCm38.p5	ENSMUSG00000022105

Fwd = forward strand. The genome coordinates provide the pre-mRNA sequence (genomic sequence). The NCBI reference provides the mRNA sequence (cDNA sequence).

Table 3. Sequence details for RB1 across species.

Species	RNA type	Length (nt)	SEQ ID NO
Human	pre-mRNA	178236	1
Human	mature mRNA	4840	2
Cynomonkey	premRNA	178136	3

**Target Sequence**

5 The term “target sequence” as used herein refers to a sequence of nucleotides present in the target nucleic acid which comprises the nucleobase sequence which is complementary to the oligonucleotide of the invention. In some embodiments, the target sequence consists of a region on the target nucleic acid with a nucleobase sequence that is complementary to the contiguous nucleotide sequence of the oligonucleotide of the invention. This region of the target  
 10 nucleic acid may interchangeably be referred to as the target nucleotide sequence, target sequence or target region. In some embodiments the target sequence is longer than the contiguous complementary sequence of a single oligonucleotide, and may, for example represent a preferred region of the target nucleic acid which may be targeted by several oligonucleotides of the invention.

15 The antisense oligonucleotide of the invention comprises a contiguous nucleotide sequence which is complementary to or hybridizes to the target nucleic acid, such as a target sequence described herein.

The target sequence to which the oligonucleotide is complementary or hybridizes to generally comprises a contiguous nucleobase sequence of at least 10 nucleotides. The contiguous  
 20 nucleotide sequence is between 10 to 50 nucleotides, such as 12 to 30, such as 14 to 20, such as 15 to 18 contiguous nucleotides.

In one embodiment of the invention the target sequence is SEQ ID NO: 15.

In one embodiment of the invention the target sequence is SEQ ID NO: 4.

In other embodiments of the invention the target sequence is SEQ ID NO: 13 (5'  
 25 TTCTACCAAACCT CACCTCCAGCATTGGGGA 3') or SEQ ID NO: 14 (5' CACATTT CAACA 3'), which are 100% identical between SEQ ID NO:1 (human RB1 pre-mRNA) and SEQ ID NO:3 (cynomolgous monkey RB1 pre-mRNA).

**Repeated Target region**

The target region or target sequence can be unique for the target nucleic acid (only present  
 30 once).

In some aspects of the invention the target region is repeated at least two times over the span of target nucleic acid. Repeated as encompassed by the present invention means that there are at least two identical nucleotide sequences (target regions) of at least 10, such as at least 11, or at least 12, nucleotides in length which occur in the target nucleic acid at different positions.

5 Each repeated target region is separated from the identical region by at least one nucleobase on the contiguous sequence of target nucleic acid and is positioned at different and non-overlapping positions within the target nucleic acid.

### *Target Cell*

The term a "target cell" as used herein refers to a cell which is expressing the target nucleic acid. In some embodiments the target cell may be *in vivo* or *in vitro*. In some embodiments the target cell is a mammalian cell such as a rodent cell, such as a mouse cell or a rat cell, or a primate cell such as a monkey cell or a human cell.

In preferred embodiments the target cell expresses RB1 mRNA, such as the RB1 pre-mRNA or RB1 mature mRNA. The poly A tail of RB1 mRNA is typically disregarded for antisense  
15 oligonucleotide targeting.

### ***Naturally occurring variant***

The term "naturally occurring variant" refers to variants of RB1 gene or transcripts which originate from the same genetic loci as the target nucleic acid, but may differ for example, by virtue of degeneracy of the genetic code causing a multiplicity of codons encoding the same  
20 amino acid, or due to alternative splicing of pre-mRNA, or the presence of polymorphisms, such as single nucleotide polymorphisms, and allelic variants. Based on the presence of the sufficient complementary sequence to the oligonucleotide, the oligonucleotide of the invention may therefore target the target nucleic acid and naturally occurring variants thereof.

In some embodiments, the naturally occurring variants have at least 95% such as at least 98%  
25 or at least 99% homology to a mammalian RB1 target nucleic acid, such as a target nucleic acid selected from the group consisting of SEQ ID NO 1, 2, and 3 or any other pre-mRNA or mRNAs disclosed herein.

### ***Modulation of expression***

The term "modulation of expression" as used herein is to be understood as an overall term for  
30 an oligonucleotide's ability to alter the amount of RB1 when compared to the amount of RB1 before administration of the oligonucleotide. Alternatively modulation of expression may be determined by reference to a control experiment. It is generally understood that the control is an individual or target cell treated with a saline composition or an individual or target cell treated with a non-targeting oligonucleotide (mock). It may however also be an individual treated with  
35 the standard of care.

One type of modulation is an oligonucleotide's ability to inhibit, down-regulate, reduce, suppress, remove, stop, block, prevent, lessen, lower, avoid or terminate expression of RB1, e.g. by degradation of mRNA or blockage of transcription. Another type of modulation is an oligonucleotide's ability to restore, increase or enhance expression of RB1, e.g. by repair of splice sites or prevention of splicing or removal or blockage of inhibitory mechanisms such as microRNA repression.

### ***High affinity modified nucleosides***

A high affinity modified nucleoside is a modified nucleotide which, when incorporated into the oligonucleotide enhances the affinity of the oligonucleotide for its complementary target, for example as measured by the melting temperature ( $T^m$ ). A high affinity modified nucleoside of the present invention preferably result in an increase in melting temperature between +0.5 to +12°C, more preferably between +1.5 to +10°C and most preferably between +3 to +8°C per modified nucleoside. Numerous high affinity modified nucleosides are known in the art and include for example, many 2' substituted nucleosides as well as locked nucleic acids (LNA) (see e.g. Freier & Altmann; Nucl. Acid Res., 1997, 25, 4429-4443 and Uhlmann; Curr. Opinion in Drug Development, 2000, 3(2), 293-213).

### ***Sugar modifications***

The oligomer of the invention may comprise one or more nucleosides which have a modified sugar moiety, *i.e.* a modification of the sugar moiety when compared to the ribose sugar moiety found in DNA and RNA.

Numerous nucleosides with modification of the ribose sugar moiety have been made, primarily with the aim of improving certain properties of oligonucleotides, such as affinity and/or nuclease resistance.

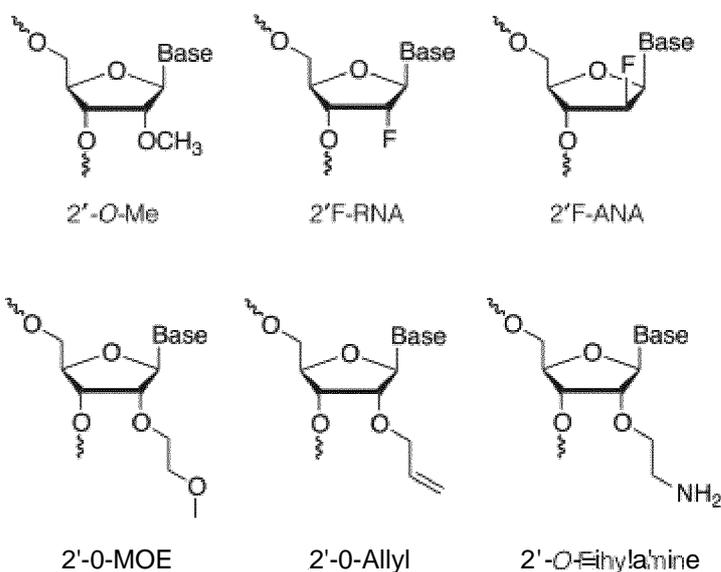
Such modifications include those where the ribose ring structure is modified, e.g. by replacement with a hexose ring (HNA), or a bicyclic ring, which typically have a biradicle bridge between the C2 and C4 carbons on the ribose ring (LNA), or an unlinked ribose ring which typically lacks a bond between the C2 and C3 carbons (e.g. UNA). Other sugar modified nucleosides include, for example, bicyclohexose nucleic acids (WO201 1/01 7521) or tricyclic nucleic acids (WO201 3/1 54798). Modified nucleosides also include nucleosides where the sugar moiety is replaced with a non-sugar moiety, for example in the case of peptide nucleic acids (RNA), or morpholino nucleic acids.

### ***2' sugar modified nucleosides.***

A 2' sugar modified nucleoside is a nucleoside which has a substituent other than H or -OH at the 2' position (2' substituted nucleoside) or comprises a 2' linked biradicle capable of forming a

bridge between the 2' carbon and a second carbon in the ribose ring, such as LNA (2' - 4' biradicle bridged) nucleosides.

Indeed, much focus has been spent on developing 2' substituted nucleosides, and numerous 2' substituted nucleosides have been found to have beneficial properties when incorporated into oligonucleotides. For example, the 2' modified sugar may provide enhanced binding affinity and/or increased nuclease resistance to the oligonucleotide. Examples of 2' substituted modified nucleosides are 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA (MOE), 2'-amino-DNA, 2'-Fluoro-RNA, and 2'-F-ANA nucleoside. For further examples, please see e.g. Freier & Altmann; Nucl. Acid Res., 1997, 25, 4429-4443 and Uhlmann; Curr. Opinion in Drug Development, 2000, 3(2), 293-213, and Deleavey and Damha, Chemistry and Biology 2012, 19, 937. Below are illustrations of some 2' substituted modified nucleosides.



In relation to the present invention 2' substituted sugar modified nucleosides does not include 2' bridged nucleosides like LNA.

### ***Locked Nucleic Acid Nucleosides (LNA)***

A "LNA nucleoside" is a 2'-modified nucleoside which comprises a biradical linking the C2' and C4' of the ribose sugar ring of said nucleoside (also referred to as a "2'- 4' bridge"), which restricts or locks the conformation of the ribose ring. These nucleosides are also termed bridged nucleic acid or bicyclic nucleic acid (BNA) in the literature. The locking of the conformation of the ribose is associated with an enhanced affinity of hybridization (duplex stabilization) when the LNA is incorporated into an oligonucleotide for a complementary RNA or DNA molecule. This can be routinely determined by measuring the melting temperature of the oligonucleotide/complement duplex.

Non limiting, exemplary LNA nucleosides are disclosed in WO 99/014226, WO 00/66604, WO 98/039352, WO 2004/046160, WO 00/047599, WO 2007/134181, WO 2010/077578, WO 2010/036698, WO 2007/090071, WO 2009/006478, WO 2011/156202, WO 2008/154401, WO 2009/067647, WO 2008/150729, Morita et al., Bioorganic & Med.Chem. Lett. 12, 73-76, Seth et al. J. Org. Chem. 2010, Vol 75(5) pp. 1569-81, and Mitsuoka et al., Nucleic Acids Research 2009, 37(4), 1225-1238.

The 2'-4' bridge comprises 2 to 4 bridging atoms and is in particular of formula -X-Y- wherein

X is oxygen, sulfur,  $-CR^aR^b-$ ,  $-C(R^a)=C(R^b)-$ ,  $-C(=CR^aR^b)-$ ,  $-C(R^a)=N-$ ,  $-Si(R^a)_2-$ ,  $-SO_2-$ ,  $-NR^a-$ ;  $-O-NR^a-$ ,  $-NR^a-O-$ ,  $>C=J$ , Se,  $-O-NR^a-$ ,  $-NR^a-CR^aR^b-$ ,  $-N(R^a)-O-$  or  $-O-CR^aR^b-$ ;

10 Y is oxygen, sulfur,  $-(CR^aR^b)_n-$ ,  $-CR^aR^b-O-CR^aR^b-$ ,  $-C(R^a)=C(R^b)-$ ,  $-C(R^a)=N-$ ,  $-Si(R^a)_2-$ ,  $-SO_2-$ ,  $-NR^a-$ ,  $>C=J$ , Se,  $-O-NR^a-$ ,  $-NR^a-CR^aR^b-$ ,  $-N(R^a)-O-$  or  $-O-CR^aR^b-$ ;

with the proviso that -X-Y- is not  $-O-O-$ ,  $Si(R^a)_2-Si(R^a)_2-$ ,  $-SO_2-SO_2-$ ,  $-C(R^a)=C(R^b)-C(R^a)=C(R^b)-$ ,  $-C(R^a)=N-C(R^a)=N-$ ,  $-C(R^a)=N-C(R^a)=C(R^b)-$ ,  $-C(R^a)=C(R^b)-C(R^a)=N-$  or  $-Se-Se-$ ;

15 J is oxygen, sulfur,  $=CH_2$  or  $=N(R^a)$ ;

$R^a$  and  $R^b$  are independently selected from hydrogen, halogen, hydroxyl, cyano, thiohydroxyl, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, alkoxy, substituted alkoxy, alkoxyalkyl, alkenyloxy, carboxyl, alkoxy-carbonyl, alkyl-carbonyl, formyl, aryl, heterocyclyl, amino, alkylamino, carbamoyl, alkylaminocarbonyl, aminoalkylaminocarbonyl, alkylaminoalkylaminocarbonyl, alkylcarbonylamino, carbamido, alkanoyloxy, sulfonyl, alkylsulfonyloxy, nitro, azido, thiohydroxylsulfidealkylsulfanyl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl,  $-OC(=X^a)R^c$ ,  $-OC(=X^a)NR^cR^d$  and  $-NR^eC(=X^a)NR^cR^d$ ;

25 or two geminal  $R^a$  and  $R^b$  together form optionally substituted methylene;

or two geminal  $R^a$  and  $R^b$ , together with the carbon atom to which they are attached, form cycloalkyl or halocycloalkyl, with only one carbon atom of -X-Y-;

wherein substituted alkyl, substituted alkenyl, substituted alkynyl, substituted alkoxy and substituted methylene are alkyl, alkenyl, alkynyl and methylene substituted with 1 to 3 substituents independently selected from halogen, hydroxyl, alkyl, alkenyl, alkynyl, alkoxy, alkoxyalkyl, alkenyloxy, carboxyl, alkoxy-carbonyl, alkyl-carbonyl, formyl, heterocyclyl, aryl and heteroaryl;

30  $X^a$  is oxygen, sulfur or  $-NR^c$ ;

$R^c$ ,  $R^d$  and  $R^e$  are independently selected from hydrogen and alkyl; and

n is 1, 2 or 3.

In a further particular embodiment of the invention, X is oxygen, sulfur,  $-NR^a$ ,  $-CR^aR^b$ - or  $-C(=CR^aR^b)-$ , particularly oxygen, sulfur,  $-NH-$ ,  $-CH_2-$  or  $-C(=CH_2)-$ , more particularly oxygen.

In another particular embodiment of the invention, Y is  $-CR^aR^b$ -,  $-CR^aR^b-CR^aR^b$ - or  $-CR^aR^b-CR^aR^b-CR^aR^b$ -, particularly  $-CH_2-CHCH_3-$ ,  $-CHCH_3-CH_2-$ ,  $-CH_2-CH_2-$  or  $-CH_2-CH_2-CH_2-$ .

In a particular embodiment of the invention,  $-X-Y-$  is  $-O-(CR^aR^b)_n-$ ,  $-S-CR^aR^b$ -,  $-N(R^a)CR^aR^b$ -,  $-CR^aR^b-CR^aR^b$ -,  $-O-CR^aR^b-O-CR^aR^b$ -,  $-CR^aR^b-O-CR^aR^b$ -,  $-C(=CR^aR^b)-CR^aR^b$ -,  $-N(R^a)CR^aR^b$ -,  $-O-N(R^a)-CR^aR^b$ - or  $-N(R^a)-O-CR^aR^b$ .

In a particular embodiment of the invention,  $R^a$  and  $R^b$  are independently selected from the group consisting of hydrogen, halogen, hydroxyl, alkyl and alkoxyalkyl, in particular hydrogen, halogen, alkyl and alkoxyalkyl.

In another embodiment of the invention,  $R^a$  and  $R^b$  are independently selected from the group consisting of hydrogen, fluoro, hydroxyl, methyl and  $-CH_2-O-CH_3$ , in particular hydrogen, fluoro, methyl and  $-CH_2-O-CH_3$ .

Advantageously, one of  $R^a$  and  $R^b$  of  $-X-Y-$  is as defined above and the other ones are all hydrogen at the same time.

In a further particular embodiment of the invention,  $R^a$  is hydrogen or alkyl, in particular hydrogen or methyl.

In another particular embodiment of the invention,  $R^b$  is hydrogen or or alkyl, in particular hydrogen or methyl.

In a particular embodiment of the invention, one or both of  $R^a$  and  $R^b$  are hydrogen.

In a particular embodiment of the invention, only one of  $R^a$  and  $R^b$  is hydrogen.

In one particular embodiment of the invention, one of  $R^a$  and  $R^b$  is methyl and the other one is hydrogen.

In a particular embodiment of the invention,  $R^a$  and  $R^b$  are both methyl at the same time.

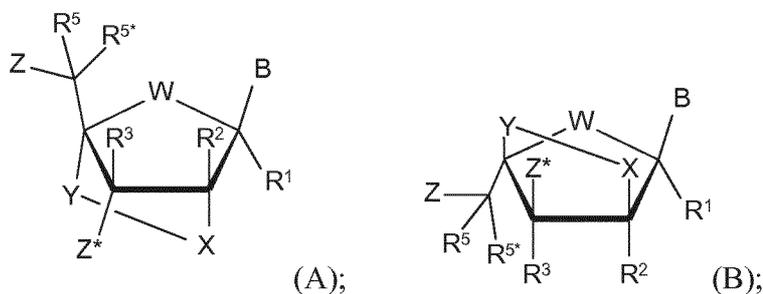
In a particular embodiment of the invention,  $-X-Y-$  is  $-O-CH_2-$ ,  $-S-CH_2-$ ,  $-S-CH(CH_3)-$ ,  $-NH-CH_2-$ ,  $-O-CH_2CH_2-$ ,  $-O-CH(CH_2-O-CH_3)-$ ,  $-O-CH(CH_2CH_3)-$ ,  $-O-CH(CH_3)-$ ,  $-O-CH_2-O-CH_2-$ ,  $-O-CH_2-O-CH_2-$ ,  $-CH_2-O-CH_2-$ ,  $-C(=CH_2)CH_2-$ ,  $-C(=CH_2)CH(CH_3)-$ ,  $-N(OCH_3)CH_2-$  or  $-N(CH_3)CH_2-$ ;

In a particular embodiment of the invention,  $-X-Y-$  is  $-O-CR^aR^b$ - wherein  $R^a$  and  $R^b$  are independently selected from the group consisting of hydrogen, alkyl and alkoxyalkyl, in particular hydrogen, methyl and  $-CH_2-O-CH_3$ .

In a particular embodiment,  $-X-Y-$  is  $-O-CH_2-$  or  $-O-CH(CH_3)-$ , particularly  $-O-CH_2-$ .

The 2'- 4' bridge may be positioned either below the plane of the ribose ring (beta-D-configuration), or above the plane of the ring (alpha-L- configuration), as illustrated in formula (A) and formula (B) respectively.

The LNA nucleoside according to the invention is in particular of formula (A) or (B)



wherein

W is oxygen, sulfur, -N(R<sup>a</sup>)- or -CR<sup>a</sup>R<sup>b</sup>-, in particular oxygen;

B is a nucleobase or a modified nucleobase;

Z is an internucleoside linkage to an adjacent nucleoside or a 5'-terminal group;

10 Z\* is an internucleoside linkage to an adjacent nucleoside or a 3'-terminal group;

R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5'</sup> are independently selected from hydrogen, halogen, alkyl, haloalkyl, alkenyl, alkynyl, hydroxy, alkoxy, alkoxyalkyl, azido, alkenyloxy, carboxyl, alkoxy carbonyl, alkyl carbonyl, formyl and aryl; and

X, Y, R<sup>a</sup> and R<sup>b</sup> are as defined above.

15 In a particular embodiment, in the definition of -X-Y-, R<sup>a</sup> is hydrogen or alkyl, in particular hydrogen or methyl. In another particular embodiment, in the definition of -X-Y-, R<sup>b</sup> is hydrogen or alkyl, in particular hydrogen or methyl. In a further particular embodiment, in the definition of -X-Y-, one or both of R<sup>a</sup> and R<sup>b</sup> are hydrogen. In a particular embodiment, in the definition of -X-Y-, only one of R<sup>a</sup> and R<sup>b</sup> is hydrogen. In one particular embodiment, in the definition of -X-Y-,  
 20 one of R<sup>a</sup> and R<sup>b</sup> is methyl and the other one is hydrogen. In a particular embodiment, in the definition of -X-Y-, R<sup>a</sup> and R<sup>b</sup> are both methyl at the same time.

In a further particular embodiment, in the definition of X, R<sup>a</sup> is hydrogen or alkyl, in particular hydrogen or methyl. In another particular embodiment, in the definition of X, R<sup>b</sup> is hydrogen or alkyl, in particular hydrogen or methyl. In a particular embodiment, in the definition of X, one or  
 25 both of R<sup>a</sup> and R<sup>b</sup> are hydrogen. In a particular embodiment, in the definition of X, only one of R<sup>a</sup> and R<sup>b</sup> is hydrogen. In one particular embodiment, in the definition of X, one of R<sup>a</sup> and R<sup>b</sup> is methyl and the other one is hydrogen. In a particular embodiment, in the definition of X, R<sup>a</sup> and R<sup>b</sup> are both methyl at the same time.

In a further particular embodiment, in the definition of Y, R<sup>a</sup> is hydrogen or alkyl, in particular hydrogen or methyl. In another particular embodiment, in the definition of Y, R<sup>b</sup> is hydrogen or alkyl, in particular hydrogen or methyl. In a particular embodiment, in the definition of Y, one or both of R<sup>a</sup> and R<sup>b</sup> are hydrogen. In a particular embodiment, in the definition of Y, only one of R<sup>a</sup> and R<sup>b</sup> is hydrogen. In one particular embodiment, in the definition of Y, one of R<sup>a</sup> and R<sup>b</sup> is methyl and the other one is hydrogen. In a particular embodiment, in the definition of Y, R<sup>a</sup> and R<sup>b</sup> are both methyl at the same time.

In a particular embodiment of the invention R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are independently selected from hydrogen and alkyl, in particular hydrogen and methyl.

10 In a further particular advantageous embodiment of the invention, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time.

In another particular embodiment of the invention, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, are all hydrogen at the same time, one of R<sup>5</sup> and R<sup>5\*</sup> is hydrogen and the other one is as defined above, in particular alkyl, more particularly methyl.

15 In a particular embodiment of the invention, R<sup>5</sup> and R<sup>5\*</sup> are independently selected from hydrogen, halogen, alkyl, alkoxyalkyl and azido, in particular from hydrogen, fluoro, methyl, methoxyethyl and azido. In particular advantageous embodiments of the invention, one of R<sup>5</sup> and R<sup>5\*</sup> is hydrogen and the other one is alkyl, in particular methyl, halogen, in particular fluoro, alkoxyalkyl, in particular methoxyethyl or azido; or R<sup>5</sup> and R<sup>5\*</sup> are both hydrogen or halogen at the same time, in particular both hydrogen or fluoro at the same time. In such particular  
20 embodiments, W can advantageously be oxygen, and -X-Y- advantageously -O-CH<sub>2</sub>-.

In a particular embodiment of the invention, -X-Y- is -O-CH<sub>2</sub>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such LNA nucleosides are disclosed in WO 99/014226, WO 00/66604, WO 98/039352 and WO 2004/046160 which are all hereby  
25 incorporated by reference, and include what are commonly known in the art as beta-D-oxy LNA and alpha-L-oxy LNA nucleosides.

In another particular embodiment of the invention, -X-Y- is -S-CH<sub>2</sub>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such thio LNA nucleosides are disclosed in WO 99/014226 and WO 2004/046160 which are hereby incorporated by reference.

30 In another particular embodiment of the invention, -X-Y- is -NH-CH<sub>2</sub>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such amino LNA nucleosides are disclosed in WO 99/014226 and WO 2004/046160 which are hereby incorporated by reference.

In another particular embodiment of the invention, -X-Y- is -O-CH<sub>2</sub>CH<sub>2</sub>- or -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-, W is oxygen, and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such LNA nucleosides  
35 are disclosed in WO 00/047599 and Morita *et al.*, Bioorganic & Med.Chem. Lett. 12, 73-76,

which are hereby incorporated by reference, and include what are commonly known in the art as 2'-0-4'C-ethylene bridged nucleic acids (ENA).

In another particular embodiment of the invention, -X-Y- is -O-CH<sub>2</sub>-, W is oxygen, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> are all hydrogen at the same time, one of R<sup>5</sup> and R<sup>5\*</sup> is hydrogen and the other one is not hydrogen, such as alkyl, for example methyl. Such 5' substituted LNA nucleosides are disclosed in WO 2007/134181 which is hereby incorporated by reference.

In another particular embodiment of the invention, -X-Y- is -O-CR<sup>a</sup>R<sup>b</sup>-, wherein one or both of R<sup>a</sup> and R<sup>b</sup> are not hydrogen, in particular alkyl such as methyl, W is oxygen, R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> are all hydrogen at the same time, one of R<sup>5</sup> and R<sup>5\*</sup> is hydrogen and the other one is not hydrogen, in particular alkyl, for example methyl. Such bis modified LNA nucleosides are disclosed in WO 2010/077578 which is hereby incorporated by reference.

In another particular embodiment of the invention, -X-Y- is -O-CHR<sup>a</sup>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such 6'-substituted LNA nucleosides are disclosed in WO 2010/036698 and WO 2007/090071 which are both hereby incorporated by reference. In such 6'-substituted LNA nucleosides, R<sup>a</sup> is in particular CrC<sub>6</sub> alkyl, such as methyl.

In another particular embodiment of the invention, -X-Y- is -O-CH(CH<sub>2</sub>-O-CH<sub>3</sub>)- ("2' O-methoxyethyl bicyclic nucleic acid", Seth *et al.* J. Org. Chem. 2010, Vol 75(5) pp. 1569-81).

In another particular embodiment of the invention, -X-Y- is -O-CH(CH<sub>2</sub>CH<sub>3</sub>)- ("2'O-ethyl bicyclic nucleic acid", Seth *at al.*, J. Org. Chem. 2010, Vol 75(5) pp. 1569-81).

In another particular embodiment of the invention, -X-Y- is -O-CH(CH<sub>2</sub>-O-CH<sub>3</sub>)-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such LNA nucleosides are also known in the art as cyclic MOEs (cMOE) and are disclosed in WO 2007/090071.

In another particular embodiment of the invention, -X-Y- is -O-CH(CH<sub>3</sub>)-.

In another particular embodiment of the invention, -X-Y- is -O-CH<sub>2</sub>-O-CH<sub>2</sub>- (Seth *et al.*, J. Org. Chem 2010 op. cit.)

In another particular embodiment of the invention, -X-Y- is -O-CH(CH<sub>3</sub>)-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such 6'-methyl LNA nucleosides are also known in the art as cET nucleosides, and may be either (S)-cET or (R)-cET diastereoisomers, as disclosed in WO 2007/090071 (beta-D) and WO 2010/036698 (alpha-L) which are both hereby incorporated by reference.

In another particular embodiment of the invention, -X-Y- is -O-CR<sup>a</sup>R<sup>b</sup>-, wherein neither R<sup>a</sup> nor R<sup>b</sup> is hydrogen, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. In a particular embodiment, R<sup>a</sup> and R<sup>b</sup> are both alkyl at the same time, in particular both methyl at

the same time. Such 6'-di-substituted LNA nucleosides are disclosed in WO 2009/006478 which is hereby incorporated by reference.

In another particular embodiment of the invention, -X-Y- is -S-CHR<sup>a</sup>, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. Such 6'-substituted thio LNA nucleosides are disclosed in WO 201 1/156202 which is hereby incorporated by reference. In a particular embodiment of such 6'-substituted thio LNA, R<sup>a</sup> is alkyl, in particular methyl.

In a particular embodiment of the invention, -X-Y- is -C(=CH<sub>2</sub>)C(R<sup>a</sup>R<sup>b</sup>)-, -C(=CHF)C(R<sup>a</sup>R<sup>b</sup>)- or -C(=CF<sub>2</sub>)C(R<sup>a</sup>R<sup>b</sup>)-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. R<sup>a</sup> and R<sup>b</sup> are advantageously independently selected from hydrogen, halogen, alkyl and alkoxyalkyl, in particular hydrogen, methyl, fluoro and methoxymethyl. R<sup>a</sup> and R<sup>b</sup> are in particular both hydrogen or methyl at the same time or one of R<sup>a</sup> and R<sup>b</sup> is hydrogen and the other one is methyl. Such vinyl carbo LNA nucleosides are disclosed in WO 2008/154401 and WO 2009/067647 which are both hereby incorporated by reference.

In a particular embodiment of the invention, -X-Y- is -N(OR<sup>a</sup>)-CH<sub>2</sub>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. In a particular embodiment, R<sup>a</sup> is alkyl such as methyl. Such LNA nucleosides are also known as N substituted LNAs and are disclosed in WO 2008/150729 which is hereby incorporated by reference.

In a particular embodiment of the invention, -X-Y- is -O-N(R<sup>a</sup>)-, -N(R<sup>a</sup>)-O-, -NR<sup>a</sup>-CR<sup>a</sup>R<sup>b</sup>-CR<sup>a</sup>R<sup>b</sup>- or -NR<sup>a</sup>-CR<sup>a</sup>R<sup>b</sup>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. R<sup>a</sup> and R<sup>b</sup> are advantageously independently selected from hydrogen, halogen, alkyl and alkoxyalkyl, in particular hydrogen, methyl, fluoro and methoxymethyl. In a particular embodiment, R<sup>a</sup> is alkyl, such as methyl, R<sup>b</sup> is hydrogen or methyl, in particular hydrogen. (Seth *et al.*, J. Org. Chem 2010 *op. cit.*).

In a particular embodiment of the invention, -X-Y- is -O-N(CH<sub>3</sub>)- (Seth *et al.*, J. Org. Chem 2010 *op. cit.*).

In a particular embodiment of the invention, R<sup>5</sup> and R<sup>5\*</sup> are both hydrogen at the same time. In another particular embodiment of the invention, one of R<sup>5</sup> and R<sup>5\*</sup> is hydrogen and the other one is alkyl, such as methyl. In such embodiments, R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup> can be in particular hydrogen and -X-Y- can be in particular -O-CH<sub>2</sub>- or -O-CHC(R<sup>a</sup>)<sub>3</sub>-, such as -O-CH(CH<sub>3</sub>)-

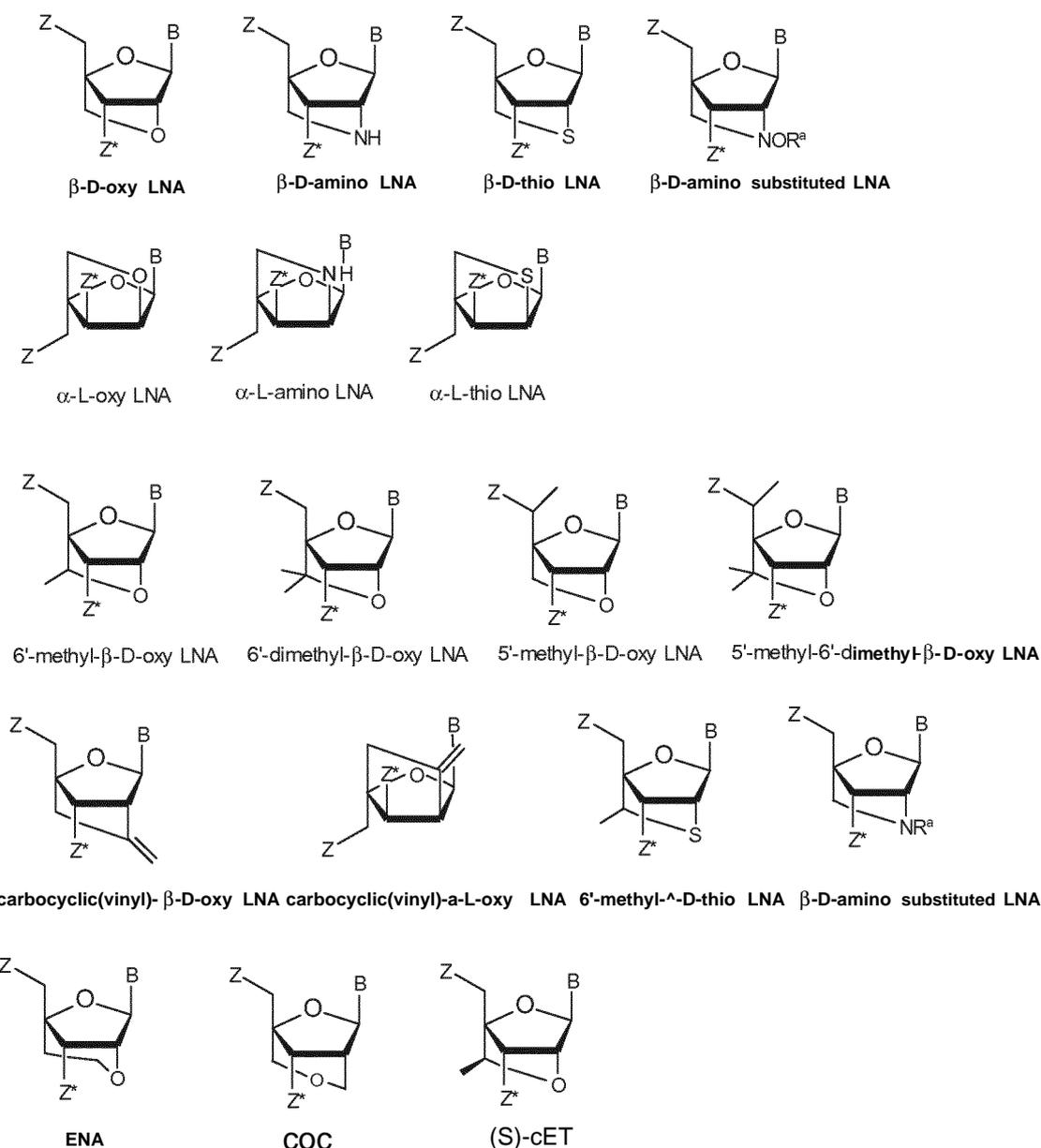
In a particular embodiment of the invention, -X-Y- is -CR<sup>a</sup>R<sup>b</sup>-O-CR<sup>a</sup>R<sup>b</sup>-, such as -CH<sub>2</sub>-O-CH<sub>2</sub>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5\*</sup> are all hydrogen at the same time. In such particular embodiments, R<sup>a</sup> can be in particular alkyl such as methyl, R<sup>b</sup> hydrogen or methyl, in particular hydrogen. Such LNA nucleosides are also known as conformationally restricted nucleotides (CRNs) and are disclosed in WO 2013/036868 which is hereby incorporated by reference.

In a particular embodiment of the invention, -X-Y- is -O-CR<sup>a</sup>R<sup>b</sup>-O-CR<sup>a</sup>R<sup>b</sup>-, such as -O-CH<sub>2</sub>-O-CH<sub>2</sub>-, W is oxygen and R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>5</sup> and R<sup>5'</sup> are all hydrogen at the same time. R<sup>a</sup> and R<sup>b</sup> are advantageously independently selected from hydrogen, halogen, alkyl and alkoxyalkyl, in particular hydrogen, methyl, fluoro and methoxymethyl. In such a particular embodiment, R<sup>a</sup> can be in particular alkyl such as methyl, R<sup>b</sup> hydrogen or methyl, in particular hydrogen. Such LNA nucleosides are also known as COC nucleotides and are disclosed in Mitsuoka *et al.*, Nucleic Acids Research 2009, 37(4), 1225-1238, which is hereby incorporated by reference.

It will be recognized that, unless specified, the LNA nucleosides may be in the beta-D or alpha-L stereoisomeric form.

10 Particular examples of LNA nucleosides of the invention are presented in Scheme 1 (wherein B is as defined above).

Scheme 1



Particular LNA nucleosides are beta-D-oxy-LNA, 6'-methyl-beta-D-oxy LNA such as (S)-6'-methyl-beta-D-oxy-LNA (ScET) and ENA.

If one of the starting materials or compounds of the invention contain one or more functional groups which are not stable or are reactive under the reaction conditions of one or more reaction steps, appropriate protecting groups (as described e.g. in "Protective Groups in Organic Chemistry" by T. W. Greene and P. G. M. Wuts, 3rd Ed., 1999, Wiley, New York) can be introduced before the critical step applying methods well known in the art. Such protecting groups can be removed at a later stage of the synthesis using standard methods described in the literature. Examples of protecting groups are tert-butoxycarbonyl (Boc), 9-fluorenylmethyl carbamate (Fmoc), 2-trimethylsilylethyl carbamate (Teoc), carbobenzyloxy (Cbz) and p-methoxybenzyloxycarbonyl (Moz).

The compounds described herein can contain several asymmetric centers and can be present in the form of optically pure enantiomers, mixtures of enantiomers such as, for example, racemates, mixtures of diastereoisomers, diastereoisomeric racemates or mixtures of diastereoisomeric racemates.

The term "asymmetric carbon atom" means a carbon atom with four different substituents. According to the Cahn-Ingold-Prelog Convention an asymmetric carbon atom can be of the "R" or "S" configuration.

### ***Chemical group definitions***

In the present description the term "alkyl", alone or in combination, signifies a straight-chain or branched-chain alkyl group with 1 to 8 carbon atoms, particularly a straight or branched-chain alkyl group with 1 to 6 carbon atoms and more particularly a straight or branched-chain alkyl group with 1 to 4 carbon atoms. Examples of straight-chain and branched-chain  $C_{1-8}$  alkyl groups are methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert.-butyl, the isomeric pentyls, the isomeric hexyls, the isomeric heptyls and the isomeric octyls, particularly methyl, ethyl, propyl, butyl and pentyl. Particular examples of alkyl are methyl, ethyl and propyl.

The term "cycloalkyl", alone or in combination, signifies a cycloalkyl ring with 3 to 8 carbon atoms and particularly a cycloalkyl ring with 3 to 6 carbon atoms. Examples of cycloalkyl are cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl, more particularly cyclopropyl and cyclobutyl. A particular example of "cycloalkyl" is cyclopropyl.

The term "alkoxy", alone or in combination, signifies a group of the formula alkyl-O- in which the term "alkyl" has the previously given significance, such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec.butoxy and tert.butoxy. Particular "alkoxy" are methoxy and ethoxy. Methoxyethoxy is a particular example of "alkoxyalkoxy".

The term "oxy", alone or in combination, signifies the -O- group.

The term "alkenyl", alone or in combination, signifies a straight-chain or branched hydrocarbon residue comprising an olefinic bond and up to 8, preferably up to 6, particularly preferred up to 4 carbon atoms. Examples of alkenyl groups are ethenyl, 1-propenyl, 2-propenyl, isopropenyl, 1-butenyl, 2-butenyl, 3-butenyl and isobutenyl.

- 5 The term "alkynyl", alone or in combination, signifies a straight-chain or branched hydrocarbon residue comprising a triple bond and up to 8, preferably up to 6, particularly preferred up to 4 carbon atoms.

The terms "halogen" or "halo", alone or in combination, signifies fluorine, chlorine, bromine or iodine and particularly fluorine, chlorine or bromine, more particularly fluorine. The term "halo",  
10 in combination with another group, denotes the substitution of said group with at least one halogen, particularly substituted with one to five halogens, particularly one to four halogens, i.e. one, two, three or four halogens.

The term "haloalkyl", alone or in combination, denotes an alkyl group substituted with at least one halogen, particularly substituted with one to five halogens, particularly one to three  
15 halogens. Examples of haloalkyl include monofluoro-, difluoro- or trifluoro-methyl, -ethyl or -propyl, for example 3,3,3-trifluoropropyl, 2-fluoroethyl, 2,2,2-trifluoroethyl, fluoromethyl or trifluoromethyl. Fluoromethyl, difluoromethyl and trifluoromethyl are particular "haloalkyl".

The term "halocycloalkyl", alone or in combination, denotes a cycloalkyl group as defined above substituted with at least one halogen, particularly substituted with one to five halogens,  
20 particularly one to three halogens. Particular example of "halocycloalkyl" are halocyclopropyl, in particular fluorocyclopropyl, difluorocyclopropyl and trifluorocyclopropyl.

The terms "hydroxyl" and "hydroxy", alone or in combination, signify the -OH group.

The terms "thiohydroxyl" and "thiohydroxy", alone or in combination, signify the -SH group.

The term "carbonyl", alone or in combination, signifies the -C(O)- group.

- 25 The term "carboxy" or "carboxyl", alone or in combination, signifies the -COOH group.

The term "amino", alone or in combination, signifies the primary amino group (-NH<sub>2</sub>), the secondary amino group (-NH-), or the tertiary amino group (-N-).

The term "alkylamino", alone or in combination, signifies an amino group as defined above substituted with one or two alkyl groups as defined above.

- 30 The term "sulfonyl", alone or in combination, means the -SO<sub>2</sub> group.

The term "sulfinyl", alone or in combination, signifies the -SO- group.

The term "sulfanyl", alone or in combination, signifies the -S- group.

The term "cyano", alone or in combination, signifies the -CN group.

The term "azido", alone or in combination, signifies the  $-N_3$  group.

The term "nitro", alone or in combination, signifies the  $NO_2$  group.

The term "formyl", alone or in combination, signifies the  $-C(O)H$  group.

The term "carbamoyl", alone or in combination, signifies the  $-C(O)NH_2$  group.

5 The term "cabamido", alone or in combination, signifies the  $-NH-C(O)-NH_2$  group.

The term "aryl", alone or in combination, denotes a monovalent aromatic carbocyclic mono- or bicyclic ring system comprising 6 to 10 carbon ring atoms, optionally substituted with 1 to 3 substituents independently selected from halogen, hydroxyl, alkyl, alkenyl, alkynyl, alkoxy, alkoxyalkyl, alkenyloxy, carboxyl, alkoxy carbonyl, alkyl carbonyl and formyl. Examples of aryl  
10 include phenyl and naphthyl, in particular phenyl.

The term "heteroaryl", alone or in combination, denotes a monovalent aromatic heterocyclic mono- or bicyclic ring system of 5 to 12 ring atoms, comprising 1, 2, 3 or 4 heteroatoms selected from N, O and S, the remaining ring atoms being carbon, optionally substituted with 1 to 3 substituents independently selected from halogen, hydroxyl, alkyl, alkenyl, alkynyl, alkoxy,  
15 alkoxyalkyl, alkenyloxy, carboxyl, alkoxy carbonyl, alkyl carbonyl and formyl. Examples of heteroaryl include pyrrolyl, furanyl, thienyl, imidazolyl, oxazolyl, thiazolyl, triazolyl, oxadiazolyl, thiadiazolyl, tetrazolyl, pyridinyl, pyrazinyl, pyrazolyl, pyridazinyl, pyrimidinyl, triazinyl, azepinyl, diazepinyl, isoxazolyl, benzofuranyl, isothiazolyl, benzothienyl, indolyl, isoindolyl, isobenzofuranyl, benzimidazolyl, benzoxazolyl, benzoisoxazolyl, benzothiazolyl,  
20 benzoisothiazolyl, benzooxadiazolyl, benzothiadiazolyl, benzotriazolyl, purinyl, quinolinyl, isoquinolinyl, quinazolinyl, quinoxalyl, carbazolyl or acridinyl.

The term "heterocyclyl", alone or in combination, signifies a monovalent saturated or partly unsaturated mono- or bicyclic ring system of 4 to 12, in particular 4 to 9 ring atoms, comprising 1, 2, 3 or 4 ring heteroatoms selected from N, O and S, the remaining ring atoms being carbon,  
25 optionally substituted with 1 to 3 substituents independently selected from halogen, hydroxyl, alkyl, alkenyl, alkynyl, alkoxy, alkoxyalkyl, alkenyloxy, carboxyl, alkoxy carbonyl, alkyl carbonyl and formyl. Examples for monocyclic saturated heterocyclyl are azetidyl, pyrrolidinyl, tetrahydrofuranyl, tetrahydro-thienyl, pyrazolidinyl, imidazolidinyl, oxazolidinyl, isoxazolidinyl, thiazolidinyl, piperidinyl, tetrahydropyranyl, tetrahydrothiopyranyl, piperazinyl, morpholinyl, thiomorpholinyl, 1,1-dioxo-thiomorpholin-4-yl, azepanyl, diazepanyl, homopiperazinyl, or  
30 oxazepanyl. Examples for bicyclic saturated heterocycloalkyl are 8-aza-bicyclo[3.2.1]octyl, quinuclidinyl, 8-oxa-3-aza-bicyclo[3.2.1]octyl, 9-aza-bicyclo[3.3.1]nonyl, 3-oxa-9-aza-bicyclo[3.3.1]nonyl, or 3-thia-9-aza-bicyclo[3.3.1]nonyl. Examples for partly unsaturated heterocycloalkyl are dihydrofuryl, imidazolyl, dihydro-oxazolyl, tetrahydro-pyridinyl or  
35 dihydropyranyl.

**Pharmaceutically acceptable salts**

The term “pharmaceutically acceptable salts” refers to those salts which retain the biological effectiveness and properties of the free bases or free acids, which are not biologically or otherwise undesirable. The salts are formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, particularly hydrochloric acid, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid, N-acetylcystein. In addition these salts may be prepared from addition of an inorganic base or an organic base to the free acid. Salts derived from an inorganic base include, but are not limited to, the sodium, potassium, lithium, ammonium, calcium, magnesium salts. Salts derived from organic bases include, but are not limited to salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, lysine, arginine, N-ethylpiperidine, piperidine, polyamine resins. The compound of formula (I) can also be present in the form of zwitterions. Particularly preferred pharmaceutically acceptable salts of compounds of formula (I) are the salts of hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid and methanesulfonic acid.

**Protecting group**

The term “protecting group”, alone or in combination, signifies a group which selectively blocks a reactive site in a multifunctional compound such that a chemical reaction can be carried out selectively at another unprotected reactive site. Protecting groups can be removed. Exemplary protecting groups are amino-protecting groups, carboxy-protecting groups or hydroxy-protecting groups. Nuclease mediated degradation

Nuclease mediated degradation refers to an oligonucleotide capable of mediating degradation of a complementary nucleotide sequence when forming a duplex with such a sequence.

In some embodiments, the oligonucleotide may function via nuclease mediated degradation of the target nucleic acid, where the oligonucleotides of the invention are capable of recruiting a nuclease, particularly an endonuclease, preferably an endoribonuclease (RNase), such as RNase H. Examples of oligonucleotide designs which operate via nuclease mediated mechanisms are oligonucleotides which typically comprise a region of at least 5 or 6 DNA nucleosides and are flanked on one side or both sides by affinity enhancing nucleosides, for example gapmers, headmers and tailmers.

**Nuclease mediated degradation**

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**RNase H Activity and Recruitment**

The RNase H activity of an antisense oligonucleotide refers to its ability to recruit RNase H when in a duplex with a complementary RNA molecule. WO01/23613 provides *in vitro* methods for determining RNaseH activity, which may be used to determine the ability to recruit RNaseH. Typically an oligonucleotide is deemed capable of recruiting RNase H if it, when provided with a complementary target nucleic acid sequence, has an initial rate, as measured in pmol/l/min, of at least 5%, such as at least 10% or more than 20% of the initial rate determined when using a oligonucleotide having the same base sequence as the modified oligonucleotide being tested, but containing only DNA monomers with phosphorothioate linkages between all monomers in the oligonucleotide, and using the methodology provided by Example 91 - 95 of WO01/23613 (hereby incorporated by reference).

**Gapmer**

The antisense oligonucleotide of the invention, or contiguous nucleotide sequence thereof, may be a gapmer, also termed gapmer oligonucleotide or gapmer designs. The antisense gapmers are commonly used to inhibit a target nucleic acid via RNase H mediated degradation. A gapmer oligonucleotide comprises at least three distinct structural regions a 5'-flank, a gap and a 3'-flank, F-G-F' in the '5 -> 3' orientation. The "gap" region (G) comprises a stretch of contiguous DNA nucleotides which enable the oligonucleotide to recruit RNase H. The gap region is flanked by a 5' flanking region (F) comprising one or more sugar modified nucleosides, advantageously high affinity sugar modified nucleosides, and by a 3' flanking region (F') comprising one or more sugar modified nucleosides, advantageously high affinity sugar modified nucleosides. The one or more sugar modified nucleosides in region F and F' enhance the affinity of the oligonucleotide for the target nucleic acid (*i.e.* are affinity enhancing sugar modified nucleosides). In some embodiments, the one or more sugar modified nucleosides in region F and F' are 2' sugar modified nucleosides, such as high affinity 2' sugar modifications, such as independently selected from LNA and 2'-MOE.

In a gapmer design, the 5' and 3' most nucleosides of the gap region are DNA nucleosides, and are positioned adjacent to a sugar modified nucleoside of the 5' (F) or 3' (F') region respectively. The flanks may further defined by having at least one sugar modified nucleoside at the end most distant from the gap region, i.e. at the 5' end of the 5' flank and at the 3' end of the 3' flank.

- 5 Regions F-G-F' form a contiguous nucleotide sequence. Antisense oligonucleotides of the invention, or the contiguous nucleotide sequence thereof, may comprise a gapmer region of formula F-G-F'.

The overall length of the gapmer design F-G-F' may be, for example 12 to 32 nucleosides, such as 13 to 24, such as 14 to 22 nucleosides, Such as from 14 to17, such as 16 to18 nucleosides.

- 10 By way of example, the gapmer oligonucleotide of the present invention can be represented by the following formulae:

$F_{1-8}-G_{7-16}-F'_{2-8}$ , such as

$F_{1-8}-G_{7-16}-F'_{2-8}$

- 15 with the proviso that the overall length of the gapmer regions F-G-F' is at least 12, such as at least 14 nucleotides in length.

Regions F, G and F' are further defined below and can be incorporated into the F-G-F' formula.

### **Gapmer - Region G**

Region G (gap region) of the gapmer is a region of nucleosides which enables the oligonucleotide to recruit RNaseH, such as human RNase H1, typically DNA nucleosides.

- 20 RNaseH is a cellular enzyme which recognizes the duplex between DNA and RNA, and enzymatically cleaves the RNA molecule. Suitably gapmers may have a gap region (G) of at least 5 or 6 contiguous DNA nucleosides, such as 5 - 16 contiguous DNA nucleosides, such as 6 - 15 contiguous DNA nucleosides, such as 7-14 contiguous DNA nucleosides, such as 8 - 12 contiguous DNA nucleotides, such as 8 - 12 contiguous DNA nucleotides in length. The gap
- 25 region G may, in some embodiments consist of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 contiguous DNA nucleosides. Cytosine (C) DNA in the gap region may in some instances be methylated, such residues are either annotated as 5'-methyl-cytosine (<sup>m</sup>eC or with an e instead of a c). Methylation of cytosine DNA in the gap is advantageous if eg dinucleotides are present in the gap to reduce potential toxicity, the modification does not have significant impact on
- 30 efficacy of the oligonucleotides. 5' substituted DNA nucleosides, such as 5' methyl DNA nucleoside have been reported for use in DNA gap regions (EP 2 742 136).

In some embodiments the gap region G may consist of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 contiguous phosphorothioate linked DNA nucleosides. In some embodiments, all internucleoside linkages in the gap are phosphorothioate linkages.

Whilst traditional gapmers have a DNA gap region, there are numerous examples of modified nucleosides which allow for RNaseH recruitment when they are used within the gap region.

Modified nucleosides which have been reported as being capable of recruiting RNaseH when included within a gap region include, for example, alpha-L-LNA, C4' alkylated DNA (as

5 described in PCT/EP2009/050349 and Vester *et al*, Bioorg. Med. Chem. Lett. 18 (2008) 2296 - 2300, both incorporated herein by reference), arabinose derived nucleosides like ANA and 2'F-ANA (Mangos *et al.* 2003 J. AM. CHEM. SOC. 125, 654-661 ), UNA (unlocked nucleic acid) (as described in Fluiter *et al.*, Mol. Biosyst, 2009, 10, 1039 incorporated herein by reference). UNA is unlocked nucleic acid, typically where the bond between C2 and C3 of the ribose has been  
10 removed, forming an unlocked "sugar" residue. The modified nucleosides used in such gapmers may be nucleosides which adopt a 2' endo (DNA like) structure when introduced into the gap region, *i.e.* modifications which allow for RNaseH recruitment). In some embodiments the DNA Gap region (G) described herein may optionally contain 1 to 3 sugar modified nucleosides which adopt a 2' endo (DNA like) structure when introduced into the gap region.

#### 15 *Region G - "Gap-breaker"*

Alternatively, there are numerous reports of the insertion of a modified nucleoside which confers a 3' endo conformation into the gap region of gapmers, whilst retaining some RNaseH activity.

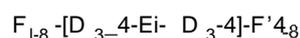
Such gapmers with a gap region comprising one or more 3' endo modified nucleosides are referred to as "gap-breaker" or "gap-disrupted" gapmers, see for example WO2013/022984.

20 Gap-breaker oligonucleotides retain sufficient region of DNA nucleosides within the gap region to allow for RNaseH recruitment. The ability of gapbreaker oligonucleotide design to recruit RNaseH is typically sequence or even compound specific - see Rukov *et al.* 2015 Nucl. Acids Res. Vol. 43 pp. 8476-8487, which discloses "gapbreaker" oligonucleotides which recruit RNaseH which in some instances provide a more specific cleavage of the target RNA.

25 Modified nucleosides used within the gap region of gap-breaker oligonucleotides may for example be modified nucleosides which confer a 3' endo confirmation, such as 2'-O-methyl (OMe) or 2'-O-MOE (MOE) nucleosides, or beta-D LNA nucleosides (the bridge between C2' and C4' of the ribose sugar ring of a nucleoside is in the beta conformation), such as beta-D-oxy LNA or ScET nucleosides.

30 As with gapmers containing region G described above, the gap region of gap-breaker or gap-disrupted gapmers, have a DNA nucleosides at the 5' end of the gap (adjacent to the 3' nucleoside of region F), and a DNA nucleoside at the 3' end of the gap (adjacent to the 5' nucleoside of region F'). Gapmers which comprise a disrupted gap typically retain a region of at least 3 or 4 contiguous DNA nucleosides at either the 5' end or 3' end of the gap region.

35 Exemplary designs for gap-breaker oligonucleotides include



F<sub>1-8</sub>- [D<sub>1-4</sub>-E<sub>1</sub>- D<sub>3-4</sub>]-F'<sub>1-8</sub>

F<sub>1-8</sub>- [D<sub>3-4</sub>-E<sub>r</sub> D<sub>1-4</sub>]-F'<sub>4-8</sub>

wherein region G is within the brackets [D<sub>n</sub>-E<sub>r</sub>- D<sub>m</sub>], D is a contiguous sequence of DNA nucleosides, E is a modified nucleoside (the gap-breaker or gap-disrupting nucleoside), and F and F' are the flanking regions as defined herein, and with the proviso that the overall length of the gapmer regions F-G-F' is at least 12, such as at least 14 nucleotides in length.

In some embodiments, region G of a gap disrupted gapmer comprises at least 6 DNA nucleosides, such as 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 DNA nucleosides. As described above, the DNA nucleosides may be contiguous or may optionally be interspersed with one or more modified nucleosides, with the proviso that the gap region G is capable of mediating RNaseH recruitment.

***Gapmer - flanking regions, F and F'***

Region F is positioned immediately adjacent to the 5' DNA nucleoside of region G. The 3' most nucleoside of region F is a sugar modified nucleoside, such as a high affinity sugar modified nucleoside, for example a 2' substituted nucleoside, such as a MOE nucleoside, or an LNA nucleoside.

Region F' is positioned immediately adjacent to the 3' DNA nucleoside of region G. The 5' most nucleoside of region F' is a sugar modified nucleoside, such as a high affinity sugar modified nucleoside, for example a 2' substituted nucleoside, such as a MOE nucleoside, or an LNA nucleoside.

Region F is 1 - 8 contiguous nucleotides in length, such as 2-6, such as 3-4 contiguous nucleotides in length. Advantageously the 5' most nucleoside of region F is a sugar modified nucleoside. In some embodiments the two 5' most nucleoside of region F are sugar modified nucleoside. In some embodiments the 5' most nucleoside of region F is an LNA nucleoside. In some embodiments the two 5' most nucleoside of region F are LNA nucleosides. In some embodiments the two 5' most nucleoside of region F are 2' substituted nucleoside nucleosides, such as two 3' MOE nucleosides. In some embodiments the 5' most nucleoside of region F is a 2' substituted nucleoside, such as a MOE nucleoside.

Region F' is 2 - 8 contiguous nucleotides in length, such as 3-6, such as 4-5 contiguous nucleotides in length. Advantageously, embodiments the 3' most nucleoside of region F' is a sugar modified nucleoside. In some embodiments the two 3' most nucleoside of region F' are sugar modified nucleoside. In some embodiments the two 3' most nucleoside of region F' are LNA nucleosides. In some embodiments the 3' most nucleoside of region F' is an LNA nucleoside. In some embodiments the two 3' most nucleoside of region F' are 2' substituted nucleoside nucleosides, such as two 3' MOE nucleosides. In some embodiments the 3' most nucleoside of region F' is a 2' substituted nucleoside, such as a MOE nucleoside.

It should be noted that when the length of region F or F' is one, it is advantageously an LNA nucleoside.

In some embodiments, region F and F' independently consists of or comprises a contiguous sequence of sugar modified nucleosides. In some embodiments, the sugar modified

5 nucleosides of region F may be independently selected from 2'-O-alkyl-RNA units, 2'-O-methyl-RNA, 2'-amino-DNA units, 2'-fluoro-DNA units, 2'-alkoxy-RNA, MOE units, LNA units, arabinonucleic acid (ANA) units and 2'-fluoro-ANA units.

In some embodiments, region F and F' independently comprises both LNA and a 2' substituted modified nucleosides (mixed wing design).

10 In some embodiments, region F and F' consists of only one type of sugar modified nucleosides, such as only MOE or only beta-D-oxy LNA or only ScET. Such designs are also termed uniform flanks or uniform gapmer design.

In some embodiments, all the nucleosides of region F or F', or F and F' are LNA nucleosides, such as independently selected from beta-D-oxy LNA, ENA or ScET nucleosides. In some  
15 embodiments region F consists of 1-5, such as 2-4, such as 3-4 such as 1, 2, 3, 4 or 5 contiguous LNA nucleosides. In some embodiments, all the nucleosides of region F and F' are beta-D-oxy LNA nucleosides.

In some embodiments, all the nucleosides of region F or F', or F and F' are 2' substituted nucleosides, such as OMe or MOE nucleosides. In some embodiments region F consists of 1,  
20 2, 3, 4, 5, 6, 7, or 8 contiguous OMe or MOE nucleosides. In some embodiments only one of the flanking regions can consist of 2' substituted nucleosides, such as OMe or MOE nucleosides. In some embodiments it is the 5' (F) flanking region that consists 2' substituted nucleosides, such as OMe or MOE nucleosides whereas the 3' (F') flanking region comprises at least one LNA nucleoside, such as beta-D-oxy LNA nucleosides or cET nucleosides. In some embodiments it  
25 is the 3' (F') flanking region that consists 2' substituted nucleosides, such as OMe or MOE nucleosides whereas the 5' (F) flanking region comprises at least one LNA nucleoside, such as beta-D-oxy LNA nucleosides or cET nucleosides.

In some embodiments, all the modified nucleosides of region F and F' are LNA nucleosides, such as independently selected from beta-D-oxy LNA, ENA or ScET nucleosides, wherein  
30 region F or F', or F and F' may optionally comprise DNA nucleosides (an alternating flank, see definition of these for more details). In some embodiments, all the modified nucleosides of region F and F' are beta-D-oxy LNA nucleosides, wherein region F or F', or F and F' may optionally comprise DNA nucleosides (an alternating flank, see definition of these for more details).

In some embodiments the 5' most and the 3' most nucleosides of region F and F' are LNA nucleosides, such as beta-D-oxy LNA nucleosides or ScET nucleosides.

In some embodiments, the internucleoside linkage between region F and region G is a phosphorothioate internucleoside linkage. In some embodiments, the internucleoside linkage between region F' and region G is a phosphorothioate internucleoside linkage. In some 5 embodiments, the internucleoside linkages between the nucleosides of region F or F', F and F' are phosphorothioate internucleoside linkages.

### **LNA Gapmer**

An LNA gapmer is a gapmer wherein either one or both of region F and F' comprises or 10 consists of LNA nucleosides. A beta-D-oxy gapmer is a gapmer wherein either one or both of region F and F' comprises or consists of beta-D-oxy LNA nucleosides.

In some embodiments the LNA gapmer is of formula: [LNA]<sup>n</sup>-[region G]-[LNA]<sub>1-5</sub>, wherein region G is as defined in the Gapmer region G definition.

### **MOE Gapmers**

15 A MOE gapmers is a gapmer wherein regions F and F' consist of MOE nucleosides. In some embodiments the MOE gapmer is of design [MOE]<sub>1-8</sub>-[Region G]-[MOE]<sub>1-8</sub>, such as [MOE]<sub>2-7</sub>-[Region G]<sub>5-16</sub>-[MOE]<sub>2-7</sub>, such as [MOE]<sub>3-6</sub>-[Region G]-[MOE]<sub>3-6</sub>, wherein region G is as defined in the Gapmer definition. MOE gapmers with a 5-10-5 design (MOE-DNA-MOE) have been widely used in the art.

### **Mixed Wing Gapmer**

20 A mixed wing gapmer is an LNA gapmer wherein one or both of region F and F' comprise a 2' substituted nucleoside, such as a 2' substituted nucleoside independently selected from the group consisting of 2'-O-alkyl-RNA units, 2'-O-methyl-RNA, 2'-amino-DNA units, 2'-fluoro-DNA units, 2'-alkoxy-RNA, MOE units, arabino nucleic acid (ANA) units and 2'-fluoro-ANA units, such 25 as a MOE nucleosides. In some embodiments wherein at least one of region F and F', or both region F and F' comprise at least one LNA nucleoside, the remaining nucleosides of region F and F' are independently selected from the group consisting of MOE and LNA. In some embodiments wherein at least one of region F and F', or both region F and F' comprise at least two LNA nucleosides, the remaining nucleosides of region F and F' are independently selected 30 from the group consisting of MOE and LNA. In some mixed wing embodiments, one or both of region F and F' may further comprise one or more DNA nucleosides.

Mixed wing gapmer designs are disclosed in W02008/049085 and WO201 2/1 09395, both of which are hereby incorporated by reference.

*Alternating Flank Gapmers*

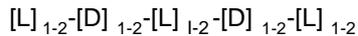
Flanking regions may comprise both LNA and DNA nucleoside and are referred to as "alternating flanks" as they comprise an alternating motif of LNA-DNA-LNA nucleosides. Gapmers comprising such alternating flanks are referred to as "alternating flank gapmers".

5 "Alternative flank gapmers" are thus LNA gapmer oligonucleotides where at least one of the flanks (F or F') comprises DNA in addition to the LNA nucleoside(s). In some embodiments at least one of region F or F', or both region F and F', comprise both LNA nucleosides and DNA nucleosides. In such embodiments, the flanking region F or F', or both F and F' comprise at least three nucleosides, wherein the 5' and 3' most nucleosides of the F and/or F' region are  
 10 LNA nucleosides..

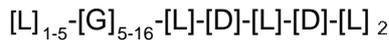
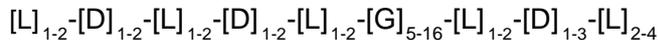
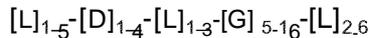
Alternating flank LNA gapmers are disclosed in WO201 6/1 27002.

An alternating flank region may comprise up to 3 contiguous DNA nucleosides, such as 1 to 2 or 1 or 2 or 3 contiguous DNA nucleosides.

The alternating flank can be annotated as a series of integers, representing a number of LNA  
 15 nucleosides (L) followed by a number of DNA nucleosides (D), for example



In oligonucleotide designs these will often be represented as numbers such that 2-2-1 represents 5' [L]<sub>2</sub>-[D]<sub>2</sub>-[L] 3', and 1-1-1-1-1 represents 5' [L]-[D]-[L]-[D]-[L] 3'. The length of the  
 20 flank (region F and F') in oligonucleotides with alternating flanks may independently be 3 to 10 nucleosides, such as 4 to 8, such as 5 to 6 nucleosides, such as 4, 5, 6 or 7 modified nucleosides. In some embodiments only one of the flanks in the gapmer oligonucleotide is alternating while the other is constituted of LNA nucleotides. It may be advantageous to have at least two LNA nucleosides at the 3' end of the 3' flank (F'), to confer additional exonuclease  
 25 resistance. Some examples of oligonucleotides with alternating flanks are:



with the proviso that the overall length of the gapmer is at least 12, such as at least 14  
 30 nucleotides in length.

*Region D' or D'' in an oligonucleotide*

The oligonucleotide of the invention may in some embodiments comprise or consist of the contiguous nucleotide sequence of the oligonucleotide which is complementary to the target nucleic acid, such as the gapmer F-G-F', and further 5' and/or 3' nucleosides. The further 5'

and/or 3' nucleosides may or may not be fully complementary to the target nucleic acid. Such further 5' and/or 3' nucleosides may be referred to as region D' and D'' herein.

The addition of region D' or D'' may be used for the purpose of joining the contiguous nucleotide sequence, such as the gapmer, to a conjugate moiety or another functional group. When used for joining the contiguous nucleotide sequence with a conjugate moiety it can serve as a biocleavable linker. Alternatively it may be used to provide exonuclease protection or for ease of synthesis or manufacture.

Region D' and D'' can be attached to the 5' end of region F or the 3' end of region F', respectively to generate designs of the following formulas D'-F-G-F', F-G-F'-D'' or D'-F-G-F'-D''. In this instance the F-G-F' is the gapmer portion of the oligonucleotide and region D' or D'' constitute a separate part of the oligonucleotide.

Region D' or D'' may independently comprise or consist of 1, 2, 3, 4 or 5 additional nucleotides, which may be complementary or non-complementary to the target nucleic acid. The nucleotide adjacent to the F or F' region is not a sugar-modified nucleotide, such as a DNA or RNA or base modified versions of these. The D' or D'' region may serve as a nuclease susceptible biocleavable linker (see definition of linkers). In some embodiments the additional 5' and/or 3' end nucleotides are linked with phosphodiester linkages, and are DNA or RNA. Nucleotide based biocleavable linkers suitable for use as region D' or D'' are disclosed in WO2014/076195, which include by way of example a phosphodiester linked DNA dinucleotide. The use of biocleavable linkers in poly-oligonucleotide constructs is disclosed in WO2015/113922, where they are used to link multiple antisense constructs (e.g. gapmer regions) within a single oligonucleotide.

In one embodiment the oligonucleotide of the invention comprises a region D' and/or D'' in addition to the contiguous nucleotide sequence which constitutes the gapmer.

In some embodiments, the oligonucleotide of the present invention can be represented by the following formulae:

F-G-F'; in particular  $F_{1-8}-G_{5-16}-F'_{2-8}$

D'-F-G-F', in particular  $D'_{1,y}-F_{1-8}-G_{5-i}-F'_{2,e}$

F-G-F'-D'', in particular  $F_{1-8}-G_{5-i6}-F'_{2-8}-D''_{i,3}$

D'-F-G-F'-D'', in particular  $D'_{1-3}-F_{1-8}-G_{5-i6}-F'_{2-8}-D''_{1-3}$

In some embodiments the internucleoside linkage positioned between region D' and region F is a phosphodiester linkage. In some embodiments the internucleoside linkage positioned between region F' and region D'' is a phosphodiester linkage.

**Conjugate**

The term conjugate as used herein refers to an oligonucleotide which is covalently linked to a non-nucleotide moiety (conjugate moiety or region C or third region).

5 In some embodiments, the non-nucleotide moiety selected from the group consisting of a protein, such as an enzyme, an antibody or an antibody fragment or a peptide; a lipophilic moiety such as a lipid, a phospholipid, a sterol; a polymer, such as polyethyleneglycol or polypropylene glycol; a receptor ligand; a small molecule; a reporter molecule; and a non-nucleosidic carbohydrate.

10 WO 93/07883 and WO2013/033230 provides suitable conjugate moieties, which are hereby incorporated by reference. Further suitable conjugate moieties are those capable of binding to the asialoglycoprotein receptor (ASGPr). In particular tri-valent N-acetylgalactosamine conjugate moieties are suitable for binding to the ASGPr, see for example WO 2014/076196, WO 2014/207232 and WO 2014/179620 (hereby incorporated by reference, in particular, figure 13 of WO2014/076196 or claims 158-164 of WO2014/179620). Such  
15 conjugates serve to enhance uptake of the oligonucleotide to the liver while reducing its presence in the kidney, thereby increasing the liver/kidney ratio of a conjugated oligonucleotide compared to the unconjugated version of the same oligonucleotide.

Oligonucleotide conjugates and their synthesis has also been reported in comprehensive reviews by Manoharan in Antisense Drug Technology, Principles, Strategies, and Applications, S.T. Crooke, ed., Ch. 16, Marcel Dekker, Inc., 2001 and Manoharan, Antisense and Nucleic  
20 Acid Drug Development, 2002, 12, 103, each of which is incorporated herein by reference in its entirety.

**Linkers**

A linkage or linker is a connection between two atoms that links one chemical group or segment  
25 of interest to another chemical group or segment of interest via one or more covalent bonds.

Conjugate moieties can be attached to the oligonucleotide directly or through a linking moiety (e.g. linker or tether). Linkers serve to covalently connect a third region (region C), e.g. a conjugate moiety to an oligonucleotide (region A) (e.g. connecting one of the termini of region A to C).

30 In some embodiments of the invention the conjugate or oligonucleotide conjugate of the invention may optionally, comprise a linker region (second region or region B) which is positioned between the oligonucleotide or the contiguous nucleotide sequence complementary to the target nucleic acid (region A or first region) and the conjugate moiety (region C or third region).

Region B refers to biocleavable linkers comprising or consisting of a physiologically labile bond that is cleavable under conditions normally encountered or analogous to those encountered within a mammalian body. Conditions under which physiologically labile linkers undergo chemical transformation (e.g., cleavage) include chemical conditions such as pH, temperature, oxidative or reductive conditions or agents, and salt concentration found in or analogous to those encountered in mammalian cells. Mammalian intracellular conditions also include the presence of enzymatic activity normally present in a mammalian cell such as from proteolytic enzymes or hydrolytic enzymes or nucleases. In one embodiment the biocleavable linker is susceptible to S1 nuclease cleavage. In a preferred embodiment the nuclease susceptible linker comprises between 1 and 10 nucleosides, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleosides, more preferably between 2 and 6 nucleosides and most preferably between 2 and 4 linked nucleosides comprising at least two consecutive phosphodiester linkages, such as at least 3 or 4 or 5 consecutive phosphodiester linkages. Preferably the nucleosides are DNA or RNA. Phosphodiester containing biocleavable linkers are described in more detail in WO 2014/076195 (hereby incorporated by reference).

Conjugates may also be linked to the oligonucleotide via non-biocleavable linkers, or in some embodiments the conjugate may comprise a non-cleavable linker which is covalently attached to the biocleavable linker (region Y). Linkers that are not necessarily biocleavable but primarily serve to covalently connect a conjugate moiety (region C or third region), to an oligonucleotide (region A or first region), may comprise a chain structure or an oligomer of repeating units such as ethylene glycol, amino acid units or amino alkyl groups. The oligonucleotide conjugates of the present invention can be constructed of the following regional elements A-C, A-B-C, A-B-Y-C, A-Y-B-C or A-Y-C. In some embodiments the non-cleavable linker (region Y) is an amino alkyl, such as a C2 - C36 amino alkyl group, including, for example C6 to C12 amino alkyl groups. In a preferred embodiment the linker (region Y) is a C6 amino alkyl group. Conjugate linker groups may be routinely attached to an oligonucleotide via use of an amino modified oligonucleotide, and an activated ester group on the conjugate group.

### ***Treatment***

The term 'treatment' as used herein refers to both treatment of an existing disease (e.g. a disease or disorder as herein referred to), or prevention of a disease, *i.e.* prophylaxis. It will therefore be recognized that treatment as referred to herein may, in some embodiments, be prophylactic.

## **DETAILED DESCRIPTION OF THE INVENTION**

### ***The Oligonucleotides of the Invention***

The invention relates to oligonucleotides capable of inhibiting expression of RB1. The modulation is achieved by hybridizing an oligonucleotide (antisense) to a target nucleic acid

encoding RB1 . The target nucleic acid may be a mammalian RB1 sequence, such as a sequence selected from the group consisting of SEQ's ID NO: 1, 2, and 3. In some embodiments, the antisense oligonucleotide hybridizes to both human RB1 pre-mRNA SEQ ID No: 1 and SEQ ID NO:3. In some embodiments, the antisense oligonucleotides of the invention hybridizes to human mRNA (SEQ ID NO: 2).

In some embodiments, the antisense oligonucleotide of the invention hybridize to SEQ ID NO: 15 which correspond to the sequence between positions 123361 and 124720 in SEQ ID NO: 1. In some embodiments, the antisense oligonucleotides of the invention hybridize to SEQ ID NO: 4.

In some preferred embodiments of the invention antisense oligonucleotides of the invention hybridize to SEQ ID NO: 13 (5' CTTCTACCAAAC CTCACCTCCAGCATTGGGGA 3') or to SEQ ID NO: 14 (5' CTTCTACCAAACCT CACCTC 3'), which have at least 95% identity to human RB1 pre-mRNA (SEQ ID NO: 1) and cynomolgous monkey RB1 pre-mRNA (SEQ ID NO: 3).

The oligonucleotide of the invention is an antisense oligonucleotide which targets RB1 .

In some embodiments the antisense oligonucleotide of the invention is capable of modulating the expression of the target by inhibiting or reducing target transcript in a cell. Preferably, such modulation produces an inhibition of expression of at least 20% compared to the normal expression level of the target, more preferably at least 30%, at least 40%, at least 50%, at least at least 60%, at least 70%, at least 80%, or at least 90% inhibition compared to the normal expression level of the target. In some embodiments oligonucleotides of the invention may be capable of inhibiting expression levels of RB1 mRNA by at least 60% or at least 70% *in vitro* using Hela cell line cells. In some embodiments compounds of the invention may be capable of inhibiting expression levels of RB1 protein by at least 50% *in vitro* using HeLa cells. Suitably, the examples provide assays which may be used to measure RB1 RNA or protein inhibition (e.g. example 1). The target modulation is triggered by the hybridization between a contiguous nucleotide sequence of the oligonucleotide and the target nucleic acid. In some embodiments the oligonucleotide of the invention comprises mismatches between the oligonucleotide and the target nucleic acid. Despite mismatches hybridization to the target nucleic acid may still be sufficient to show a desired modulation of RB1 expression. Reduced binding affinity resulting from mismatches may advantageously be compensated by increased number of nucleotides in the oligonucleotide and/or an increased number of modified nucleosides capable of increasing the binding affinity to the target, such as 2' sugar modified nucleosides, including LNA, present within the oligonucleotide sequence.

The oligonucleotide of the invention comprises a contiguous nucleotide sequence which is complementary to or hybridizes to the target nucleic acid, such as a target sequence as described herein.

An aspect of the present invention relates to an antisense oligonucleotide of 10 to 50, such as 10 - 30, nucleotides in length, which comprises a contiguous nucleotide sequence of 10 to 30 nucleotides in length with at least 90% complementarity, such as fully complementarity to a mammalian RB1 encoding target nucleic acid. Such antisense oligonucleotide are capable of  
5 reducing the expression of the mammalian RB1 encoding target nucleic acid in a cell.

An aspect of the present invention relates to an antisense oligonucleotide of 10 to 30 nucleotides in length, which comprises a contiguous nucleotide sequence of 12 to 22 nucleotides in length with at least 90% complementarity, such as full complementarity, to a mammalian RB1 encoding target nucleic acid of SEQ ID NO: 1, wherein the antisense  
10 oligonucleotide is capable of reducing the expression of the mammalian RB1 encoding target nucleic acid in a cell.

In some embodiments, the oligonucleotide comprises a contiguous sequence of 10 to 30 nucleotides in length, such as 12-22 nucleotides in length, which is at least 90% complementary, such as at least 91%, such as at least 92%, such as at least 93%, such as at  
15 least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, or 100% complementary with a region of the target nucleic acid or a target sequence.

In a preferred embodiment the oligonucleotide of the invention, or contiguous nucleotide sequence thereof is fully complementary (100% complementary) to a region of the target nucleic acid, or in some embodiments may comprise one or two mismatches between the  
20 oligonucleotide and the target nucleic acid.

In some embodiments the oligonucleotide sequence is at least 90% complementary or at least 95% complementary, such as fully complementary to a corresponding target sequence present in SEQ ID NO: 1 and SEQ ID NO: 3. In some embodiments the contiguous sequence of the antisense oligonucleotide is fully complementary to the mammalian FNDC3B encoding target  
25 nucleic acid.

In a preferred embodiment the oligonucleotide sequence is 95% complementary, such as fully complementary, to a corresponding target nucleic acid region present in SEQ ID NO: 1, 3 and 15.

A further aspect of the present invention relates to the antisense oligonucleotide, wherein the  
30 contiguous nucleotide sequence of 10 to 30 nucleotides in length is at least 90% complementary, such as 95% complementary, such as fully (or 100%) complementary, to an intron region present in the pre-mRNA of mammalian RB1 encoding target nucleic acid (e.g. SEQ ID NO: 1 and/or SEQ ID NO: 3).

It shall be understood that intron positions on SEQ ID NO: 1 may vary depending on different  
35 splicing of FNDC3B pre-mRNA. In the context of the present invention any nucleotide sequence in

the gene sequence or pre-mRNA that is removed from the pre-mRNA by RNA splicing during maturation of the final RNA product (mature mRNA) are introns irrespectively on their position on SEQ ID NO: 1. Table 1 provides the most common intron regions in SEQ ID NO: 1.

5 In one embodiment the present invention relates to the antisense oligonucleotide, wherein the contiguous nucleotide sequence is at least 95% complementary, such as fully complementary, to position 77694 to 149242 of the pre-mRNA of human RB1 (SEQ ID NO: 1).

In a preferred embodiment the present invention relates to the antisense oligonucleotide, wherein the contiguous nucleotide sequence is at least 95% complementary, such as fully complementary, to SEQ ID NO: 15.

10 In another preferred embodiment the present invention relates to the antisense oligonucleotide, wherein the contiguous nucleotide sequence is at least 95% complementary, such as fully complementary, to SEQ ID NO: 4.

In another preferred embodiment the present invention relates to the antisense oligonucleotide, wherein the contiguous nucleotide sequence is at least 95% complementary, such as fully  
15 complementary, to SEQ ID NO: 13.

In another preferred embodiment the present invention relates to the antisense oligonucleotide, wherein the contiguous nucleotide sequence is at least 95% complementary, such as fully complementary, to SEQ ID NO: 14.

In some embodiments, the oligonucleotide or contiguous nucleotide sequence is  
20 complementary to a region of the target nucleic acid, wherein the target nucleic acid region is selected from the group consisting of position 123370-123389, 123473-123492, 123526-123545, 123579-123598, 123632-123651, 123685-123704, 123738-123757, 123791-123810, 123897-123916, 123950-123969, 124003-124022, 124056-124075, 124109-124128, 124162-124181, 124215-124234, 124268-124287, 124321-124340, 124374-124393, 124427-124446,  
25 124480-124499, 124533-124552, 124586-124605, 124639-124658, 124692-12471 1; 123370-123387, 123473-123490, 123526-123543, 123579-123596, 123632-123649, 123685-123702, 123738-123755, 123791-123808, 123897-123914, 123950-123967, 124003-124020, 124056-124073, 124109-124126, 124162-124179, 124215-124232, 124268-124285, 124321-124338, 124374-124391, 124427-124444, 124480-124497, 124533-124550, 124586-124603, 124639-  
30 124656, 124692-124709; 123372-123387, 123422-123437, 123475-123490, 123528-123543, 123581-123596, 123634-123649, 123687-123702, 123740-123755, 123793-123808, 123899-123914, 123952-123967, 124005-124020, 124058-124073, 1241 11-124126, 124164-124179, 124217-124232, 124270-124285, 124323-124338, 124376-124391, 124429-124444, 124482-124497, 124535-124550, 124588-124603, 124641-124656, 124694-124709; 123375-123388,  
35 123425-123438, 123478-123491, 123531-123544, 123584-123597, 123637-123650, 123690-123703, 123743-123756, 123796-123809, 123902-123915, 123955-123968, 124008-124021,

124061-124074, 124114-124127, 124167-124180, 124220-124233, 124273-124286, 124326-124339, 124379-124392, 124432-124445, 124485-124498, 124538-124551, 124591-124604, 124644-124657, 124697-124710 of SEQ ID NO:1.

5 According to one aspect of the invention, the target sequence is repeated within the target nucleic acid, i.e. at least two identical target nucleotide sequences (target regions) of at least 10 nucleotides in length occur in the target nucleic acid at different positions. A repeated target region is generally between 10 and 50 nucleotides, such as between 11 and 30 nucleotides, such as between 12 and 25 nucleotides, such as between 13 and 22 nucleotides, such as between 14 and 20 nucleotides, such as between 15 and 19 nucleotides, such as between 16  
10 and 18 nucleotides. In a preferred embodiment the repeated target region is between 14 and 20 nucleotides.

In one aspect the invention provides antisense oligonucleotides wherein the contiguous nucleotide sequence is at least 90% complementary, such as fully complementary, to a target region that is repeated at least 2 times across the target nucleic acid of SEQ ID NO: 1. The  
15 effect of this is that several oligonucleotide compounds (with the same sequence) can hybridize to one or more target regions on the same target nucleic acid (at the same time), which may result in multiple cleavage events of the target nucleic acid when the oligonucleotide is administered to a cell or an animal or a human.

In some embodiments the oligonucleotide or the contiguous nucleotide sequence is at least  
20 90% complementary, such as fully complementary to a target region that is repeated at least at least 3 repeated target regions, such as at least 4, 5, 6, 7, 8, 9 or 10 repeated target regions, or more than 10 repeated target regions.

In a further embodiment the antisense oligonucleotide comprises a contiguous nucleotide sequence that is at least 95% complementary, such as fully complementary, to a target region  
25 of 10-22, such as 14-20, nucleotides in length of the target nucleic acid of SEQ ID NO: 1, wherein the target region is repeated at least 6 or more times across the intons of the target nucleic acid.

In some embodiments, the antisense oligonucleotide of the invention or the contiguous nucleotide sequence thereof is complementary to at least 24 repeated target regions in SEQ ID  
30 NO: 15.

In some embodiments, the oligonucleotide of the invention comprises or consists of 10 to 35 nucleotides in length, such as from 10 to 30, such as 11 to 22, such as from 12 to 20, such as from such as from 14 to 20, such as from 14 to 18 such as from 14 to 16. Advantageously, the oligonucleotide comprises or consists of 14 to 20 contiguous nucleotides in length.

35 It is to be understood that any range given herein includes the range endpoints.

In some embodiments, the oligonucleotide or contiguous nucleotide sequence thereof comprises or consists of 22 or less nucleotides, such as 20 or less nucleotides, such as 18 or less nucleotides, such as less than 19, such as 14, 15, 16, 17 or 18 nucleotides.

5 In some embodiments, the oligonucleotide or contiguous nucleotide sequence comprises or consists of a sequence selected from the group consisting of sequences listed in Table 4.

In some embodiments, the antisense oligonucleotide or contiguous nucleotide sequence thereof, comprises or consists of 10 to 30 nucleotides in length with at least 90% identity, preferably 100% identity, to a sequence selected from the group consisting of SEQ ID NO: 5 to 12 .

10 In some embodiments, the antisense oligonucleotide or contiguous nucleotide sequence comprises or consists of 10 to 30 nucleotides in length with at least 90% identity, preferably 100% identity, to a sequence selected from the group consisting of SEQ ID NO: 9, 10, 11 and 12 (see motif sequences listed in Table 4).

15 In some embodiments, the antisense oligonucleotide or contiguous nucleotide sequence thereof comprises or consists of 14 to 20 contiguous nucleotides in length with at least 90% identity, preferably 100% identity, to a sequence selected from SEQ ID NO: 9, 10, 11, 12 and 18 .

In some embodiments the contiguous nucleotide sequence consists of a sequence (motif sequence) selected from the group consisting of SEQ ID NO: 9, 10, 11 and 12.

20 In some embodiments, the antisense oligonucleotide or contiguous nucleotide sequence comprises or consists of 10 to 30 nucleotides in length with at least 90% identity, preferably 100% identity to a sequence selected from the group consisting of SEQ ID NO: 5, 6, 7 and 8.

In some embodiments, the antisense oligonucleotide or contiguous nucleotide sequence thereof comprises or consists of 14 to 20 contiguous nucleotides in length with at least 90% identity, preferably 100% identity, to a sequence selected from SEQ ID NO: 5, 6, 7 and 8.

25 In some embodiments the contiguous nucleotide sequence (motif sequence) consists of a sequence selected from the group consisting of SEQ ID NO: 5, 6, 7 and 8.

It is understood that the contiguous nucleobase sequences (motif sequence) can be modified to for example increase nuclease resistance and/or binding affinity to the target nucleic acid.

30 Modifications are described in the definitions and in the following paragraphs. Table 4 lists preferred designs of each motif sequence.

The pattern in which the modified nucleosides (such as high affinity modified nucleosides) are incorporated into the oligonucleotide sequence is generally termed oligonucleotide design.

The oligonucleotides of the invention are designed with modified nucleosides and DNA nucleosides. Advantageously, high affinity modified nucleosides are used.

In an embodiment, the oligonucleotide comprises at least 1 modified nucleoside, such as at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15 or at least 16 modified nucleosides. In an embodiment the oligonucleotide comprises from 1 to 10 modified nucleosides, such as from 2 to 9 modified nucleosides, such as from 3 to 8 modified nucleosides, such as from 4 to 7 modified nucleosides, such as 6 or 7 modified nucleosides. Suitable modifications are described in the "Definitions" section under "modified nucleoside", "high affinity modified nucleosides", "sugar modifications", "2' sugar modifications" and Locked nucleic acids (LNA)".

In an embodiment, the oligonucleotide comprises one or more sugar modified nucleosides, such as 2' sugar modified nucleosides. Preferably the oligonucleotide of the invention comprise one or more 2' sugar modified nucleoside independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA, 2'-fluoro-DNA, arabino nucleic acid (ANA), 2'-fluoro-ANA and LNA nucleosides. It is advantageous if one or more of the modified nucleoside(s) is a locked nucleic acid (LNA).

In a further embodiment the oligonucleotide comprises at least one modified internucleoside linkage. Suitable internucleoside modifications are described in the "Definitions" section under "Modified internucleoside linkage". It is advantageous if at least 75%, such as all, the internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate or boranophosphate internucleoside linkages. In some embodiments all the internucleotide linkages in the contiguous sequence of the oligonucleotide are phosphorothioate linkages.

In some embodiments, the oligonucleotide of the invention comprises at least one LNA nucleoside, such as 1, 2, 3, 4, 5, 6, 7, or 8 LNA nucleosides, such as from 2 to 6 LNA nucleosides, such as from 3 to 7 LNA nucleosides, 4 to 8 LNA nucleosides or 3, 4, 5, 6, 7 or 8 LNA nucleosides. In some embodiments, at least 75% of the modified nucleosides in the oligonucleotide are LNA nucleosides, such as 80%, such as 85%, such as 90% of the modified nucleosides are LNA nucleosides. In a still further embodiment all the modified nucleosides in the oligonucleotide are LNA nucleosides. In a further embodiment, the oligonucleotide may comprise both beta-D-oxy-LNA, and one or more of the following LNA nucleosides: thio-LNA, amino-LNA, oxy-LNA, ScET and/or ENA in either the beta-D or alpha-L configurations or combinations thereof. In a further embodiment, all LNA cytosine units are 5-methyl-cytosine. It is advantageous for the nuclease stability of the oligonucleotide or contiguous nucleotide sequence to have at least 1 LNA nucleoside at the 5' end and at least 2 LNA nucleosides at the 3' end of the nucleotide sequence.

In an embodiment of the invention the oligonucleotide of the invention is capable of recruiting RNase H.

In the current invention an advantageous structural design is a gapmer design as described in the "Definitions" section under for example "Gapmer", "LNA Gapmer", "MOE gapmer" and "Mixed Wing Gapmer" "Alternating Flank Gapmer". The gapmer design includes gapmers with uniform flanks, mixed wing flanks, alternating flanks, and gapbreaker designs. In the present invention it is advantageous if the oligonucleotide of the invention is a gapmer with an F-G-F' design. In some embodiments the gapmer is an LNA gapmer with uniform flanks.

In some embodiments of the invention the LNA gapmer is selected from the following uniform flank designs 2-16-2, 3-13-2, 3-12-3, , 3-1 1-2 or 4-8-2.

### Exemplary Compounds of the Invention

In the exemplified oligonucleotide compounds, capital letters represent beta-D-oxy LNA nucleosides, lowercase letters represent DNA nucleosides, all LNA C are 5-methyl cytosine, and 5-methyl DNA cytosines may be present in the gap region and would be presented by "e" or <sup>m</sup>c, all internucleoside linkages are phosphorothioate internucleoside linkages. For some embodiments of the invention, the oligonucleotide is selected from the group of oligonucleotide compounds with CMP-ID-NO: 5\_1 , 6\_1 , 7\_1 , 8\_1 , 9\_1 , 10\_1 , 11\_1 , 12\_1 .

For some embodiments of the invention, the oligonucleotide is selected from the group of oligonucleotide compounds with CMP-ID-NO: 9\_1 , 10\_1 , 11\_1 and 12\_1 .

For some embodiments of the invention, the oligonucleotide is CMP-ID-NO: 5\_1 or 6\_1 .

### Method of manufacture

In a further aspect, the invention provides methods for manufacturing the oligonucleotides of the invention comprising reacting nucleotide units and thereby forming covalently linked contiguous nucleotide units comprised in the oligonucleotide. Preferably, the method uses phosphoramidite chemistry (see for example Caruthers et al, 1987, Methods in Enzymology vol. 154, pages 287-313). In a further embodiment the method further comprises reacting the contiguous nucleotide sequence with a conjugating moiety (ligand). In a further aspect a method is provided for manufacturing the composition of the invention, comprising mixing the oligonucleotide or conjugated oligonucleotide of the invention with a pharmaceutically acceptable diluent, solvent, carrier, salt and/or adjuvant.

### Pharmaceutical salt

In a further aspect the invention provides a pharmaceutically acceptable salt of the antisense oligonucleotide or a conjugate thereof. In a preferred embodiment, the pharmaceutically acceptable salt is a sodium or a potassium salt.

### Pharmaceutical Composition

In a further aspect, the invention provides pharmaceutical compositions comprising any of the aforementioned oligonucleotides and/or oligonucleotide conjugates or salts thereof and a pharmaceutically acceptable diluent, carrier, salt and/or adjuvant. A pharmaceutically

5 acceptable diluent includes phosphate-buffered saline (PBS) and pharmaceutically acceptable salts include, but are not limited to, sodium and potassium salts. In some embodiments the pharmaceutically acceptable diluent is sterile phosphate buffered saline. In some embodiments the oligonucleotide is used in the pharmaceutically acceptable diluent at a concentration of 50 - 300  $\mu$ M solution.

10 Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed., 1985. For a brief review of methods for drug delivery, see, e.g., Langer (Science 249:1527-1533, 1990). WO 2007/031091 provides further suitable and preferred examples of pharmaceutically acceptable diluents, carriers and adjuvants (hereby incorporated by reference). Suitable dosages, formulations, 15 administration routes, compositions, dosage forms, combinations with other therapeutic agents, pro-drug formulations are also provided in W02007/031091 .

Oligonucleotides or oligonucleotide conjugates of the invention may be mixed with pharmaceutically acceptable active or inert substances for the preparation of pharmaceutical compositions or formulations. Compositions and methods for the formulation of pharmaceutical 20 compositions are dependent upon a number of criteria, including, but not limited to, route of administration, extent of disease, or dose to be administered.

These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration.

25 The pH of the preparations typically will be between 3 and 11, more preferably between 5 and 9 or between 6 and 8, and most preferably between 7 and 8, such as 7 to 7.5. The resulting compositions in solid form may be packaged in multiple single dose units, each containing a fixed amount of the above-mentioned agent or agents, such as in a sealed package of tablets or capsules. The composition in solid form can also be packaged in a container for a flexible 30 quantity, such as in a squeezable tube designed for a topically applicable cream or ointment.

In some embodiments, the oligonucleotide or oligonucleotide conjugate of the invention is a prodrug. In particular with respect to oligonucleotide conjugates the conjugate moiety is cleaved of the oligonucleotide once the prodrug is delivered to the site of action, e.g. the target cell.

### Applications

35 The oligonucleotides of the invention may be utilized as research reagents for, for example, diagnostics, therapeutics and prophylaxis.

In research, such oligonucleotides may be used to specifically modulate the synthesis of RB1 protein in cells (e.g. *in vitro* cell cultures) and experimental animals thereby facilitating functional analysis of the target or an appraisal of its usefulness as a target for therapeutic intervention. Typically the target modulation is achieved by degrading or inhibiting the pre-mRNA or mRNA producing the protein, thereby prevent protein formation or by degrading or inhibiting a modulator of the gene or mRNA producing the protein.

If employing the oligonucleotide of the invention in research or diagnostics the target nucleic acid may be a cDNA or a synthetic nucleic acid derived from DNA or RNA.

The present invention provides an *in vivo* or *in vitro* method for modulating RB1 expression in a target cell which is expressing RB1, said method comprising administering an oligonucleotide of the invention in an effective amount to said cell.

In some embodiments, the target cell, is a mammalian cell in particular a human cell. The target cell may be an *in vitro* cell culture or an *in vivo* cell forming part of a tissue in a mammal. In preferred embodiments the target cell is present in neurons, such as peripheral neurons, or ganglion, such as dorsal root ganglia or basal ganglia or nerve fibers.

In diagnostics the oligonucleotides may be used to detect and quantitate RB1 expression in cell and tissues by northern blotting, *in-situ* hybridisation or similar techniques.

For therapeutics, an animal or a human, suspected of having a disease or disorder, which can be treated by modulating the expression of RB1.

The invention provides methods for treating or preventing a disease, comprising administering a therapeutically or prophylactically effective amount of an oligonucleotide, an oligonucleotide conjugate or a pharmaceutical composition of the invention to a subject suffering from or susceptible to the disease.

The invention also relates to an oligonucleotide, a composition or a conjugate as defined herein for use as a medicament.

The oligonucleotide, oligonucleotide conjugate or a pharmaceutical composition according to the invention is typically administered in an effective amount.

The invention also provides for the use of the oligonucleotide or oligonucleotide conjugate of the invention as described for the manufacture of a medicament for the treatment of a disorder as referred to herein, or for a method of the treatment of as a disorder as referred to herein.

The disease or disorder, as referred to herein, can be improved by decreasing the expression of RB1.

The invention further relates to use of an oligonucleotide, oligonucleotide conjugate or a pharmaceutical composition as defined herein for the manufacture of a medicament for the reducing levels and/or activity of RB1 .

5 In one embodiment, the invention relates to oligonucleotides, oligonucleotide conjugates or pharmaceutical compositions for use in the treatment of diseases or disorders selected from neurological injury or neuronal degeneration.

Neurological injury can either be in the brain or in the peripheral nervous system. Peripheral nerve injuries (i.e. peripheral neuropathy) may result in loss of motor function, sensory function, or both and can occur as a result of trauma (blunt or penetrating) or acute compression, other  
10 causes maybe diabetes, vitamin deficiency, medication like chemotherapy and antibiotics, immune system diseases or viral infections. In one embodiment of the present invention the antisense oligonucleotide of the present invention is used to treat injuries to the peripheral nervous system. In particular the treatment of traumatic injuries to the peripheral nervous system is an object of the invention. The antisense oligonucleotides of the invention may  
15 stimulate the proliferation of sensory neurons, axons or dorsal root ganglion and/or Schwann cells to induce nerve regeneration.

One embodiment of the invention is the use of an antisense oligonucleotide which comprises a contiguous nucleotide sequence of 10 to 30 nucleotides in length with at least 90% complementarity to a target nucleic acid of SEQ ID NO: 1, or natural occurring variants thereof  
20 in promoting adult neuron regeneration. In particular following acute injury to the peripheral nervous system.

Diseases that involve neuronal degeneration are for example Alzheimer's, diabetes, Huntington's, motor neuron disease, Spinocerebellar ataxia, olivopontocerebellar atrophy, sclerosis, and Parkinson's.

## 25 **Administration**

The oligonucleotides or pharmaceutical compositions of the present invention may be administered topically (such as, to the skin, by inhalation, ophthalmic or otic) or enteral (such as, orally or through the gastrointestinal tract) or parenteral (such as, intravenous, subcutaneous, intra-muscular, intracerebral, intracerebroventricular or intrathecal).

30 It is advantageous to administer the oligonucleotides of the invention such that RB1 is not reduced systemically. This can either be achieved by targeted delivery of the oligonucleotides using for example conjugates or formulation technology. Alternatively, the oligonucleotides can be administered locally, e.g. via injection into the affected neurons such as peripheral neurons or injection into ganglion, such as dorsal root ganglia or basal ganglia or injection in the area  
35 surrounding the affected neurons or nerve fibers or injection into a joint with neuronal damage.

The oligonucleotides can also be administered locally into the central nervous system (CNS) for example via intracranial, e.g. intracerebral or intraventricular, intravitreal administration or via the cerebrospinal fluid (CSF) using intrathecal administration or lumbar puncture. In one embodiment the active oligonucleotide or oligonucleotide conjugate is administered locally. In another embodiment the active oligonucleotide or oligonucleotide conjugate is administered to the CNS.

In some embodiments, the oligonucleotide, oligonucleotide conjugate or pharmaceutical composition of the invention is administered at a dose of 0.1 - 15 mg/kg, such as from 0.2 - 10 mg/kg, such as from 0.25 - 5 mg/kg. The administration can be every 2<sup>nd</sup> week, every third week, once a month or even every 3<sup>rd</sup> month.

The invention also provides for the use of the oligonucleotide or oligonucleotide conjugate of the invention as described for the manufacture of a medicament wherein the medicament is in a dosage form for local administration. The invention also provides for the use of the oligonucleotide or oligonucleotide conjugate of the invention as described for the manufacture of a medicament wherein the medicament is in a dosage form for CNS or CSF administration.

The invention also provides for the use of the oligonucleotide or oligonucleotide conjugate of the invention as described for the manufacture of a medicament wherein the medicament is in a dosage form for intrathecal administration.

## EMBODIMENTS

The following embodiments of the present invention may be used in combination with any other embodiments described herein.

1. An antisense oligonucleotide which comprises a contiguous nucleotide sequence of 10 to 30 nucleotides in length, wherein the contiguous nucleotide sequence is complementary to an intron of the target nucleic acid of SEQ ID NO: 1.

2. The antisense oligonucleotide according to embodiment 1, wherein the intron is intron 17 within SEQ ID NO: 1.

3. The antisense oligonucleotide of embodiment 1 or 2, wherein the contiguous nucleotide sequence is at least 80%, or at least 85%, or at least 90%, or at least 95% or fully complementary to the target nucleic acid.

4. The antisense oligonucleotide according to any one of embodiments 1 to 3, wherein the contiguous nucleotide sequence is at least 100% complementary to the target nucleic acid of SEQ ID NO:1 .

5. The antisense oligonucleotide of embodiment 1 to 3, wherein the contiguous nucleotide sequence is at least 95% complementary to both SEQ ID NO: 1 and SEQ ID NO: 3.

6. The antisense oligonucleotide according to any one of embodiments 1 to 5, wherein the contiguous nucleotide sequence is complementary to a target sequence of 10-22 such as 14-20 nucleotides in length of the target nucleic acid of SEQ ID NO: 1, wherein the target sequence is repeated at least 6 or more times across the target nucleic acid.
- 5 7. The antisense oligonucleotide according to embodiment 6, wherein the target sequence is repeated 24 to 25 times.
8. The antisense oligonucleotide according to any one of embodiments 1 to 7, wherein the target sequence is within SEQ ID NO: 15.
9. The antisense oligonucleotide according to any one of embodiments 1 to 7, wherein the  
10 target sequence is within SEQ ID NO: 4.
10. The antisense oligonucleotide according to any one of embodiments 1 to 7, wherein the target sequence is within SEQ ID NO: 13.
11. The antisense oligonucleotide according to any one of embodiments 1 to 7, wherein the target sequence is within SEQ ID NO: 14.
- 15 12. The antisense oligonucleotide according to any one of embodiments 1 to 11, which is capable of reducing the expression of RB1 in a cell.
13. The antisense oligonucleotide of anyone of embodiements 1 to 12, wherein said repeated target sequences have at least at least 90%, or at least 95% or at least 100% identity with each other.
- 20 14. The antisense oligonucleotide of any one of embodiments 1 to 13, wherein the contiguous nucleotide sequence is 100% complementary to at least 14 nucleosides within SEQ ID NO: 13 or SEQ ID NO:14.
15. The antisense oligonucleotide of any one of embodiments 1 to 14, wherein the contiguous nucleotide sequence is complementary to a target region of the target nucleic acid, selected  
25 from the group consisting of position 123370-123389, 123473-123492, 123526-123545, 123579-123598, 123632-123651, 123685-123704, 123738-123757, 123791-123810, 123897-123916, 123950-123969, 124003-124022, 124056-124075, 124109-124128, 124162-124181, 124215-124234, 124268-124287, 124321-124340, 124374-124393, 124427-124446, 124480-124499, 124533-124552, 124586-124605, 124639-124658, 124692-12471 1; 123370-123387,  
30 123473-123490, 123526-123543, 123579-123596, 123632-123649, 123685-123702, 123738-123755, 123791-123808, 123897-123914, 123950-123967, 124003-124020, 124056-124073, 124109-124126, 124162-124179, 124215-124232, 124268-124285, 124321-124338, 124374-124391, 124427-124444, 124480-124497, 124533-124550, 124586-124603, 124639-124656, 124692-124709; 123372-123387, 123422-123437, 123475-123490, 123528-123543, 123581-  
35 123596, 123634-123649, 123687-123702, 123740-123755, 123793-123808, 123899-123914,

- 123952-123967, 124005-124020, 124058-124073, 1241 11-124126, 124164-124179, 124217-124232, 124270-124285, 124323-124338, 124376-124391 , 124429-124444, 124482-124497, 124535-124550, 124588-124603, 124641-124656, 124694-124709; 123375-123388, 123425-123438, 123478-123491 , 123531-123544, 123584-123597, 123637-123650, 123690-123703, 5 123743-123756, 123796-123809, 123902-123915, 123955-123968, 124008-124021 , 124061-124074, 1241 14-124127, 124167-124180, 124220-124233, 124273-124286, 124326-124339, 124379-124392, 124432-124445, 124485-124498, 124538-124551 , 124591-124604, 124644-124657, and 124697-124710 of SEQ ID NO:1 .
16. The antisense oligonucleotide of embodiment 1 to 15, wherein the oligonucleotide is 10 capable of hybridizing to a target nucleic acid of selected from the group consisting of SEQ ID NO:1 , 3, 4, or 15 with a  $\Delta G^\circ$  below -10 kcal.
17. The antisense oligonucleotide of embodiment 1 to 16, wherein the target nucleic acid is RNA.
18. The antisense oligonucleotide of embodiment 17, wherein the mRNA is pre-mRNA.
- 15 19. The antisense oligonucleotide of any one of embodiments 1-18, wherein the contiguous nucleotide sequence comprises or consists of from 12 to 22 nucleotides.
20. The antisense oligonucleotide of embodiment 19, wherein the contiguous nucleotide sequence comprises or consists of from 14-20 nucleotides.
- 20 21. The antisense oligonucleotide of any one of embodiments 1-20, wherein the oligonucleotide comprises or consists of 12 to 25 nucleotides in length.
22. The antisense oligonucleotide of any one of embodiments 1-21 , wherein the oligonucleotide or contiguous nucleotide sequence is single stranded.
23. The antisense oligonucleotide of any one of embodiments 1-22, wherein the contiguous nucleotide sequence comprises or consists of a sequence selected from SEQ ID NO: 9, SEQ ID 25 NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.
24. The antisense oligonucleotide of any one of embodiments 1-5 or 16 to 22 when dependent on embodiment 1-5, wherein the contiguous nucleotide sequence comprises or consists of a sequence selected from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.
25. The antisense oligonucleotide of any one of embodiments 1-24, wherein the contiguous 30 nucleotide sequence has zero to three mismatches compared to the target nucleic acid it is complementary to.
26. The antisense oligonucleotide of embodiment 25, wherein the contiguous nucleotide sequence has one mismatch compared to the target nucleic acid.

27. The antisense oligonucleotide of embodiment 25, wherein the contiguous nucleotide sequence is fully complementary to the target nucleic acid sequence.
28. The antisense oligonucleotide of any one of embodiments 1-27, wherein the contiguous nucleotide sequence comprises one or more modified nucleoside.
- 5 29. The antisense oligonucleotide of embodiment 28, wherein the one or more modified nucleoside is a high-affinity modified nucleoside.
30. The antisense oligonucleotide of embodiments 28 or 29, wherein the modified nucleoside is a 2' sugar modified nucleoside.
31. The antisense oligonucleotide of embodiment 30, wherein the 2' sugar modified nucleoside  
10 is independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA, 2'-fluoro-DNA, arabino nucleic acid (ANA), 2'-fluoro-ANA and LNA nucleosides.
32. The antisense oligonucleotide of any one of embodiments 28-31, wherein the antisense oligonucleotide comprise 3 to 6 2' sugar modified nucleosides.
- 15 33. The antisense oligonucleotide of any one of embodiments 1-32, wherein the oligonucleotide comprises at least one modified internucleoside linkage.
34. The antisense oligonucleotide of embodiment 33, wherein the modified internucleoside linkage is nuclease resistant.
35. The antisense oligonucleotide of embodiment 33 or 34, wherein at least 50% of the  
20 internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate internucleoside linkages or boranophosphate internucleoside linkages.
36. The antisense oligonucleotide of embodiment 33 or 34, wherein all the internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate internucleoside linkages.
- 25 37. The antisense oligonucleotide of any one of embodiments 1 to 36, wherein the oligonucleotide is capable of recruiting RNase H.
38. The antisense oligonucleotide of embodiment 37, wherein the oligonucleotide is a gapmer.
39. The antisense oligonucleotide of embodiment 37 or 38, wherein the oligonucleotide is a gapmer of formula 5'-F-G-F'-3', where region F and F' independently comprise or consist of 1 -  
30 4 modified nucleosides and G is a region between 6 and 17 nucleosides which are capable of recruiting RNaseH.
40. The antisense oligonucleotide of embodiment 39, wherein one or more of the modified nucleosides in region F and F' is a LNA nucleoside.

41. The antisense oligonucleotide of embodiment 40, wherein all the modified nucleosides in region F and F' are LNA nucleosides.
42. The antisense oligonucleotide of embodiment 41, wherein region F and F' consist of LNA nucleosides.
- 5 43. The antisense oligonucleotide of any one of embodiments 40-42, wherein the LNA nucleoside is selected from beta-D-oxy-LNA, alpha-L-oxy-LNA, beta-D-amino-LNA, alpha-L-amino-LNA, beta-D-thio-LNA, alpha-L-thio-LNA, (S)cET, (R)cET beta-D-ENA and alpha-L-ENA.
44. The antisense oligonucleotide of any one of embodiments 40-42, wherein the LNA nucleoside is oxy-LNA.
- 10 45. The antisense oligonucleotide of any one of embodiments 40-44, wherein the LNA nucleoside is beta-D-oxy-LNA.
46. The antisense oligonucleotide of any one of embodiments 40-42, wherein the LNA nucleoside is thio-LNA.
47. The antisense oligonucleotide of any one of embodiments 40-42, wherein the LNA  
15 nucleoside is amino-LNA.
48. The antisense oligonucleotide of any one of embodiment 40-42, wherein the LNA nucleoside is cET.
49. The antisense oligonucleotide of any one of embodiment 40-42, wherein the LNA nucleoside is ENA.
- 20 50. The antisense oligonucleotide of embodiment 40, wherein at least one of region F or F' further comprises at least one 2' sugar substituted nucleoside independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA and 2'-fluoro-DNA.
51. The antisense oligonucleotide of any one of embodiment 40-50, wherein the RNaseH  
25 recruiting nucleosides in region G are independently selected from DNA, alpha-L-LNA, C4' alkylated DNA, ANA and 2'-F-ANA and UNA.
52. The antisense oligonucleotide of embodiment 51, wherein all the nucleosides in region G are DNA nucleosides.
53. The antisense oligonucleotide of any one of embodiments 1-52, wherein the oligonucleotide  
30 is selected from CMP ID's NO: 9\_1, 10\_1, 11\_1, or 12\_1.
54. The antisense oligonucleotide of any one of embodiments 24 or 25 to 52 in so far they are dependent on embodiment 24, wherein the oligonucleotide is selected from CMP ID's NO: 5\_1, 6\_1, 7\_1, or 8\_1.

55. A conjugate comprising the antisense oligonucleotide according to any one of embodiments 1-54, and at least one conjugate moiety covalently attached to said oligonucleotide.
56. The oligonucleotide conjugate of embodiment 55, wherein the conjugate moiety is selected from carbohydrates, cell surface receptor ligands, drug substances, hormones, lipophilic substances, polymers, proteins, peptides, toxins, vitamins, viral proteins or combinations thereof.
57. The oligonucleotide conjugate of embodiments 55 or 56, comprising a linker which is positioned between the oligonucleotide and the conjugate moiety.
58. The oligonucleotide conjugate of embodiment 57, wherein the linker is a physiologically labile linker.
59. The oligonucleotide conjugate of embodiment 58, wherein the physiologically labile linker is nuclease susceptible linker.
60. The oligonucleotide conjugate of embodiment 58 or 59, wherein the oligonucleotide has the formula D'-F-G-F' or F-G-F'-D'', wherein F, F' and G are as defined in embodiments 40-53 and D' or D'' comprises 1, 2 or 3 DNA nucleosides with phosphorothioate internucleoside linkages.
61. A pharmaceutical composition comprising the antisense oligonucleotide of any one of embodiments 1-54 or a conjugate of any one of embodiments 55-60 and a pharmaceutically acceptable diluent, carrier, salt and/or adjuvant.
62. An *in vivo* or *in vitro* method for modulating RB1 expression in a target cell which is expressing RB1, said method comprising administering an oligonucleotide of embodiment 1-54 or a conjugate of embodiment 55-60 or the pharmaceutical composition of embodiment 61 in an effective amount to said cell.
63. A method for alleviating or preventing a disease comprising administering a therapeutically or prophylactically effective amount of an oligonucleotide of embodiment 1-54 or a conjugate of embodiment 55-60 or the pharmaceutical composition of embodiment 61 to a subject suffering from or susceptible to the disease.
64. The antisense oligonucleotide of embodiment 1-54 or a conjugate of embodiment 55-60 or the pharmaceutical composition of embodiment 61, for use as a medicament for treatment or prevention of a disease in a subject.
65. Use of the antisense oligonucleotide of oligonucleotide of embodiment 1-54 or a conjugate of embodiment 55-60 for the preparation of a medicament for treatment or prevention of a disease in a subject.
66. The method, the antisense oligonucleotide or the use of embodiments 63 - 65, wherein the treatment is associated with reduction of *in vivo* activity of RB1.

67. The method, the oligonucleotide or the use of embodiments 66, wherein the RB1 is reduced by at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% compared to the expression without the oligonucleotide of embodiment 1-54 or a conjugate of embodiment 55-60 or the pharmaceutical composition of embodiment 61.
68. The method, the oligonucleotide or the use of embodiments 63 - 67, wherein the disease is selected from neurological injury or neuronal degeneration.
69. The method, the oligonucleotide or the use of embodiment 68, wherein the neurological injury is a traumatic injury to the peripheral nervous system.
70. The method, the oligonucleotide or the use of embodiment 68 or 69, wherein the treatment stimulate the proliferation of sensory neurons, axons or dorsal root ganglion and/or Schwann cells.
71. The method, the oligonucleotide or the use of embodiment 68 or 69, wherein the treatment induce nerve regeneration.
72. The method, the antisense oligonucleotide or the use of any one of embodiments 63 - 71, wherein the subject is a mammal.
73. The method, the antisense oligonucleotide or the use of embodiment 72, wherein the mammal is human.

## EXAMPLES

### Materials and methods

Table 4: list of oligonucleotide motif sequences (indicated by SEQ ID NO), designs of these, as well as specific oligonucleotide compounds (indicated by CMP ID NO) designed based on the motif sequence.

SEQ ID NO	Motif sequence	Design	Oligonucleotide Compound	CMP ID NO	Start position on SEQ ID NO: 1*
5	aggaggtagaagtgtgtgtt	2-16-2	AGgaggtagaagtgtgtgTT	5_1	99543
6	gaggtagaagtgtgtgtt	3-12-3	GAGgtagaagtgtgtGTT	6_1	99543
7	ggaggtagaagtgtgt	4-10-2	GGAGgtagaagtgtGT	7_1	99546
8	ggaggtagaagtgt	4-6-4	GGAGgtagaaGTGT	8_1	99548
9	gaggtgaggtttggtagaag	2-16-2	GAggtgaggtttggtagaAG	9_1	123370, 123473, 123526, 123579, 123632, 123685, 123738, 123791, 123897, 123950, 124003, 124056, 124109, 124162,

SEQ ID NO	Motif sequence	Design	Oligonucleotide Compound	CMP ID NO	Start position on SEQ ID NO: 1*
					124215, 124268, 124321, 124374, 124427, 124480, 124533, 124586, 124639, 124692
10	ggtgaggtttgtagaag	3-13-2	GGTgaggtttgtagaAG	10_1	123370, 123473, 123526, 123579, 123632, 123685, 123738, 123791, 123897, 123950, 124003, 124056, 124109, 124162, 124215, 124268, 124321, 124374, 124427, 124480, 124533, 124586, 124639, 124692
11	ggtgaggtttgtaga	3-11-2	GGTgaggtttgtagaGA	11_1	123372, 123422, 123475, 123528, 123581, 123634, 123687, 123740, 123793, 123899, 123952, 124005, 124058, 124111, 124164, 124217, 124270, 124323, 124376, 124429, 124482, 124535, 124588, 124641, 124694
12	aggtgaggtttggt	4-8-2	AGGTgaggtttgGT	12_1	123375, 123425, 123478, 123531, 123584, 123637, 123690, 123743, 123796, 123902, 123955, 124008, 124061, 124114, 124167, 124220, 124273, 124326, 124379, 124432, 124485, 124538, 124591, 124644, 124697

\*multiple numbers refers to repeat targeting compounds

Motif sequences represent the contiguous sequence of nucleobases present in the oligonucleotide.

Designs refer to the gapmer design, F-G-F', where each number represents the number of consecutive modified nucleosides, e.g 2' modified nucleosides (first number=5' flank), followed by the number of DNA nucleosides (second number= gap region), followed by the number of modified nucleosides, e.g 2'

5 modified nucleosides (third number=3' flank), optionally preceded by or followed by further repeated regions of DNA and LNA, which are not necessarily part of the contiguous sequence that is complementary to the target nucleic acid.

Oligonucleotide compounds represent specific designs of a motif sequence. Capital letters represent beta-D-oxy LNA nucleosides, lowercase letters represent DNA nucleosides, all LNA C are 5-methyl cytosine, and 5-methyl DNA cytosines are presented by "e", all internucleoside linkages are phosphorothioate internucleoside linkages.

### ***Oligonucleotide synthesis***

Oligonucleotide synthesis is generally known in the art. Below is a protocol which may be applied. The oligonucleotides of the present invention may have been produced by slightly  
15 varying methods in terms of apparatus, support and concentrations used.

Oligonucleotides are synthesized on uridine universal supports using the phosphoramidite approach on an Oligomaker 48 at 1  $\mu$ mol scale. At the end of the synthesis, the oligonucleotides are cleaved from the solid support using aqueous ammonia for 5-16hours at 60°C. The oligonucleotides are purified by reverse phase HPLC (RP-HPLC) or by solid phase extractions  
20 and characterized by UPLC, and the molecular mass is further confirmed by ESI-MS.

#### *Elongation of the oligonucleotide:*

The coupling of  $\beta$ -cyanoethyl- phosphoramidites (DNA-A(Bz), DNA- G(ibu), DNA- C(Bz), DNA- T, LNA-5-methyl-C(Bz), LNA-A(Bz), LNA- G(dmf), or LNA-T) is performed by using a solution of 0.1 M of the 5'-O-DMT -protected amidite in acetonitrile and DCI (4,5-dicyanoimidazole) in  
25 acetonitrile (0.25 M) as activator. For the final cycle a phosphoramidite with desired modifications can be used, e.g. a C6 linker for attaching a conjugate group or a conjugate group as such. Thiolation for introduction of phosphorothioate linkages is carried out by using xanthane hydride (0.01 M in acetonitrile/pyridine 9:1 ). Phosphordiester linkages can be introduced using 0.02 M iodine in THF/Pyridine/water 7:2:1 . The rest of the reagents are the ones typically used  
30 for oligonucleotide synthesis.

For post solid phase synthesis conjugation a commercially available C6 aminolinker phosphoramidite can be used in the last cycle of the solid phase synthesis and after deprotection and cleavage from the solid support the aminolinked deprotected oligonucleotide is isolated. The conjugates are introduced via activation of the functional group using standard  
35 synthesis methods.

#### *Purification by RP-HPLC:*

The crude compounds are purified by preparative RP-HPLC on a Phenomenex Jupiter C18 10 $\mu$  150x10 mm column. 0.1 M ammonium acetate pH 8 and acetonitrile is used as buffers at a flow rate of 5 mL/min. The collected fractions are lyophilized to give the purified compound typically as a white solid.

5 **Abbreviations:**

DCI: 4,5-Dicyanoimidazole

DCM: Dichloromethane

DMF: Dimethylformamide

DMT: 4,4'-Dimethoxytrityl

10 THF: Tetrahydrofurane

Bz: Benzoyl

Ibu: Isobutyryl

RP-HPLC: Reverse phase high performance liquid chromatography

**$T_m$  Assay:**

15 Oligonucleotide and RNA target (phosphate linked, PO) duplexes are diluted to 3 mM in 500 ml RNase-free water and mixed with 500 ml 2x  $T_m$ -buffer (200mM NaCl, 0.2mM EDTA, 20mM Naphosphate, pH 7.0). The solution is heated to 95°C for 3 min and then allowed to anneal in room temperature for 30 min. The duplex melting temperatures ( $T_m$ ) is measured on a Lambda 40 UV/VIS Spectrophotometer equipped with a Peltier temperature programmer PTP6 using PE  
20 Templab software (Perkin Elmer). The temperature is ramped up from 20°C to 95°C and then down to 25°C, recording absorption at 260 nm. First derivative and the local maximums of both the melting and annealing are used to assess the duplex  $T_m$ .

**Example 1 - Testing in vitro efficacy and potency**

Oligonucleotides targeting one region as well as oligonucleotides targeting at least two target  
25 regions being identical to each other on RB1 (SEQ ID NO: 1) were tested in an *in vitro* experiment in HeLa cells. EC50 (potency) and max kd (efficacy) was assessed for the single targeting and repeat targeting oligonucleotides.

Cell lines

The HeLa cell line was purchased from European Collection of Authenticated Cell Cultures  
30 (ECACC) and maintained as recommended by the supplier in a humidified incubator at 37°C with 5% CO<sub>2</sub>. For assays, 2,500 cells/well were seeded in a 96 multi well plate in Eagle's Minimum Essential Medium (Sigma, M4655) with 10% fetal bovine serum (FBS) as recommended by the supplier.

Oligonucleotide potency and efficacy

Cells were incubated for 24 hours before addition of oligonucleotides. The oligonucleotides were dissolved in PBS and added to the cells at final concentrations of oligonucleotides was of 0.01 , 0.031 , 0.1 , 0.31 , 1, 3.21 , 10, and 32.1  $\mu$ M, the final culture volume was 100  $\mu$ l/well. The cells were harvested 3 days after addition of oligonucleotide compounds and total RNA was extracted using the PureLink Pro 96 RNA Purification kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Target transcript levels were quantified using FAM labeled TaqMan assays from Thermo Fisher Scientific in a multiplex reaction with a VIC labelled GAPDH control probe in a technical duplex and biological triplex set up. TaqMan primer assays for the target transcript of interest RB1( Hs01078066\_m1), and a house keeping gene GAPDH (432631 7E VIC®/MGB probe). EC50 and efficacy of the oligonucleotides are shown in table 5 as % of control sample.

EC50 calculations were performed in GraphPad Prism6. The maximum [insert target] knock down level is shown in table 5 as % of control.

Table 5: EC50 and maximal knock down (Max Kd) % of control

CMP ID NO	EC50	Std	Max kd	std	Start position(s) on SEQ ID NO: 1
5_1	7.30	34.28	33.17	72.20	99543
6_1	1.55	0.28	7.63	4.85	99543
7_1	9.08	12.88	77.64	3.77	99546
8_1	5.00	1150.04	92.27	2.37	99548
9_1	3.66	1.60	13.40	12.16	123370, 123473, 123526, 123579, 123632, 123685, 123738, 123791, 123897, 123950, 124003, 124056, 124109, 124162, 124215, 124268, 124321, 124374, 124427, 124480, 124533, 124586, 124639, 124692
10_1	0.82	0.08	2.76	2.39	123370, 123473, 123526, 123579, 123632, 123685, 123738, 123791, 123897, 123950, 124003, 124056, 124109, 124162, 124215, 124268, 124321, 124374, 124427, 124480, 124533, 124586, 124639, 124692
11_1	0.72	0.06	0.00	1.90	123372, 123422, 123475, 123528, 123581, 123634, 123687, 123740, 123793, 123899, 123952, 124005, 124058, 124111, 124164, 124217, 124270, 124323, 124376, 124429, 124482, 124535, 124588, 124641, 124694
12_1	4.07	0.14	11.06	1.27	123375, 123425, 123478, 123531, 123584, 123637,

CMP ID NO	EC50	Std	Max kd	std	Start position(s) on SEQ ID NO: 1
					123690, 123743, 123796, 123902, 123955, 124008, 124061, 124114, 124167, 124220, 124273, 124326, 124379, 124432, 124485, 124538, 124591, 124644, 124697

## CLAIMS

1. An antisense oligonucleotide which comprises a contiguous nucleotide sequence of 10 to 30 nucleotides in length and has at least 95% complementarity to an intron region within the target nucleic acid of SEQ ID NO: 1, or naturally occurring variants thereof.
2. The antisense oligonucleotide according to claim 1, wherein the intron region is intron 17 corresponding to position 77694 to 149242 of SEQ ID NO: 1.
3. The antisense oligonucleotide according to any one of claims 1 or 2, which is capable of reducing the expression of RB1 in a cell.
4. The antisense oligonucleotide according to claim 1 to 3, wherein the contiguous nucleotide sequence is fully complementary to SEQ ID NO: 4, 13, 14 or 15.
5. The antisense oligonucleotide of anyone of claims 1 to 4, wherein the contiguous nucleotide sequence is fully complementary to a target region of the target nucleic acid, selected from the group consisting of position 123370-123389, 123473-123492, 123526-123545, 123579-123598, 123632-123651, 123685-123704, 123738-123757, 123791-123810, 123897-123916, 123950-123969, 124003-124022, 124056-124075, 124109-124128, 124162-124181, 124215-124234, 124268-124287, 124321-124340, 124374-124393, 124427-124446, 124480-124499, 124533-124552, 124586-124605, 124639-124658, 124692-124711; 123370-123387, 123473-123490, 123526-123543, 123579-123596, 123632-123649, 123685-123702, 123738-123755, 123791-123808, 123897-123914, 123950-123967, 124003-124020, 124056-124073, 124109-124126, 124162-124179, 124215-124232, 124268-124285, 124321-124338, 124374-124391, 124427-124444, 124480-124497, 124533-124550, 124586-124603, 124639-124656, 124692-124709; 123372-123387, 123422-123437, 123475-123490, 123528-123543, 123581-123596, 123634-123649, 123687-123702, 123740-123755, 123793-123808, 123899-123914, 123952-123967, 124005-124020, 124058-124073, 124111-124126, 124164-124179, 124217-124232, 124270-124285, 124323-124338, 124376-124391, 124429-124444, 124482-124497, 124535-124550, 124588-124603, 124641-124656, 124694-124709; 123375-123388, 123425-123438, 123478-123491, 123531-123544, 123584-123597, 123637-123650, 123690-123703, 123743-123756, 123796-123809, 123902-123915, 123955-123968, 124008-124021, 124061-124074, 124114-124127, 124167-124180, 124220-124233, 124273-124286, 124326-124339, 124379-124392, 124432-124445, 124485-124498, 124538-124551, 124591-124604, 124644-124657, and 124697-124710 of SEQ ID NO:1.
6. The antisense oligonucleotide according to anyone of claims 1 to 5, wherein the contiguous nucleotide sequence is at least 95% complementary, such as fully complementary, to a target region of 10-22, such as 14-20, nucleotides in length of the target nucleic acid of SEQ

- ID NO: 1, wherein the target region is repeated at least 6 or more times across the intons of the target nucleic acid.
7. The antisense oligonucleotide of anyone of claims 1 to 6, wherein the oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and/or SEQ ID NO: 12.
  8. The antisense oligonucleotide of anyone of claims 1-15, wherein the contiguous nucleotide sequence comprises one or more 2' sugar modified nucleosides.
  9. The antisense oligonucleotide of claim 8, wherein the one or more 2' sugar modified nucleoside is independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA, 2'-fluoro-DNA, arabino nucleic acid (ANA), 2'-fluoro-ANA and LNA nucleosides.
  10. The antisense oligonucleotide of claims 8 or 9, wherein the one or more 2' sugar modified nucleoside is a LNA nucleoside.
  11. The antisense oligonucleotide of any one of claims 1-10, where the oligonucleotide comprises at least one modified internucleoside linkage.
  12. The antisense oligonucleotide of claim 11, wherein at least 75% of the internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate internucleoside linkages.
  13. The antisense oligonucleotide of claim 1-12, wherein the oligonucleotide is capable of recruiting RNase H.
  14. The antisense oligonucleotide of any one of claims 1 to 13, wherein the antisense oligonucleotide, or contiguous nucleotide sequence thereof, consists or comprises a gapmer of formula 5'-F-G-F'-3', where region F and F' independently comprise - 8 nucleosides, of which 1-5 are 2' sugar modified and defines the 5' and 3' end of the F and F' region, and G is a region between 5 and 16 nucleosides which are capable of recruiting RNaseH, such as a region comprising 5 - 16 DNA nucleosides.
  15. The antisense oligonucleotide of anyone of claims 1 to 14, wherein the oligonucleotide, or contiguous nucleotide sequence thereof, is selected from the group of CMP ID NO: 9\_1 , 10\_1 , 11\_1 and 12\_1.
  16. A conjugate comprising the antisense oligonucleotide according to any one of claims 1 - 15, and at least one conjugate moiety covalently attached to said oligonucleotide.
  17. A pharmaceutically acceptable salt of the antisense oligonucleotide according to any one of claims 1 - 15, or the conjugate according to claim 16.

18. A pharmaceutical composition comprising the antisense oligonucleotide of claim 1-15 or the conjugate of claim 16 and a pharmaceutically acceptable diluent, solvent, carrier, salt and/or adjuvant.
19. An *in vivo* or *in vitro* method for modulating RB1 expression in a target cell which is expressing RB1, said method comprising administering an antisense oligonucleotide of any one of claims 1-15 or the conjugate of claim 16, the pharmaceutically acceptable salt of claim 17 or the pharmaceutical composition of claim 18 in an effective amount to said cell.
20. The antisense oligonucleotide of any one of claims 1-15 or the conjugate of claim 16, the pharmaceutically acceptable salt of claim 17 or the pharmaceutical composition of claim 18 for use in medicine.
21. The antisense oligonucleotide of any one of claims 1-15 or the conjugate of claim 16, the pharmaceutically acceptable salt of claim 17 or the pharmaceutical composition of claim 18 for use in the treatment neurological injury or neuronal degeneration.
22. Use of the antisense oligonucleotide of claim 1-15 or the conjugate of claim 16, the pharmaceutically acceptable salt of claim 17 or the pharmaceutical composition of claim 18, for the preparation of a medicament for treatment neurological injury or neuronal degeneration.