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An agency of Industry Canada

CA 2635187 A1 2009/12/05

(21) 2 635 187

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(22) Date de dépôt/Filing Date: 2008/06/17

(41) Mise à la disp. pub./Open to Public Insp.: 2009/12/05

(30) Priorité/Priority: 2008/06/05 (US61/059,186)

(51) Cl.Int./Int.Cl. *C12N 15/11* (2006.01), *A61K 31/7105* (2006.01), *A61K 31/712* (2006.01), *A61K 31/713* (2006.01), *C07H 21/00* (2006.01)

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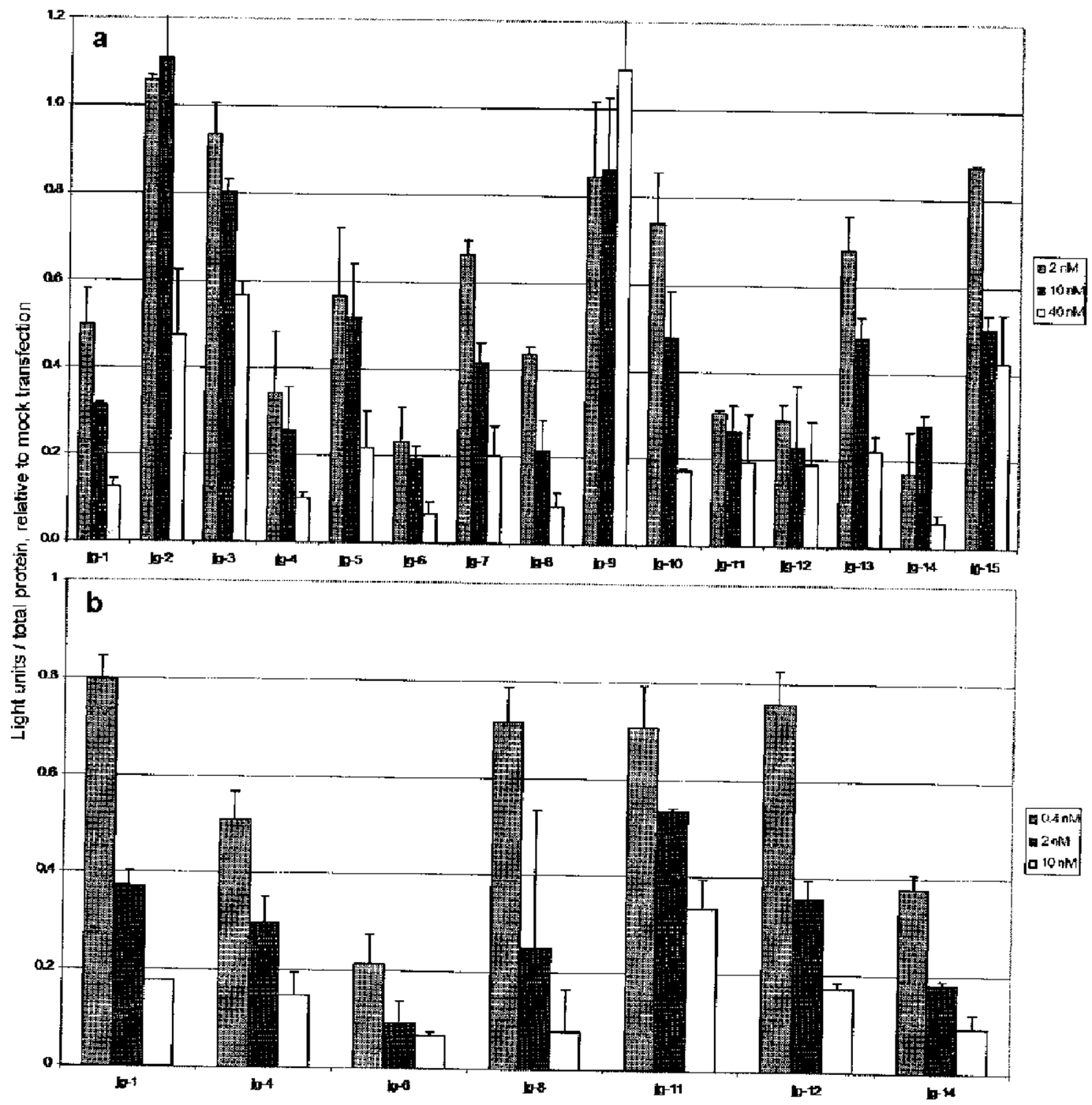
(54) Titre : DUPLEX D'OLIGONUCLEOTIDES ET LEURS UTILISATIONS
(54) Title: OLIGONUCLEOTIDE DUPLEXES AND USES THEREOF



(57) Abrégé/Abstract:
Oligonucleotides duplexes are described, as well as uses thereof for applications such as RNAi-based gene silencing.

ABSTRACT

Oligonucleotides duplexes are described, as well as uses thereof for applications such as RNAi-based gene silencing.

OLIGONUCLEOTIDE DUPLEXES AND USES THEREOF

FIELD OF THE INVENTION

The invention relates to oligonucleotides duplexes, methods for their preparation and uses thereof, such as for silencing the expression of a nucleic acid or gene of interest using small interfering RNA (siRNA) technologies.

BACKGROUND OF THE INVENTION

Gene silencing via the introduction of an antisense oligonucleotide (AON) or small interfering RNA (siRNA) into an organism is an attractive and elegant means of selectively blocking the expression of a deleterious gene.[1-4] The successful development of antisense and siRNA as therapeutics will likely require modification of the oligonucleotide sugar-phosphate backbone to enhance delivery, stability, efficacy and specificity. In fact, progress toward routine use in the clinic, especially for AON therapy, has been slow, partly due to limitations in the currently available chemical modifications.[5]

Much recent work is focusing on the chemical modification of siRNA, and it is not clear yet which chemical modifications will be the most advantageous. Dowler et al. were the first to show that 2'-deoxy-2'F-arabinonucleic acids (2'F-ANA) could be incorporated throughout the sense strand, including a fully-modified sense strand.[6] Modification of the antisense-strand 3'-overhang with 2'F-ANA brought a significant increase in potency.[6] Several of the 2'F-ANA-modified duplexes have been able to surpass the native siRNA in potency.[6] Furthermore, siRNA duplexes with extensive 2'F-ANA modification were found to have a significantly longer serum half-life than unmodified siRNAs. Modified siRNA duplexes containing 2'-fluoro-4'-thioarabinonucleotide (4'S-FANA) units were able to enter the RNAi pathway.[7] One or two inserts internally in either strand gave duplexes of potency comparable to that of the control. The 4'S-FANA modification was also able to work with good efficiency in a duplex with a modified 2'F-ANA–RNA sense strand, demonstrating that 2'F-ANA (with its preference for southern and

eastern conformations) can achieve synergy with 4'S-FANA, in RNAi gene silencing.

2'F-RNA is another siRNA modification, and partial 2'F-RNA modification is tolerated throughout both the sense and antisense strands, and some fully-modified 2'F-RNA siRNAs are also active. 2'F-RNA-modified siRNA duplexes have significantly increased serum stability.[8] 2'F-RNA also increases the binding affinity of the duplex.

It is very rare to find patterns of chemical modification that universally increase potency. On the other hand, there are known modifications that increase potency, sometimes very significantly, for particular sequences or systems. A dramatic increase was observed for a fully modified siRNA made of a combination of 2'-*O*-Me and 2'F-RNA modified nucleotides, which was 500 times more potent than unmodified RNA,[9,10] however, such a high degree of improvement was not observed for other sequences.

These techniques present significant challenges, and there is a need for improvements in for example efficacy, *in vivo* stability and reduction of "off-target" effects (e.g., the silencing of a gene other than the intended target).

There is therefore a continued need for improved oligonucleotide-based approaches.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides an oligonucleotide duplex comprising:

(a) a sense strand comprising (i) one or more DNA-like oligonucleotides, (ii) one or more RNA-like oligonucleotides, or (iii) both (i) and (ii); and

(b) an antisense strand complementary to the sense strand, the antisense strand comprising (i) one or more DNA-like oligonucleotides, (ii) one or more RNA-like oligonucleotides, or (iii) both (i) and (ii).

In an embodiment, the DNA-like oligonucleotides are DNA, 2'-deoxy-2'-fluoro-arabinonucleotide (2'F-ANA), their corresponding phosphorothioate analogs, or combinations thereof.

In an embodiment, the RNA-like oligonucleotides are RNA nucleotides, 2'F-RNA nucleotides, LNA nucleotides, 4'S-FANA nucleotides, 2'-O-alkyl-RNA, their corresponding phosphorothioate analogs, or combinations thereof.

In an embodiment, the sense strand comprises one or more DNA-like oligonucleotides.

In an embodiment, the sense strand consists of DNA-like oligonucleotides.

In an embodiment, the antisense strand comprises one or more DNA-like oligonucleotides.

In an embodiment, the sense strand comprises one or more RNA-like oligonucleotides.

In an embodiment, the sense strand consists of RNA-like oligonucleotides.

In an embodiment, the antisense strand comprises one or more RNA-like oligonucleotides.

In an embodiment, the antisense strand consists of RNA-like oligonucleotides.

In an embodiment, either or both strands are 5-100 nucleotides in length.

In an embodiment, the sense and antisense strands form a double-stranded siRNA-like molecule.

In an embodiment, one or both strands of the double-stranded siRNA-like molecule have overhangs from 1-5 nucleotides on the 3'-end.

In an embodiment, the overhang is 2 nucleotides.

In an embodiment, the overhanging nucleotides are DNA-like nucleotides.

In an embodiment, neither strand has an overhang.

In an embodiment, the sense and antisense strands are within an oligonucleotide of 15-80 nucleotides in length and such that the oligonucleotide or a portion thereof is capable of adopting an siRNA-like hairpin structure in which the sense and antisense strands form the stem of the hairpin structure.

In an embodiment, the sense and antisens strands are 19 to 29 nucleotides in length.

In an embodiment, the double-stranded siRNA or siRNA-like molecule comprises a 19-29 bp duplex portion.

In an embodiment, the double-stranded siRNA or siRNA-like molecule comprises a 1-5 nucleotide 3' overhang in one or both strands.

In an embodiment, the double-stranded siRNA or siRNA-like molecule comprises a 2 nucleotide 3' overhang in one or both strands.

The invention further provides a composition comprising the above-mentioned oligonucleotide duplex or the above-mentioned siRNA or siRNA-like molecule and a pharmaceutically acceptable carrier.

In an embodiment, the above-mentioned oligonucleotide has a sugar phosphate backbone.

In an embodiment, the above-mentioned oligonucleotide comprises at least one internucleotide linkage selected from the group consisting of phosphodiester, phosphotriester, phosphorothioate, methylphosphonate, boranophosphate and any combination thereof.

In an embodiment, the above-mentioned oligonucleotide comprises heterocyclic canonical bases selected from the group consisting of Adenine, Cytosine, Guanine, Thymine and Uracil.

In an embodiment, the above-mentioned oligonucleotide comprises a modified (non-canonical) base.

In an embodiment, the ends of the above-mentioned oligonucleotide are capped with modified nucleotides or moieties capable of conferring exonuclease resistance.

In a further aspect, the invention provides a siRNA or siRNA-like molecule comprising the above-mentioned oligonucleotide duplex.

In embodiments, the first and second oligonucleotides are 19 to 23 nucleotides in length. In an embodiment, the double-stranded siRNA or siRNA-like molecule comprises a 19-29 bp duplex portion. In an embodiment, the double-stranded siRNA or siRNA-like molecule comprises a 1-5 (e.g. 2 nucleotide) nucleotide 3' overhang in one or both strands.

In a further aspect, the invention provides a pharmaceutical composition comprising the above-mentioned oligonucleotide duplex and a pharmaceutically acceptable carrier.

In a further aspect, the invention provides a use of the above-mentioned oligonucleotide duplex, siRNA or siRNA-like molecule or composition for gene silencing.

In a further aspect, the invention provides a use of the above-mentioned oligonucleotide duplex or siRNA or siRNA-like molecule for the preparation of a medicament.

In a further aspect, the invention provides a use of the above-mentioned oligonucleotide duplex or siRNA or siRNA-like molecule for the preparation of a medicament for gene silencing.

In a further aspect, the invention provides a method of inhibiting gene expression in a biological system, comprising

introducing into the system the above-mentioned oligonucleotide duplex, siRNA or siRNA-like molecule or composition.

In a further aspect, the invention provides a method of inhibiting gene expression in a subject, comprising administering a therapeutically effective amount of the above-mentioned oligonucleotide duplex, siRNA or siRNA-like molecule or composition to the subject.

In a further aspect, the invention provides a method of treating a condition associated with expression of a gene in a subject, the method comprising administering the above-mentioned oligonucleotide duplex, siRNA or siRNA-like molecule or composition to the subject, wherein the oligonucleotide is targeted to the gene.

In a further aspect, the invention provides a kit or commercial package comprising: (i) the above-mentioned oligonucleotide duplex; (ii) the above-mentioned siRNA or siRNA-like molecule; or (iii) the above-mentioned composition; together with instructions for use of any of (i) to (iii) for:(a) gene silencing; (b) inhibiting gene expression in a biological system; (c) inhibiting gene expression in a subject; (d) treating a condition associated with expression of a gene in a subject; or (e) any combination of (a) to (d).

"DNA-like" as used herein in reference to conformation refers to a conformation of for example a modified nucleoside or nucleotide which is similar to the conformation of a corresponding unmodified DNA unit. DNA-like conformation may be expressed for example as having a southern pseudorotation (P) value. DNA-like nucleotides include for example 2'-deoxyribonucleotides, 2'-deoxy-2'-fluoroarabinonucleotides (2'F-ANA or FANA), and corresponding phosphorothioate analogs. "RNA-like" as used herein in reference to conformation refers to a conformation of for example a modified nucleoside or nucleotide which is similar to the conformation of a corresponding unmodified RNA unit. RNA-like conformation may be

expressed for example as having a northern P value. Further, RNA-like molecules tend to adopt an A-form helix while DNA-like molecules tend to adopt a B-form helix. RNA-like nucleotides include for example RNA nucleotides, 2'F-RNA nucleotides, LNA nucleotides, 4'S-FANA nucleotides, 2'-O-alkyl-RNA and corresponding phosphorothioate analogs.

In a further aspects of the invention, a double-stranded oligonucleotide duplex is provided, for example:

Sense: DNA-like nucleotide(s), RNA-like nucleotide(s), or both
Antisense: DNA-like nucleotide(s), RNA-like nucleotide(s), or both

Sense: DNA-like nucleotide(s), RNA-like nucleotide(s), or both
Antisense: RNA-like nucleotide(s)

Sense: DNA-like nucleotide(s)
Antisense: DNA-like nucleotide(s), RNA-like nucleotide(s), or both

Sense: RNA-like nucleotide(s)
Antisense: DNA-like nucleotide(s), RNA-like nucleotide(s), or both

Sense: DNA-like nucleotide(s)
Antisense: RNA-like nucleotide(s)

In embodiments, the DNA-like and RNA-like nucleotides are in alternating segments within a strand, such as in an irregular fashion (whereby there may be differences in the number of residues per segment) or a regular fashion (whereby each segment has the same number of residues), or combinations thereof.

In embodiments the double-stranded duplex may have an overhang on the 3'-end of one or both strands. Alternatively, the duplex may have one or two blunt ends.

In an embodiment, the above duplex is a hairpin duplex, that is a single strand comprising the sense and antisense strands which is self-complementary and folds back onto itself.

In embodiments, the internucleotide linkages are phosphodiesters, phosphorothioates or combination thereof.

In other embodiments of the invention, the 2'-F substituent may be substituted with a group selected from the group consisting of 2'-hydroxyl, 2'-amino, 2'-azido, 2'-alkyl, 2'-alkoxy, and 2'-alkoxyalkyl groups. In a further embodiment of the invention, the 2'-alkyl group is selected from the group consisting of methyl, ethyl, propyl, butyl, and functionalized alkyl groups such as cyanoethyl, ethylamino, propylamino and butylamino groups. In embodiments, the alkoxy group is selected from the group consisting of 2'-methoxy, 2'-ethoxy, 2'-proproxy and functionalized alkoxy groups such as $2'\text{-}O(CH_2)_q\text{-}R$, where q=2-4 and -R is a $-NH_2$, $-OCH_3$, or $-OCH_2CH_3$ group. In embodiments, the 2'-alkoxyalkyl group is selected from the group consisting of methoxyethyl, and ethoxyethyl.

In other embodiments of the invention, the oligonucleotide (or, in the case of a double-stranded oligonucleotide, either strand) is fully substituted with DNA-like or RNA-like nucleotides, in embodiments having a length of 4 to 30 nt.

The heterocyclic base moiety of any nucleotides described herein may be one of the canonical bases of DNA or RNA, for example, adenine, cytosine, guanine, thymine or uracil. In other embodiments of the invention, some of the heterocyclic base moieties may be made up of modified or non-canonical bases, for example, inosine, 5-methylcytosine, 2-thiothymine, 4-thiothymine, 7-deazaadenine, 9-deazaadenine, 3-deazaadenine, 7-deazaguanine, 9-deazaguanine, 6-thioguanine, isoguanine, 2,6-diaminopurine, hypoxanthine, and 6-thiohypoxanthine.

In other embodiments of the invention, the oligonucleotide comprises one or more internucleotide linkages selected from the

group consisting of: a) phosphodiester; b) phosphotriester; c) phosphorothioate; d) methylphosphonate; and e) boranophosphate.

According to another aspect of the invention, a method of inhibiting a deleterious gene ("gene silencing") in a patient in need thereof is provided. "Gene silencing" as used herein refers to an inhibition or reduction of the expression of the protein encoded by a particular nucleic acid sequence or gene (e.g., a deleterious gene). The method comprises administering to the patient a therapeutically effective amount of oligonucleotide, a double stranded molecule/duplex, an siRNA molecule or a composition of the invention.

According to another aspect of the invention a composition is provided, comprising the oligonucleotide, double stranded molecule/duplex, or siRNA molecule of the present invention along with a pharmaceutically acceptable carrier.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows siRNA activity of 2'-fluorinated duplexes. (a) Initial results (average of two transfections); (b) Confirmed activity of the most potent duplexes from part a, at lower concentrations (average of two transfections).

Figure 2 shows CD spectra of **jg1 - jg15**. (a) **jg1 - jg5**, in which both strands have the same chemistry; (b) **jg6 - jg9**, in which one of the two strands is a fully-modified chimeric strand; (c) **jg10 - jg13**, in which one of the two strands is a fully-modified strand of a single chemistry; and (d) fully modified heteroduplexes **jg14 - jg15**. The control duplex **jg-1** is included in all spectra for comparison.

Figure 3 shows siRNA activity of 2'-fluorinated duplexes targeting bases 515-533 of firefly luciferase.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to oligonucleotides and their uses, for example in various types of RNA-based technologies, such as gene silencing approaches.

Oligonucleotides of the invention may include those which contain intersugar backbone linkages such as phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages, phosphorothioates and those with formacetal (O-CH_2-O), CH_2--NH--O--CH_2, CH_2--N(CH_3)--O--CH_2 (known as methylene(methylimino) or MMI backbone), CH_2--O--N(CH_3)--CH_2, CH_2--N(CH_3)--N(CH_3)--CH_2 and O--N(CH_3)--CH_2 --CH_2 backbones (where phosphodiester is O--PO_2--O--CH_2). Oligonucleotides having morpholino backbone structures may also be used (U.S. Pat. No. 5,034,506). In alternative embodiments, antisense oligonucleotides may have a peptide nucleic acid (PNA, sometimes referred to as "protein nucleic acid") backbone, in which the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone wherein nucleosidic bases are bound directly or indirectly to aza nitrogen atoms or methylene groups in the polyamide backbone (Nielsen et al. 1991 and U.S. Pat. No. 5,539,082). The phosphodiester bonds may be substituted with structures which are chiral and enantiomerically specific. Persons of ordinary skill in the art will be able to select other linkages for use in practice of the invention.

Oligonucleotides of the invention may also include species which include at least one modified nucleotide base. Thus, purines and pyrimidines other than those normally found in nature may be used. Similarly, modifications on the pentofuranosyl portion of the nucleotide subunits may also be effected. Examples of such modifications are 2'-O-alkyl- and 2'-halogen-substituted nucleotides. Some specific examples of modifications at the 2'

position of sugar moieties which are useful in the present invention are OH, SH, SCH_3, F, OCN, $O(CH_2)_n NH_2$ or $O(CH_2)_n CH_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3 ; OCF_3 ; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; $SO_2 CH_3$; ONO_2 ; NO_2 ; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. One or more pentofuranosyl groups may be replaced by another sugar, by a sugar mimic such as cyclobutyl or by another moiety which takes the place of the sugar.

In some embodiments, the oligonucleotides in accordance with this invention may comprise from about 5 to about 100 nucleotide units, in further embodiments from about 10 to about 100, from about 5 to about 30, from about 10 to about 30, from about 18 to about 27, from about 19 to about 27, from about 18 to about 25, from about 19 to about 25, or from about 19 to about 23 nucleotide units. As will be appreciated, a nucleotide unit is a base-sugar combination (or a combination of analogous structures) suitably bound to an adjacent nucleotide unit through phosphodiester or other bonds forming a backbone structure.

<u>siRNA (RNAi) applications</u>

In further embodiments, the invention provides oligonucleotides of the invention and uses thereof in siRNA/RNAi applications, whereby expression of a nucleic acid encoding a polypeptide of interest, or a fragment thereof, may be inhibited or prevented using RNA interference (RNAi) technology, a type of post-transcriptional gene silencing. RNAi may be used to create a pseudo "knockout", i.e., a system in which the expression of the product encoded by a gene or coding region of interest is reduced, resulting in an overall reduction of the activity of the encoded

product in a system. As such, RNAi may be performed to target a nucleic acid of interest or fragment or variant thereof, to in turn reduce its expression and the level of activity of the product which it encodes. Such a system may be used for functional studies of the product, as well as to treat disorders related to the activity of such a product. RNAi is described in for example published US patent applications 20020173478 (Gewirtz; published November 21, 2002) and 20020132788 (Lewis et al.; published November 7, 2002), all of which are herein incorporated by reference. Reagents and kits for performing RNAi are available commercially from for example Ambion Inc. (Austin, TX, USA), New England Biolabs Inc. (Beverly, MA, USA) and Invitrogen (Carlsbad, CA, USA).

The initial agent for RNAi in some systems is thought to be dsRNA molecule corresponding to a target nucleic acid. The dsRNA is then thought to be cleaved into short interfering RNAs (siRNAs) which are for example 21-23 nucleotides in length (19-21 bp duplexes, each with 2 nucleotide 3' overhangs). The enzyme thought to effect this first cleavage step (the Drosophila version is referred to as "Dicer") is categorized as a member of the RNase III family of dsRNA-specific ribonucleases. Alternatively, RNAi may be effected via directly introducing into the cell, or generating within the cell by introducing into the cell an siRNA or siRNA-like molecule or a suitable precursor (e.g. vector encoding precursor(s), etc.) thereof. An siRNA may then associate with other intracellular components to form an RNA-induced silencing complex (RISC). The RISC thus formed may subsequently target a transcript of interest via base-pairing interactions between its siRNA component and the target transcript by virtue of homology, resulting in the cleavage of the target transcript approximately 12 nucleotides from the 3' end of the siRNA. Thus the target mRNA is cleaved and the level of protein product it encodes is reduced.

RNAi may be effected by the introduction of suitable *in vitro* synthesized siRNA or siRNA-like molecules into cells. RNAi may for example be performed using chemically-synthesized RNA.

Alternatively, suitable expression vectors may be used to transcribe such RNA either *in vitro* or *in vivo*. *In vitro* transcription of sense and antisense strands (encoded by sequences present on the same vector or on separate vectors) may be effected using for example T7 RNA polymerase, in which case the vector may comprise a suitable coding sequence operably-linked to a T7 promoter. The *in vitro*-transcribed RNA may in embodiments be processed (e.g. using E. coli RNase III) *in vitro* to a size conducive to RNAi. The sense and antisense transcripts are combined to form an RNA duplex which is introduced into a target cell of interest. Other vectors may be used, which express small hairpin RNAs (shRNAs) which can be processed into siRNA-like molecules. Various vector-based methods have been described (see e.g., Brummelkamp et al. [2002] Science **296**:550). Various methods for introducing such vectors into cells, either *in vitro* or *in vivo* (e.g. gene therapy) are known in the art.

Accordingly, in an embodiment of the invention, a nucleic acid, encoding a polypeptide of interest, or a fragment thereof, may be inhibited by introducing into or generating within a cell an siRNA or siRNA-like molecule based on an oligonucleotide of the invention, corresponding to a nucleic acid encoding a polypeptide of interest, or a fragment thereof, or to an nucleic acid homologous thereto (sometimes collectively referred to herein as a "target nucleic acid/gene"). "siRNA-like molecule" refers to a nucleic acid molecule similar to an siRNA (e.g. in size and structure) and capable of eliciting siRNA activity, i.e. to effect the RNAi-mediated inhibition of expression. In various embodiments such a method may entail the direct administration of the siRNA or siRNA-like molecule into a cell, or use of the vector-based methods described above. In an embodiment, the siRNA or siRNA-like molecule is less than about 30 nucleotides in length. In a further embodiment, the siRNA or siRNA-like molecule is about 19-23 nucleotides in length. In an embodiment, siRNA or siRNA-like molecule comprises a 19-21 bp duplex portion, each strand having a 2 nucleotide 3' overhang. In other embodiments, one or both strands may have blunt ends. In embodiments, the siRNA or siRNA-

like molecule is substantially identical to a nucleic acid encoding a polypeptide of interest, or a fragment or variant (or a fragment of a variant) thereof. Such a variant is capable of encoding a protein having activity similar to the polypeptide of interest. In embodiments, the sense strand of the siRNA or siRNA-like molecule is substantially identical to a target gene/sequence, or a fragment thereof (where, in embodiments, U may replace the T residues of the DNA sequence).

Accordingly, the invention further provides an siRNA or siRNA-like molecule comprising an oligonucleotide of the invention. In embodiments, the invention provides a double-stranded siRNA or siRNA-like molecule comprising a first oligonucleotide which is an oligonucleotide of the invention and a second oligonucleotide complementary thereto. In further embodiments, the invention provides a kit or package comprising a first oligonucleotide which is an oligonucleotide of the invention and a second oligonucleotide complementary thereto. In embodiments, the second oligonucleotide is also an oligonucleotide of the invention. In embodiments, the first and second oligonucleotides are 19-23 nucleotides in length. In embodiments, the double-stranded siRNA or siRNA-like molecule comprises a 19-21 bp duplex portion. In embodiments, the double-stranded siRNA or siRNA-like molecule comprises a 3' overhang of 1-5 nucleotides in each strand. In further embodiments, neither strand of the double-stranded siRNA or siRNA-like molecule has an overhang. In a further embodiment, the double-stranded siRNA or siRNA-like molecule comprises one or both blunt ends.

The invention further provides a method of inhibiting gene expression in a biological system, comprising introducing into the system the siRNA or siRNA-like molecule.

The invention further provides a method of inhibiting gene expression in a subject, comprising administering the siRNA or siRNA-like molecule to the subject.

The invention further provides a method of treating a condition associated with expression of a gene in a subject, the

method comprising administering the siRNA or siRNA-like molecule to the subject, wherein the siRNA or siRNA-like molecule is targeted to the gene.

The invention further provides a use of the siRNA or siRNA-like molecule for the preparation of a medicament.

The invention further provides a use of the siRNA or siRNA-like molecule for a method selected from: (a) gene silencing; (b) inhibiting gene expression in a biological system; (c) inhibiting gene expression in a subject; and (d) treating a condition associated with expression of a gene in a subject; and (e) preparation of a medicament for treating a condition associated with expression of a gene in a subject.

In various embodiments, an oligonucleotide of the invention may be used therapeutically in formulations or medicaments to prevent or treat disease associated with the expression of a target nucleic acid or gene. The invention provides corresponding methods of medical treatment, in which a therapeutic dose of an oligonucleotide of the invention is administered in a pharmacologically acceptable formulation, e.g. to a patient or subject in need thereof. Accordingly, the invention also provides therapeutic compositions comprising an oligonucleotide of the invention and a pharmacologically acceptable excipient or carrier. In one embodiment, such compositions include an oligonucleotide of the invention in a therapeutically or prophylactically effective amount sufficient to treat a disease associated with the expression of a target nucleic acid or gene. The therapeutic composition may be soluble in an aqueous solution at a physiologically acceptable pH.

A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as a reduction or reversal in progression of a disease associated with the expression of a target nucleic acid or gene. A therapeutically effective amount of an oligonucleotide of the invention may vary according to factors such

as the disease state, age, sex, and weight of the individual, and the ability of the compound to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting the rate of onset or progression of a disease associated with the expression of a target nucleic acid or gene. A prophylactically effective amount can be determined as described above for the therapeutically effective amount. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions.

As used herein "pharmaceutically acceptable carrier" or "excipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Alternatively, the carrier can be suitable for intravenous, intraperitoneal, intramuscular, topical, sublingual or oral administration, or for administration by inhalation. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, an oligonucleotide of the invention can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g. an oligonucleotide of the invention) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile

powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. In accordance with an alternative aspect of the invention, an oligonucleotide of the invention may be formulated with one or more additional compounds that enhance its solubility.

In accordance with another aspect of the invention, therapeutic compositions of the present invention, comprising an oligonucleotide of the invention, may be provided in containers or commercial packages which further comprise instructions for its use for the inhibition of target gene expression, and/or prevention and/or treatment of a disease associated with expression of a target nucleic acid or gene.

The invention further provides a use of an oligonucleotide of the invention or the above-mentioned composition for inhibition of expression of a target nucleic acid or gene or for the prevention and/or treatment of a disease associated with expression of a target nucleic acid or gene. The invention further provides a use of an oligonucleotide of the invention for the preparation of a medicament for prevention and/or treatment of a disease associated with expression of a target nucleic acid or gene.

"Nucleoside" refers to a base-sugar combination, the base being attached to the sugar via an N-glycosidic linkage. "Nucleotide" refers to a nucleoside that additionally comprises a phosphate group attached to the sugar portion of the nucleoside. "Base", "nucleic acid base" or "nucleobase" refer to a heterocyclic base moiety, which within a nucleoside or nucleotide is attached to the sugar portion thereof, generally at the 1' position of the sugar moiety. This term includes both naturally-occurring and modified bases. The two most common classes of naturally-occurring bases are purines and pyrimidines, and comprise for example guanine, cytosine, thymine, adenine and uracil. A number of other naturally-occurring bases, as well as modified bases, are known in

the art, for example, inosine, 5-methylcytosine, 2-thiothymine, 4-thiothymine, 7-deazaadenine, 9-deazaadenine, 3-deazaadenine, 7-deazaguanine, 9-deazaguanine, 6-thioguanine, isoguanine, 2,6-diaminopurine, hypoxanthine, and 6-thiohypoxanthine.

The invention further provides a method of synthesizing an oligonucleotide of the invention, the method comprising: (a) 5'-deblocking; (b) coupling; (c) capping; and (d) oxidation; wherein (a), (b), (c) and (d) are repeated under conditions suitable for the synthesis of the oligonucleotide, wherein the synthesis is carried out in the presence of a suitable nucleotide monomer described herein.

In embodiments, the synthesis is carried out on a solid phase, such as on a solid support selected from the group consisting of controlled pore glass, polystyrene, polyethylene glycol, polyvinyl, silica gel, silicon-based chips, cellulose paper, polyamide/kieselgur and polacryloylmorpholide. In further embodiments, the monomers may be used for solution phase synthesis or ionic-liquid based synthesis of oligonucleotides.

"Protecting group" as used herein refers to a moiety that is temporarily attached to a reactive chemical group to prevent the synthesis of undesired products during one or more stages of synthesis. Such a protecting group may then be removed to allow for step of the desired synthesis to proceed, or to generate the desired synthetic product. Examples of protecting groups are trityl (e.g., monomethoxytrityl, dimethoxytrityl), silyl, levulinyl and acetyl groups.

"5'-Deblocking" as used herein refers to a step in oligonucleotide synthesis wherein a protecting group is removed from a previously added nucleoside (or a chemical group linked to a solid support), to produce a reactive hydroxyl which is capable of reacting with a nucleoside molecule, such as a nucleoside phosphoramidite or H-phosphonate.

"Coupling" as used herein refers to a step in oligonucleotide synthesis wherein a nucleoside is covalently attached to the terminal nucleoside residue of the oligonucleotide (or to the solid support via for example a suitable linker), for example via nucleophilic attack of an activated nucleoside phosphoramidite, H-phosphonate, phosphotriester, pyrophosphate, or phosphate in solution by a terminal 5'-hydroxyl group of a nucleotide or oligonucleotide bound to a support. Such activation may be effected by an activating reagent such as tetrazole, 5-ethylthio-tetrazole, 4,5-dicyanoimidazole (DCI), and/or pivaloyl chloride.

"Capping" as used herein refers to a step in oligonucleotide synthesis wherein a chemical moiety is covalently attached to any free or unreacted hydroxyl groups on the support bound nucleic acid or oligonucleotide (or on a chemical linker attached to the support). Such capping is used to prevent the formation of for example sequences of shorter length than the desired sequence (e.g., containing deletions). An example of a reagent which may be used for such capping is acetic anhydride. Further, the capping step may be performed either before or after the oxidation (see below) of the phosphite bond.

"Oxidation" as used herein refers to a step in oligonucleotide synthesis wherein the newly synthesized phosphite triester or H-phosphonate diester bond is converted into pentavalent phosphate triester or diester bond. In the case where a phosphorothioate internucleotide linkage is desired, "oxidation" also refers to the addition of a sulfur atom to generate a phosphorothioate linkage.

The invention further provides a salt of any of the above-mentioned compounds where applicable.

The following examples are illustrative of various aspects of the invention, and do not limit the broad aspects of the invention as disclosed herein.

EXAMPLES

Example 1: siRNA duplexes containing combinations of 2'F-ANA and 2'F-RNA

Oligonucleotide design

We made a series of duplexes containing fully-modified 2'F-ANA and 2'F-RNA strands (Table I). A series of chimeric strands containing both 2'-fluoro epimers was also designed. One chimera consisted of 2'F-RNA pyrimidines and 2'F-ANA purines. Another pair of strands was a "1-1 altimer" structure, with alternating 2'F-ANA and 2'F-RNA residues. For all of these 2'F-ANA/2'F-RNA chimeric strands, the 3'-overhang was always made of 2'F-ANA, since this may lead to increased potency.[6] 2'F-ANA is also more resistant to 3'-exonucleases than is 2'F-RNA. [11]

Table I siRNA strands containing mixtures of 2'F-ANA and 2'F-RNA. Legend: RNA, dna, ***2'F-ANA***, **2'F-RNA**.

Name	Description	Sequence	T_m	SEQ ID NO:
jg-1	Control	5'-GCUUGAAGUCUUUAAUUAAtt-3'	61.8	**1**
		3'-ggCGAACUUCAGAAAUUAAUU-5'		**2**
jg-2	pur/pyr	5'-***G*CTT*G*AAG*T*CTTTAA*T*TAA*TT*-3'	65.6	**3**
		3'-***GG*CGAA*C*TTCAGAAA*T*TAA*T*Tp-5'		**4**
jg-3	1-1 altimer	5'-***G*C*T*T*G*AA*G*T*C*TTTAA*T*TAA*TT*-3'	36.8	**5**
		3'-***GG*C*G*AA*C*TTCAGAAA*T*TAA*T*Tp-5'		**6**
jg-4	2'F-RNA	5'-**GCTTGAAGTCTTTAATTAATT**-3'	>90	**7**
		3'-**GGCGAACTTCAGAAATTAATT**p-5'		**8**
jg-5	2'F-ANA	5'-***GCTTGAAGTCTTTAATTAATT***-3'	72.8	**9**
		3'-***GGCGAACTTCAGAAATTAATT***p-5'		**10**
jg-6	pur/pyr	5'-***G*CTT*G*AAG*T*CTTTAA*T*TAA*TT*-3'	62.5	**3**
	RNA	3'-ggCGAACUUCAGAAAUUAAUU-5'		**2**
jg-7	RNA	5'-GCUUGAAGUCUUUAAUUAAtt-3'	56.7	**1**
	pur/pyr	3'-***GG*C*G*AA*C*TTCAGAAA*T*TAA*T*Tp-5'		**4**
jg-8	1-1 altimer	5'-***G*C*T*T*G*AA*G*T*C*TTTAA*T*TAA*TT*-3'	48.2	**5**
	RNA	3'-ggCGAACUUCAGAAAUUAAUU-5'		**2**
jg-9	RNA	5'-GCUUGAAGUCUUUAAUUAAtt-3'	45.8	**1**
	1-1 altimer	3'-***GG*C*G*AA*C*T*T*C*AG*AA*A*T*TAA*T*Tp-5'		**6**

jg-10	2'F-RNA	5'-**GCTTGAAGTCTTTAATTAATT**-3'	76.5	**7**
	RNA	3'-ggCGAACUUCAGAAAUUAAUU-5'		**2**
jg-11	RNA	5'-GCUUGAAGUCUUUAAUUAAtt-3'	76.2	**1**
	2'F-RNA	3'-**GGCGAACTTCAGAAATTAATT**p-5'		**8**
jg-12	2'F-ANA	5'-***GCTTGAAGTCTTTAATTAATT***-3'	64.7	**9**
	RNA	3'-ggCGAACUUCAGAAAUUAAUU-5'		**2**
jg-13	RNA	5'-GCUUGAAGUCUUUAAUUAAtt-3'	62.8	**1**
	2'F-ANA	3'-***GGCGAACTTCAGAAATTAATT***p-5'		**10**
jg-14	2'F-ANA	5'-***GCTTGAAGTCTTTAATTAATT***-3'	80.1	**9**
	2'F-RNA	3'-**GGCGAACTTCAGAAATTAATT**p-5'		**8**
jg-15	2'F-RNA	5'-**GCTTGAAGTCTTTAATTAATT**-3'	77.5	**7**
	2'F-ANA	3'-***GGCGAACTTCAGAAATTAATT***p-5'		**10**

RNA interference

The RNAi activity of all duplexes was tested under the same conditions described below. Results are shown in Fig. 1.

Four of the duplexes (**jg-6**, **jg-8**, **jg-10** and **jg-12**) contained a modified sense strand paired with an RNA antisense strand. The best of these four duplexes is **jg-6**, containing a purine/pyrimidine chimeric sense strand. The second-best duplex is duplex **jg-8**, containing the 1-1 altimer configuration in the sense strand. Thus, combining the two 2'-F epimers in the sense strand yields better results than using either chemistry alone, and strikingly, with better results relative to the natural RNA (**jg-1**).

Comparison of the RNAi activity of duplexes **jg-6** - **jg-13** allows us to evaluate the appropriateness of each type of modified strand architecture (2'F-ANA, 2'F-RNA, purine/pyrimidine and 1-1 altimer) in the sense or antisense strands. Sense/antisense preferences are observed for all four types of modified strands. Duplexes **jg-6**, **jg-8** and **jg-12** are more active than **jg-7**, **jg-9** and **jg-13**, respectively, revealing that both chimeric constructs and the 2'F-ANA strand are better-tolerated in the sense strand than the antisense strand. The difference is particularly striking between duplexes **jg-8** and **jg-9** containing one 1-1 altimer strand; **jg-8** (1-1 altimer in the sense strand) was one of the most active duplexes

tested, while **jg-9** (1-1 altimer in the antisense strand) was totally inactive.

It is somewhat surprising is that **jg-11** is more active than **jg-10**, thus 2'F-RNA is better-tolerated in the antisense than the sense strand. We believe this is the first time a fully-modified or heavily-modified strand has been observed to be better tolerated in the antisense than the sense position.

A 2'F-ANA sense strand and a 2'F-RNA antisense strand formed a duplex that was found to be active as well. Indeed, synergy between these two modifications is observed in the case of duplex **jg-14**, which is more active than either of the duplexes **jg-11** or **jg-12** from which it is derived. On the other hand, reversing the sense/antisense combination gave **jg-15**, one of the least potent siRNAs tested in this study.

Binding affinity

The thermal stabilities of the duplexes were tested by heating the annealed duplexes, in physiological buffer, and measuring the change in A_{260}. Binding affinities of the modified duplexes vary widely. As expected, there is no correlation between RNAi activity and binding affinity. For example, two of the most active duplexes we tested were **jg-4** and **jg-8**, with T_m values of >90 °C and 48.2 °C, respectively. The most potent duplex, the fully fluorinated heteroduplex **jg-14**, had a T_m about 20 °C higher than that that of native RNA (80.1 °C vs 61.8 °C).

CD studies

The CD spectra of the modified duplexes were examined, to explore possible connections between helical structure and siRNA activity. Results are presented in Fig. 2. The changes in the Cotton effects at 210-220 nm are noteworthy. Beginning with duplexes **jg-2**-**jg-5**, which have the same chemistry in both strands, it is noteworthy that for 2'F-RNA duplex **jg-4**, this band is of maximum intensity at 227 nm, which is slightly redshifted with respect to the control duplex **jg-1** (224 nm). On the other hand, for the three duplexes containing 2'F-ANA, including the two chimeric

architectures **jg-2** and **jg-3** and the all-2'F-ANA duplex **jg-5**, this band is blueshifted and reaches maximum intensity at about 220 nm. Furthermore, duplexes **jg-1** and **jg-4** feature a more strongly negative band at 210 nm. This is consistent with the degree of A-form helicity of the duplexes.[12] 2'F-RNA duplex **jg-4** also has the highest intensity for its 270 nm band, followed by native RNA duplex **jg-1**, then the 2'F-ANA-containing strands. Fully-2'F-ANA duplex **jg-5** is quite B-form in character, as evidenced by the fact that its 270 nm band is of the lowest intensity and contains a shoulder above 280 nm, and its 245 nm negative band is significantly more negative than the other duplexes.[12]

For duplexes **jg-6**–**jg-13**, a modified sense strand corresponded to higher molar ellipticity at 220 nm than was observed for the native and antisense-modified duplexes. Thus, the intensity of the 220 nm band for the various sense antisense pairs **jg-6/jg-7**, **jg-8/jg-9**, **jg-10/jg-11** and **jg-12/jg-13** was always higher for the first member of each pair. (Because sense modification led to higher potency for 3 of the 4 modified strand architectures, this higher intensity also corresponded with higher potency, with the exception of duplexes **jg-10** and **jg-11**, for which the 2'F-RNA-modified strand was better-accepted in the antisense than the sense). It is also interesting that modifying the sense strand, but not the antisense strand, with 2'F-RNA, led to a notable increase in the intensity of the Cotton effects at 270 nm.

For duplexes **jg-14** and **jg-15**, in which both strands were modified, the more potent duplex **jg-14** featured higher intensity for its 220 nm band, and indeed, in the whole range from 205-250 nm. It is not clear why such a large difference is observed between these two duplexes at lower wavelengths. Duplex **jg-15** should have more A-form character since it has more strongly negative peaks at 210 nm,[12] but the higher T_m of **jg-14** implies that it has more A-form character than **jg-15**.[12] Therefore, the structure is too complicated to be explained by these simple rationalizations.

To investigate whether the potency and synergy obtained for 2'F-ANA-2'F-RNA combinations was applicable to other siRNA sequences, we tested another duplex against the same gene and cell line, this time targeting positions 515-533.[13,14] A series of fully or heavily 2'-fluorinated duplexes was designed, with the following principles in mind:

1) The preference of 2'F-ANA and 2'F-ANA-2'F-RNA chimeras for the sense strand, and of 2'F-RNA for the antisense strand;
2) The low binding affinity of 1-1 altimers of 2'F-ANA and 2'F-RNA (duplexes **jg-8** and **jg-9** had Tm values 23-26 °C lower than the control sequence, see Table I);
3) We wished to compare the activity of a fully-modified 2'F-ANA sense strand with that of the "fr-type" 2'F-ANA sense strand described above, which includes five RNA inserts near its 3'-end, when paired with a 2'F-RNA antisense strand.

The resulting duplexes are presented in Table II. Each of two antisense strands (either RNA or 2'F-RNA) was paired with each of six modified sense strands (2'F-ANA or a 2'F-ANA-2'F-RNA chimera). The potency of these strands to induce RNAi was evaluated and the results are given in Fig. 3.

Table II. Modification of a second siRNA sequence with combinations of 2'F-ANA and 2'F-RNA. *Legend:* RNA, dna, ***2'F-ANA***, ***2'F-RNA***.

Name	Description	Sequence	SEQ ID NO:
kl-ctl	Control	5'-CGUACGCGGAAUACUUCGAtt-3'	**11**
		3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**
kl-1	2'F-RNA	5'-**CGUACGCGGAAUACUUCGAUU**-3'	**13**
	RNA	3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**
kl-2	2'F-ANA	5'-***CGTACGCGGAATACTTCGATT***-3'	**14**
	RNA	3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**
kl-3	"fr" type	5'-***CGTACGCGGAATAC***UUCGA***TT***-3'	**15**
	RNA	3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**
kl-4	3-3 altimer	5'-***CGTACGCGGAAUACTUCGATT***-3'	**16**
	RNA	3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**

kl-5	3-3/1-1 alt	5'-*CGT**A**C**G**C**GG**A**A**U**A**C**T**U**C**G**ATT*-3'	**17**
	RNA	3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**
kl-6	1-1 altimer	5'-*C**G**T**A**C**G**C**G**G**A**A**U**A**C**T**U**C**G**ATT*-3'	**18**
	RNA	3'-ttGCAUGCGCCUUAUGAAGCU-5'	**12**
kl-7	2'F-RNA	5'-*G**C**T**T**G**A**A**G**T**C**T**T**T**A**A**T**T**A**ATT*-3'	**13**
	2'F-RNA	3'-**UUGCAUGCGCCUUAUGAAGCU**p-5'	**19**
kl-8	2'F-ANA	5'-*CGTACGCGGAATACTTCGATT*-3'	**14**
	2'F-RNA	3'-**UUGCAUGCGCCUUAUGAAGCU**p-5'	**19**
kl-9	"fr" type	5'-*CGTACGCGGAATAC*UUCGA*TT*-3'	**15**
	2'F-RNA	3'-**UUGCAUGCGCCUUAUGAAGCU**p-5'	**19**
kl-10	3-3 altimer	5'-*CGT**A**C**G**C**GG**A**A**U**A**C**T**U**C**G**ATT*-3'	**16**
	2'F-RNA	3'-**UUGCAUGCGCCUUAUGAAGCU**p-5'	**19**
kl-11	3-3/1-1 alt	5'-*CGT**A**C**G**C**GG**A**A**U**A**C**T**U**C**G**ATT*-3'	**17**
	2'F-RNA	3'-**UUGCAUGCGCCUUAUGAAGCU**p-5'	**19**
kl-12	1-1 altimer	5'-*C**G**T**A**C**G**C**G**G**A**A**U**A**C**T**U**C**G**ATT*-3'	**18**
	2'F-RNA	3'-**UUGCAUGCGCCUUAUGAAGCU**p-5'	**19**

Several results are clear from this set of duplexes. Perhaps the most striking is that nearly all of the duplexes are able to beat the control siRNA. Four fully-modified duplexes (**kl-7**, **kl-9**, **kl-10**, **kl-11**) and five other heavily-modified duplexes (**kl-4**, **kl-5**, **kl-6**, **kl-8**, **kl-12**) have greater potency than the control for this second sequence.

Furthermore, synergy between 2'F-RNA and 2'F-ANA is again visible. These duplexes can be thought of as belonging to two sub-series, the first with an RNA antisense strand (**kl-1** to **kl-6**) and the second with a 2'F-RNA antisense strand (**kl-7** to **kl-12**). Comparing the corresponding members of each series (**kl-1** to **kl-7**, **kl-2** to **kl-8**, etc), it is clear that all of the modified sense strands show better potency when paired to a 2'F-RNA antisense strand than an RNA antisense strand.

Taking each sub-series separately, and ranking the duplexes in order of potency, a pattern can be observed: the sense strands follow the same order, with either antisense strand. Thus, the "worst" sense strand is all 2'F-ANA (**kl-2** and **kl-8**), followed by

the "fr-type" sense strand containing five RNA inserts (**kl-3** and **kl-9**). We observe, however, that both kl-8 and kl-9 are nonetheless more potent than the control.

Use of the chimeric 2'F-ANA-2'F-RNA sense strands led to better potency, again irrespective of the antisense strand used. The best sense strand was the 3-3/1-1 altimer strand (**kl-5** and **kl-11**), suggesting that rational design for controlling thermodynamic bias does indeed improve potency. Duplex **kl-11** was unsurpassed in both potency and efficacy. It is not possible even to estimate an IC50 value for this duplex, since at 2 nM, the lowest concentration used for these transfections, the silencing is still at its maximal level.

Finally, it is worth noting that both duplexes **kl-7** and **kl-11** seem to be silencing at their maximum efficacy, since the dose response is essentially flat. The chimeric sense strand of **kl-11** thus allows higher efficacy silencing (relative luciferase level of 0.12-0.15 instead of 0.21-0.24).

As described herein, for example 2'F-ANA and 2'F-RNA can be combined in various ways in siRNA duplexes. Two types of combinations of these two modifications lead to increased potency: combining both chemistries in the sense strand, and combining an 2'F-RNA strand with a 2'F-ANA or chimeric sense strand. Examples of both of these types of synergistic combinations led to increased potency for two siRNA sequences.

Example 2: Experimental methods

Oligonucleotide synthesis. Standard conditions for solid-phase oligonucleotide synthesis were used for the synthesis of all oligonucleotides, at a 0.8 to 1.0 mmol scale. 4,5-Dicyanoimidazole (0.50 M in acetonitrile) or 5-ethylthiotetrazole (0.25 M in acetonitrile) were used as activators, and 0.10 M iodine in 1:2:10 pyridine:water:THF was used as oxidant (wait time during the oxidation step was 24 seconds). Phosphoramidites were prepared as 0.15 M solutions (RNA amidites) or 0.08-0.10 M solutions (DNA, 2'-fluoro amidites). Coupling times were extended to 10-30 minutes for modified nucleotides. The oligonucleotides were treated with

3:1 ammonium hydroxide:ethanol for 16 h at 55 °C to cleave them from the solid support and deprotect the phosphates and bases. Sequences containing ribonucleotides were concentrated and desilylated with $Et_3N \bullet 3HF$ (100 μL) for 48 h at room temperature. Sequence purification was accomplished by anion exchange HPLC using 0 - 0.2 M $LiClO_4$ solution as eluent, or by preparative denaturing PAGE. Desalting was effected on Sephadex G-25 or NAP-25 columns. Sequence purity was verified using denaturing PAGE.

5'-phosphorylation of oligonucleotides was generally accomplished on the CPG solid support, by treating the newly-synthesized oligonucleotide with bis(2-cyanoethyl)-diisopropylaminophosphoramidite and ethylthiotetrazole, followed by normal deprotection conditions. However, the antisense strand of duplex **T3p** was phosphorylated using enzymatic methods (treatment with T4 polynucleotide kinase and ATP under conditions recommended by the enzyme supplier. In all cases, ESI-MS was used to confirm the success of the phosphorylation reaction.

Thermal denaturation and CD studies. Equimolar amounts of complementary sequences were combined, dried and rediluted in pH 7.2 buffer containing 140 mM KCl, 1 mM $MgCl_2$ and 5 mM $NaHPO_4$ (1 mL). After heating to 90 °C, the samples were slowly cooled to room temperature and refrigerated overnight. They were then transferred into cold cuvettes in a Cary 300 UV spectrophotometer. The change in absorbance at 260 nm was then monitored upon heating from 15 °C to 90 °C. Melting temperatures were determined as the maxima of the first derivatives or using the baseline method, as implemented in the Varian software.

CD spectra were obtained on a Jasco J-720 spectropolarimeter at 20 °C using samples annealed in the same buffer and under the same conditions as for the thermal denaturation studies. Spectra were baseline-corrected with respect to a blank containing the buffer but no duplex. Smoothing and adjustment for duplex concentration were effected using the Spectra-Manager program (Jasco).

siRNA assays. HelaX1/5 cells that stably express firefly luciferase were grown as previously described.[15] The day prior to

transfection, 0.5 x 10^5 cells were plated in each well of a 24-well plate. The next day, the cells were incubated with increasing amounts of siRNAs premixed with lipofectamine-plus reagent (Invitrogen) using 1 μL of lipofectamine and 4 μL of the plus reagent per 20 pmol of siRNA (for the highest concentration tested). For the siRNA titrations, each siRNA was diluted into dilution buffer (30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 2 mM $MgOAc_2$) and the amount of lipofectamine-plus reagent used relative to the siRNAs remained constant. 24 hours after transfection, the cells were lysed in hypotonic lysis buffer (15 mM K_3PO_4, 1 mM EDTA, 1% Triton, 2 mM NaF, 1 mg/ml BSA, 1 mM DTT, 100 mM NaCl, 4 μg/mL aprotinin, 2 μg/mL leupeptin and 2 μg/mL pepstatin) and the firefly light units were determined using a Fluostar Optima 96-well plate bioluminescence reader (BMG Labtech) using firefly substrate as described.[16] The luciferase counts were normalized to the protein concentration of the cell lysate as determined by the DC protein assay (BioRad). Error bars represent the standard deviation of at least four transfections. Cotransfecting the siRNAs and the plasmid pCI-hRL-con expressing the *Renilla* luciferase mRNA[17] in the same cell line showed no difference in expression of this reporter, demonstrating the specificity of the RNAi effects (data not shown).

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to".

Throughout this application, various references are referred to describe more fully the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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WHAT IS CLAIMED IS:

1. An oligonucleotide duplex comprising:

(a) a sense strand comprising (i) one or more DNA-like oligonucleotides, (ii) one or more RNA-like oligonucleotides, or (iii) both (i) and (ii); and

(b) an antisense strand complementary to the sense strand, the antisense strand comprising (i) one or more DNA-like oligonucleotides, (ii) one or more RNA-like oligonucleotides, or (iii) both (i) and (ii).

2. The oligonucleotide duplex of claim 1, wherein the DNA-like oligonucleotides are DNA, 2'-deoxy-2'-fluoro-arabinonucleotide (2'F-ANA), their corresponding phosphorothioate analogs, or combinations thereof.

3. The oligonucleotide duplex of claim 1 or 2, wherein the RNA-like oligonucleotides are RNA nucleotides, 2'F-RNA nucleotides, LNA nucleotides, 4'S-FANA nucleotides, 2'-O-alkyl-RNA, their corresponding phosphorothioate analogs, or combinations thereof.

4. The oligonucleotide duplex according to any one of claims 1 to 3, wherein the sense strand comprises one or more DNA-like oligonucleotides.

5. The oligonucleotide duplex of claim 4, wherein the sense strand consists of DNA-like oligonucleotides.

6. The oligonucleotide duplex according to any one of claims 1 to 3, wherein the antisense strand comprises one or more DNA-like oligonucleotides.

7. The oligonucleotide duplex according to any one of claims 1 to 3, wherein the sense strand comprises one or more RNA-like oligonucleotides.

8. The oligonucleotide duplex of claim 4, wherein the sense strand consists of RNA-like oligonucleotides.

9. The oligonucleotide duplex according to any one of claims 1 to 3, wherein the antisense strand comprises one or more RNA-like oligonucleotides.

10. The oligonucleotide duplex of claim 6, wherein the antisense strand consists of RNA-like oligonucleotides.

11. The oligonucleotide duplex according to any one of claims 1 to 10, wherein either or both strands are 5-100 nucleotides in length.

12. The oligonucleotide duplex according to any one of claims 1 to 11, wherein the sense and antisense strands form a double-stranded siRNA-like molecule.

13. The oligonucleotide duplex of claim 12, where one or both strands of the double-stranded siRNA-like molecule have overhangs from 1-5 nucleotides on the 3'-end.

14. The oligonucleotide duplex of claim 13, wherein the overhang is 2 nucleotides.

15. The oligonucleotide duplex of claim 13 or 14, wherein the overhanging nucleotides are DNA-like nucleotides.

16. The oligonucleotide duplex of claim 15 wherein the DNA-like nucleotides are 2'-deoxyribonucleotides, 2'-deoxy-2'-fluoroarabinonucleotides or combinations thereof.

17. The oligonucleotide duplex of claim 12, wherein neither strand has an overhang.

18. The oligonucleotide duplex according to any one of claims 1 to 11, wherein the sense and antisense strands are within an oligonucleotide of 15-80 nucleotides in length and such that the oligonucleotide or a portion thereof is capable of

adopting an siRNA-like hairpin structure in which the sense and antisense strands form the stem of the hairpin structure.

19. The double-stranded siRNA or siRNA-like molecule according to claim 12, wherein the first and second oligonucleotides are 19 to 29 nucleotides in length.

20. The double-stranded siRNA or siRNA-like molecule according to claim 12, wherein the double-stranded siRNA or siRNA-like molecule comprises a 19-29 bp duplex portion.

21. The double-stranded siRNA or siRNA-like molecule according to claim 19 or 20 wherein the double-stranded siRNA or siRNA-like molecule comprises a 1-5 nucleotide 3′ overhang in one or both strands.

22. The double-stranded siRNA or siRNA-like molecule of claim 21, wherein the double-stranded siRNA or siRNA-like molecule comprises a 2 nucleotide 3′ overhang in one or both strands.

23. A composition comprising the oligonucleotide duplex according to any one of claims 1-18 or the siRNA or siRNA-like molecule according to any one of claims 19 to 22 and a pharmaceutically acceptable carrier.

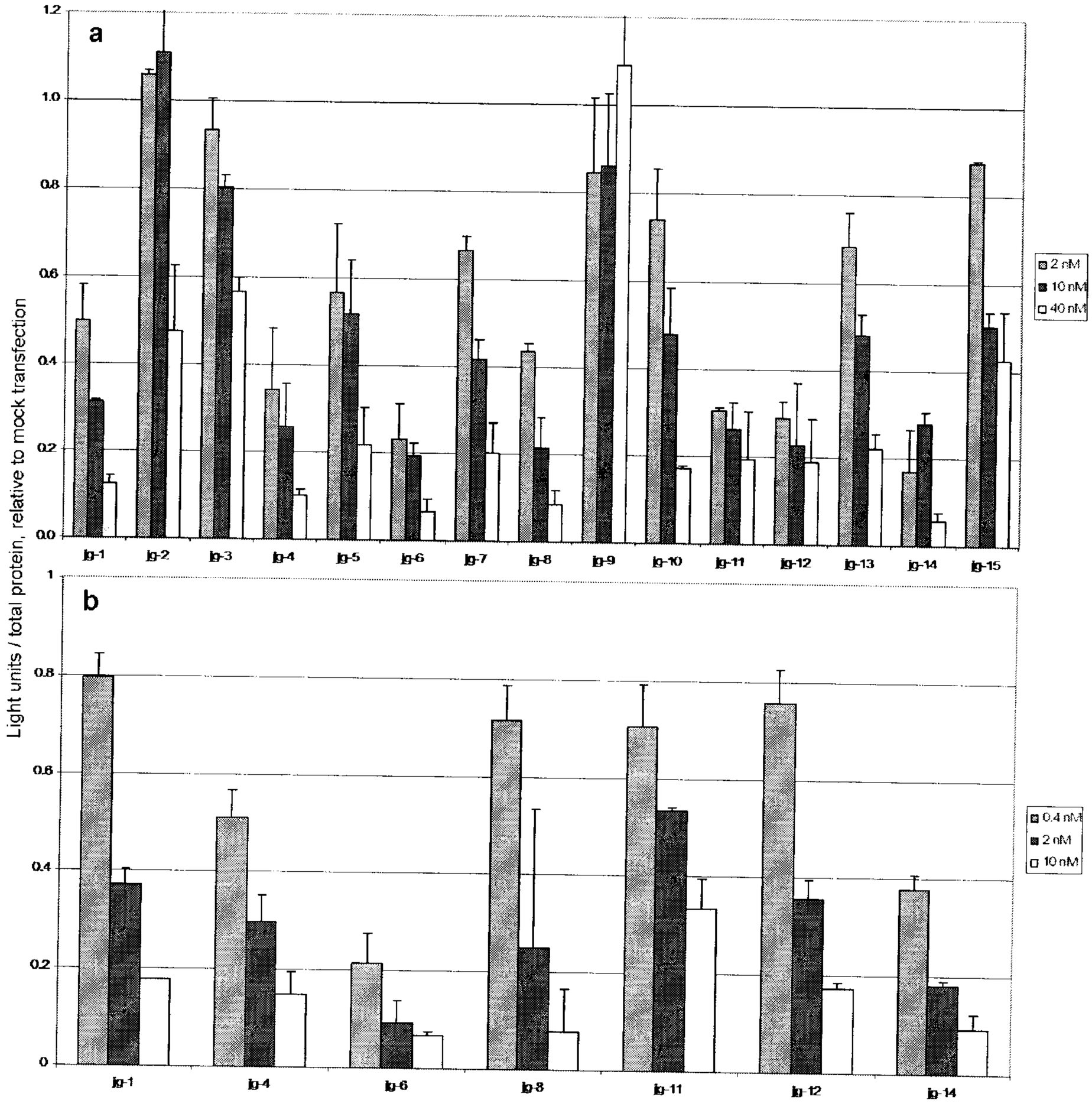

a
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Light units / total protein, relative to mock transfection
2 nM
10 nM
40 nM
Ig-1
Ig-2
Ig-3
Ig-4
Ig-5
Ig-6
Ig-7
Ig-8
Ig-9
Ig-10
Ig-11
Ig-12
Ig-13
Ig-14
Ig-15
b
1
0.8
0.6
0.4
0.2
0
0.4 nM
2 nM
10 nM
Ig-1
Ig-4
Ig-6
Ig-8
Ig-11
Ig-12
Ig-14

Fig. 1

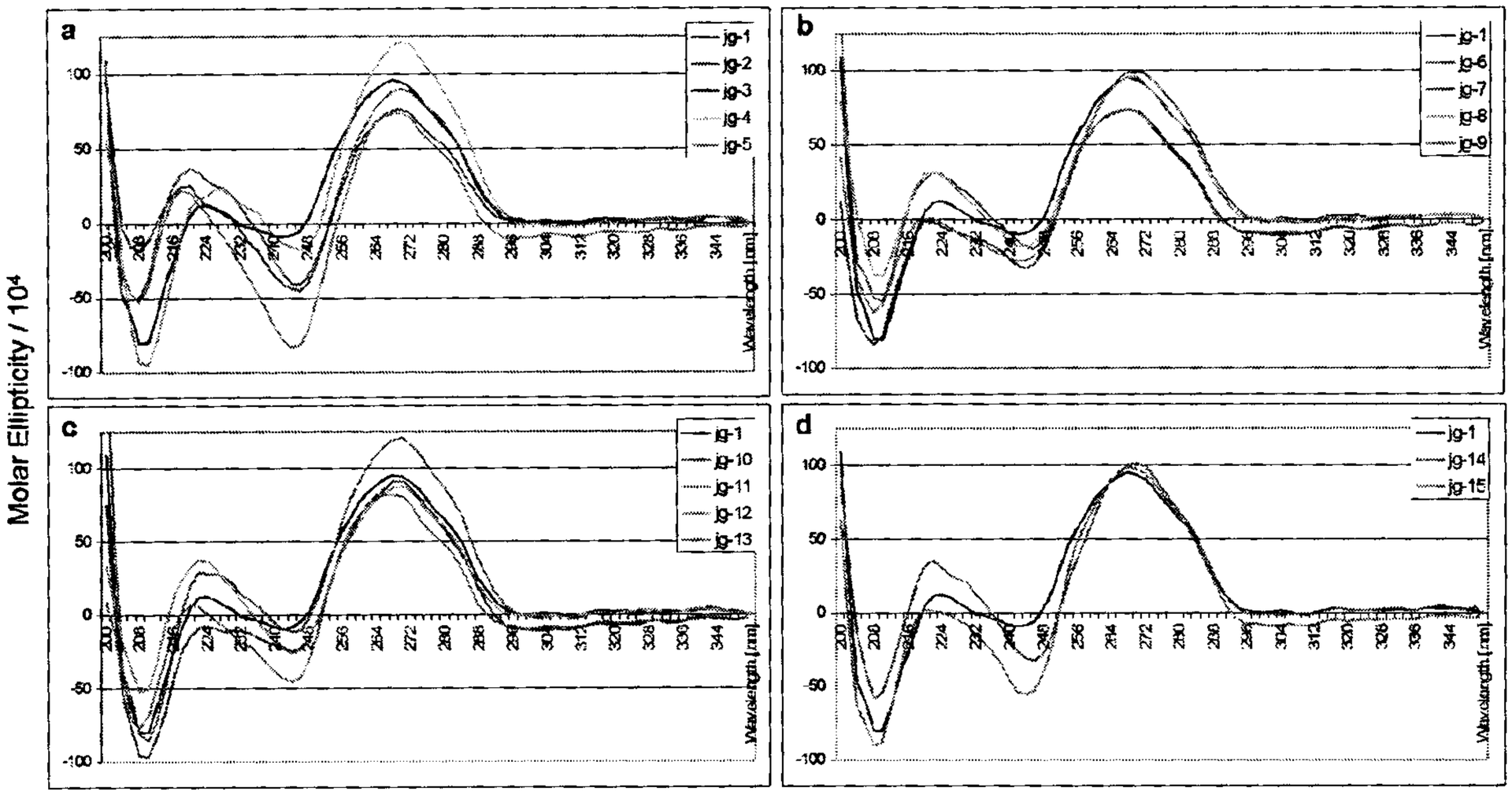

a
b
c
d
jg-1
jg-2
jg-3
jg-4
jg-5
jg-6
jg-7
jg-8
jg-9
jg-10
jg-11
jg-12
jg-13
jg-14
jg-15
Molar Ellipticity / 10⁴
Wavelength [nm]

Fig. 2

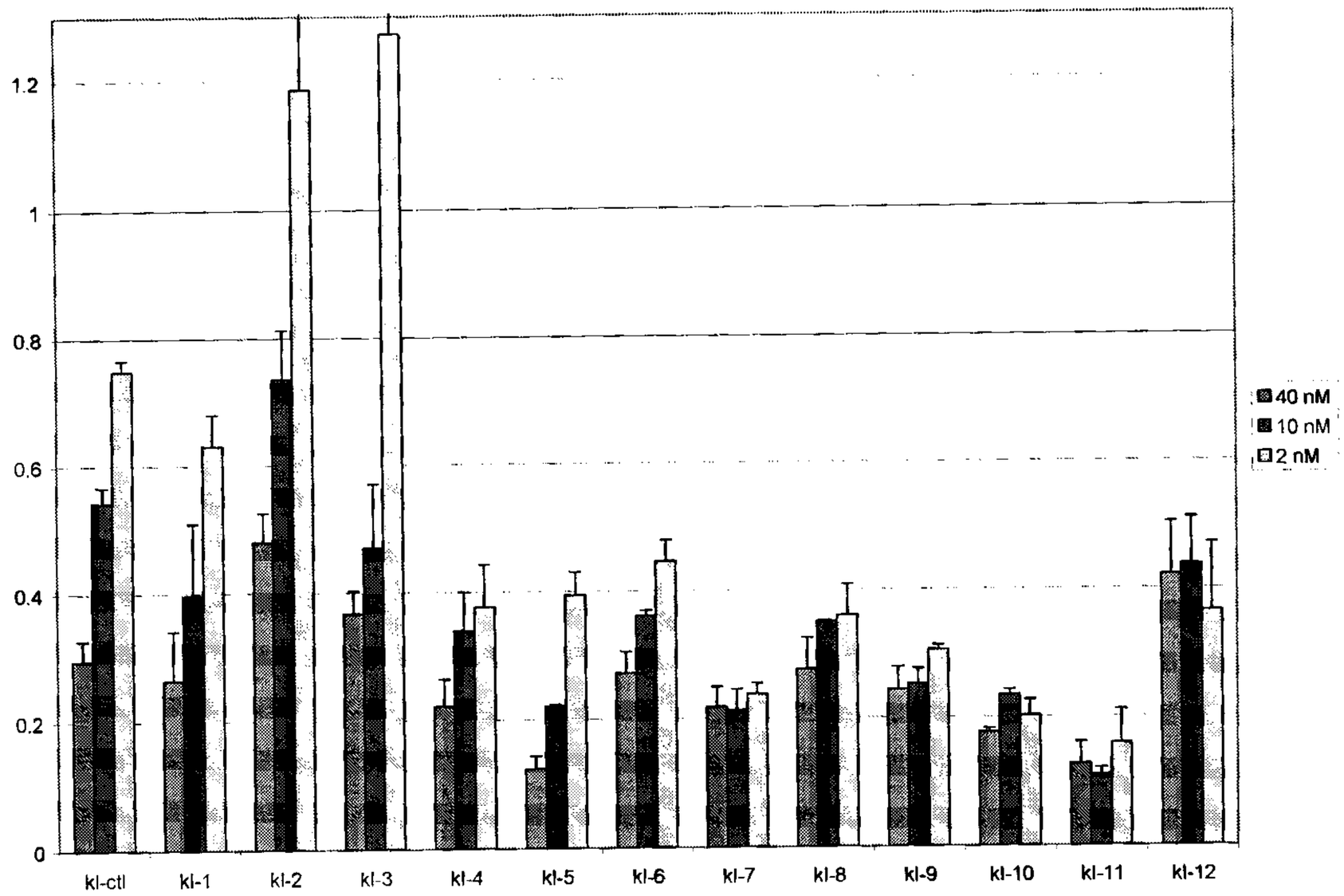
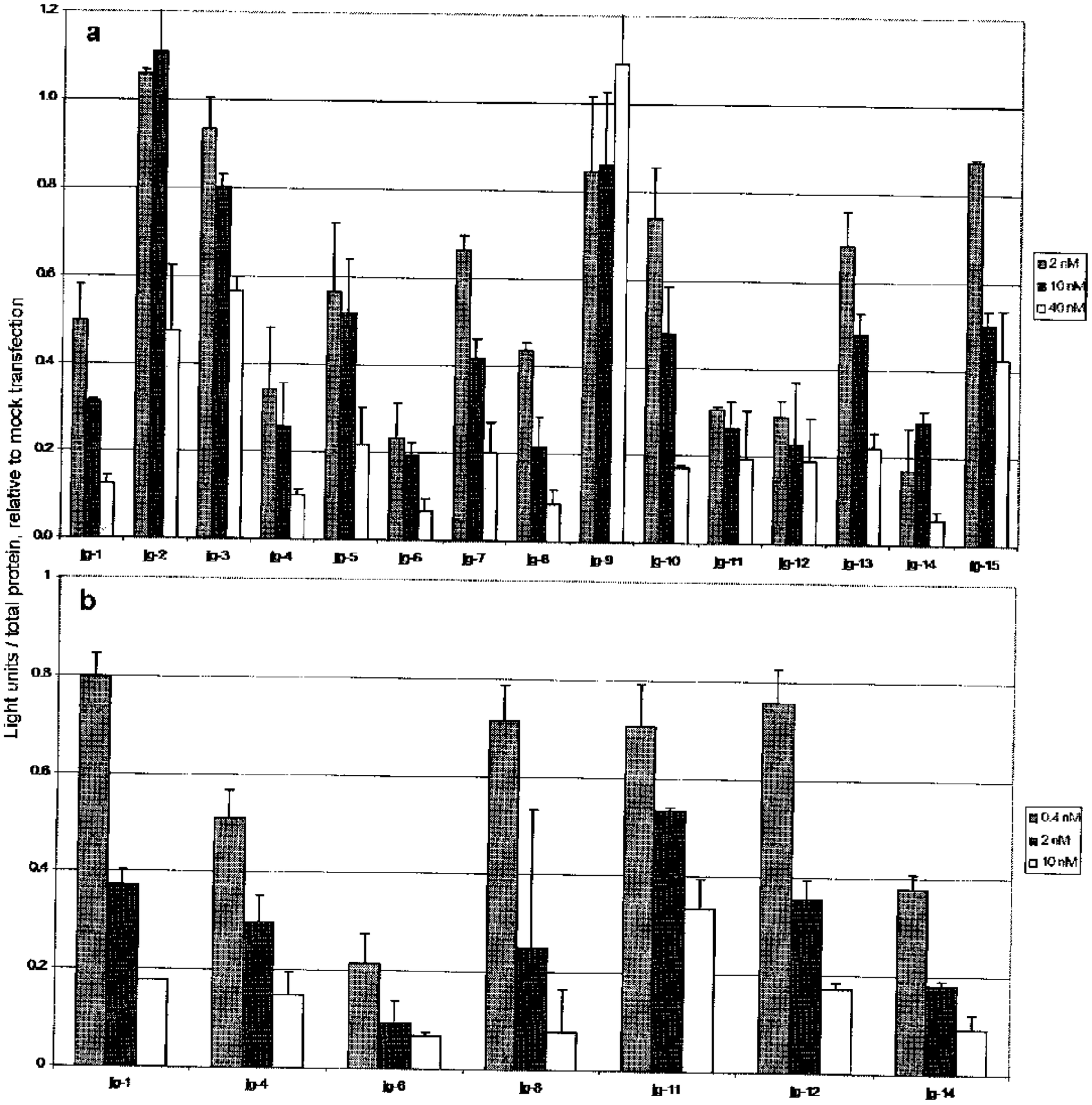

1.2
1
0.8
0.6
0.4
0.2
0
kl-ctl
kl-1
kl-2
kl-3
kl-4
kl-5
kl-6
kl-7
kl-8
kl-9
kl-10
kl-11
kl-12
40 nM
10 nM
2 nM

Fig. 3


a
Light units / total protein, relative to mock transfection
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ig-1
Ig-2
Ig-3
Ig-4
Ig-5
Ig-6
Ig-7
Ig-8
Ig-9
Ig-10
Ig-11
Ig-12
Ig-13
Ig-14
Ig-15
2 nM
10 nM
40 nM
b
Light units / total protein, relative to mock transfection
1
0.8
0.6
0.4
0.2
0
Ig-1
Ig-4
Ig-6
Ig-8
Ig-11
Ig-12
Ig-14
0.4 nM
2 nM
10 nM