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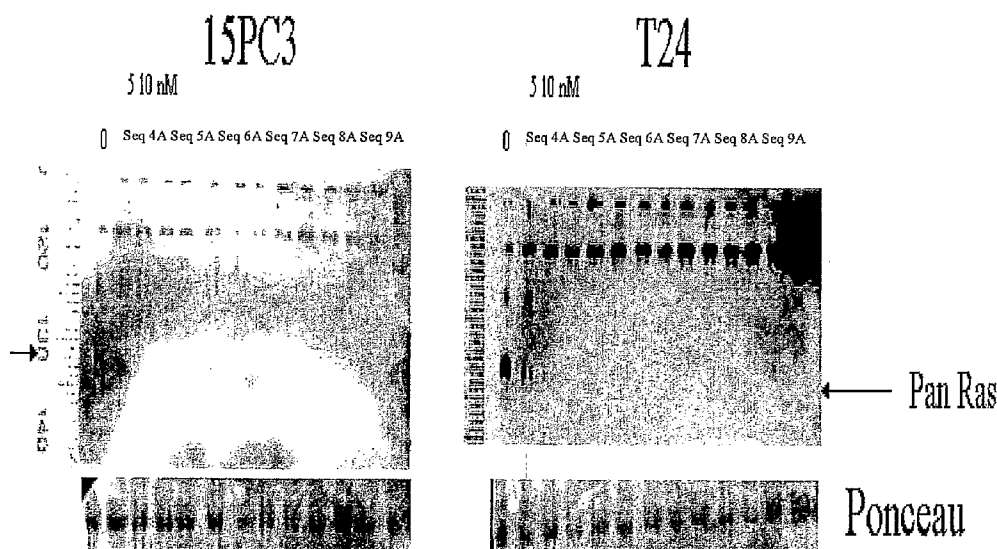
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(54) Title: RNA ANTAGONIST COMPOUNDS FOR THE MODULATION OF P21 RAS EXPRESSION



(57) Abstract: Oligonucleotides directed against the p21 Ras genes are provided for modulating the expression of p21 Ras. The compositions comprise oligonucleotides, particularly antisense oligonucleotides, targeted to nucleic acids encoding Ha-ras, Ki-ras and N-ras. Methods of using these compounds for modulation of p21 Ras expression and for the treatment of diseases associated with either overexpression of p21 Ras, expression of mutated p21 Ras or both are provided. Examples of diseases are cancer such as lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries cancers. The oligonucleotides may be composed of deoxyribonucleotides or a nucleic acid analogue such as for example locked nucleic acid or a combination thereof.



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RNA ANTAGONIST COMPOUNDS FOR THE MODULATION OF P21 RAS EXPRESSION

FIELD OF THE INVENTION

The present invention provides compounds, compositions and methods for modulating the expression of the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras. In particular, this invention relates to oligomeric compounds, preferred such compounds being oligonucleotides, which are hybridisable with target nucleic acids encoding *ras*, and methods for the preparation of such oligomeric compounds. The oligonucleotide compounds have been shown to modulate the expression of multiple members of the *ras* protein family, and pharmaceutical preparations thereof and their use as treatment of cancer diseases are disclosed.

BACKGROUND OF THE INVENTION

The *ras* proto-oncogenes encode a group of plasma membrane-associated G-proteins that bind guanine nucleotides with high affinity and activate several downstream effector proteins including raf-1, PI3-K etc. that are known to activate several distinct signalling cascades that are involved in the regulation of cellular survival, proliferation and differentiation in response to extracellular stimuli such as growth factors or hormones. The "classical" p21 *ras* family of mammalian proto-oncogenes consisting of Harvey-*ras* (Ha-*ras*), Kirsten-*ras* (Ki-*ras*) 4a and 4b and Neuroblastoma-*ras* (N-*ras*) are the most well known members of the rapidly expanding Ras superfamily of small GTPases.

These three *ras* family members are closely related sharing 85% amino acid sequence homology and function in very similar ways.

Several *in vitro* (and *in vivo*) studies have demonstrated that the Ras family of proto-oncogenes are involved in the induction of malignant transformation; see for example Chin *et al.*, (1999) Nature 400, 468-472. Consequently, the p21 Ras family are regarded as important targets in development of anticancer drugs and it has been found that the Ras proteins are either over-expressed or mutated (often leading to constitutively active Ras proteins) in approximately 25% of all human cancers. Interestingly, the *ras* gene mutations in most cancer types are frequently limited to only one of the *ras* genes and are dependent on tumour type and tissue.

Mutation of Ki-ras is the most frequent occurring in approximately 85% of ras-mutated cancers, while N-ras is mutated in about 15% and Ha-ras in less than 1% of ras-mutated cancers.

Ras oncogenic activating mutations have been identified at codon 12, 13 and 61.

Mutated Ki-ras has been shown in lung, pancreas and colorectal cancer, N-ras is mutated in melanoma, acute myelogenous leukaemia and liver cancer while activating mutations in the Ha-ras gene are mainly restricted to thyroid, kidney, urinary tract and bladder cancer.

Aberrant ras signalling can also be activated by overexpression of growth factor receptors such as EGFR and amplification of upstream activators such as PI3K and Akt or mutation of inhibitors of ras activation such as PTEN.

Because of the evidence of ras involvement in cancer development, interruption of the ras pathway has been a major focus for drug development. Efforts have concentrated on either inhibiting ras maturation and thereby membrane localization, or inhibiting ras protein expression.

Several studies have been published showing tumour growth inhibition in xenograft mouse models treated with antisense oligonucleotides targeted to Ha-ras. Gray *et al.* (1993) Cancer Research 53, 577-580 showed inhibition of tumour growth of oncogenic Ha-ras transformed NIH-3T3 cells pretreated with antisense oligonucleotides targeting an intron in the 5' UTR of the Ha-ras mRNA. Using a similar model, Wickstrom *et al.* (1997), in Oligonucleotides as Therapeutic Agents, Wiley, London, 124-141, showed an 80% inhibition of tumour growth of oncogenic Ha-ras transformed NIH-3T3 cells treated by subcutaneous injection of antisense oligonucleotides targeting Ha-ras codon 12 mutation.

An antisense phosphorothioate oligo targeted to the AUG start codon of Ha-Ras (ISIS 2503) developed by Isis Pharmaceuticals has shown potent Ha-ras downregulation *in vitro* and tumour growth inhibition in human tumour xenografts *in vivo*. This antisense oligo was selected as the most potent inhibitor of ras mRNA assayed by Northern blot and it was shown to have an $IC_{50} = 45$ nM (Bennett *et al.* (1996) Antisense Therapeutics, Humana Press, Totowa, New Jersey, 13-47).

Interestingly, the anti-tumour effect of the ISIS 2503 Ha-ras antisense oligo in mouse models was not limited to Ha-ras mutated xenografts, but also showed tumour growth inhibition in Ki-ras mutated tumour xenografts (Cowser et al. (1997) *Anti-Cancer Drug Design* 12, 359-371).

Modification of ISIS 2503 with second-generation compounds conferring enhanced affinity and nuclease resistance has been shown to significantly improve the antisense effect. Incorporation of 2'-methoxyethyl (MOE) into ISIS 2503 increased the potency (IC_{50} = 14.7nM), and the duration of antisense effect *in vitro* (Cowser et al. (1997) *Anti-Cancer Drug Design* 12, 359-371). ISIS 2503 has been tested in phase I/II clinical trials either alone or in combination with chemotherapeutic agents against a variety of advanced cancers, but development of this compound has been terminated.

Casey-Cunningham *et al.* (2001) *Cancer* 92, 1265-1271, reported that in a phase I study of ISIS 2503 in advanced carcinoma, the compound was well tolerated but none of the 23 patients showed either complete or partial response. However, 4 patients had stable disease for 2 months or longer.

The above-mentioned phosphorothioate and MOE antisense compounds, typically between 20 and 25 base pairs, have been described in several patent applications (WO 92/22651, WO 94/08003, WO 94/28720, WO 98/49349, WO 99/02732, WO 99/227723). However, a majority of the disclosed compounds are targeted to two sites on Ha-ras, namely the codon 12 mutation or the AUG start codon, which only constitute a very small portion of the whole target. The codon 12 mutation is also targeted by one antisense sequence disclosed in WO 98/500540, which was tested with different phosphorothioate contents.

Fluiter *et al.* (2005) *ChemBioChem* 6, 1-6, reported very high serum stability and *in vivo* growth inhibition with low doses (down to 0.5 mg/kg/day) of LNA containing antisense oligonucleotides targeting Ha-ras.

WO 2004/069992 discloses antisense oligonucleotides containing LNA targeted specifically to Ha-ras.

US 6,117,848 discloses a number of Ki-ras antisense oligonucleotides based on phosphorothioate chemistry or O'-2-methyl and in US 5,872,242 several N-ras phosphorothioate oligonucleotides were disclosed.

US 5,874,416 discloses a single 26-mer antisense oligonucleotide targeted to a portion of the 5'-UTR region of Ha-ras where all cytosine bases in CG dinucleotide pairs are 5-methylcytosine.

OBJECT OF THE INVENTION

The development of cancer typically involves a series of discrete changes in numerous genes in a cell.

As described above, all three major forms of ras have been indicated in various forms of cancer, and the prior art provides examples of oligonucleotides which target specific ras targets, for use as therapeutics.

p21 Ras is involved in a number of basic biological mechanisms including red blood cell proliferation, cellular proliferation, ion metabolism, glucose and energy metabolism, pH regulation and matrix metabolism

However, as ras clearly plays an important role in healthy cells, it has not previously been considered to attempt to down-regulate all three major forms of ras.

The use of oligonucleotides to target all three major forms of ras are limited by the limited conserved sequence between the nucleic acids which encode Ha-ras, Ki-ras and N-ras, and the length of oligonucleotide which is required to develop an oligonucleotide which specifically hybridises to all three targets whilst retaining the pharmacological properties required for in vivo delivery to the required site of treatment.

By the use of nucleotide analogues which increase the T_m of the oligonucleotide/target RNA hybrid, we have successfully created a collection of single oligonucleotides, each of which retains high specificity for all three of the major forms of ras, whilst retaining excellent penetration *in vivo*. Most surprisingly of all, we have found that at dosages of the oligonucleotides which are effective in treating cancer tumors, there was little or no adverse effect on healthy cells. This is eloquently illustrated in Figures 2 and 3.

The present invention therefore provides a remarkable therapeutic agent which can be used for treatment of cancers which are either characterised in having mutations in more than one ras family member, or for example, for the treatment of cancers which are likely to be associated with mutations in one or more ras family, but where the cancer has not been genotyped for specific ras mutations, or such genotyping has proved inconclusive.

The use of the oligonucleotides which target all three major forms of ras have been found to be so effective, that it is also considered that cancer cells may be able to survive the down regulation of perhaps one or two ras proteins, but concurrent down regulation of all three is intolerable for the cancer cell. Indeed, it appears that, whereas normal cells are able to tolerate severe down regulation of all three ras proteins, cancer cells are not – which has led us to the concept that cancer cells are truly 'addicted' and their survival is dependant upon their sufficient supply of at least one of the three major forms of ras.

Therefore, it is a principal object of the present invention to provide novel oligomeric compounds, targeting, at least two, such as at least three, such as the 3 major mutant forms of ras, namely Ha-ras, Ki-ras and N-ras mRNA. The compounds of the invention have been found to exhibit efficient down-regulation of p21 ras protein and growth inhibition in both Ha-ras and Ki-ras mutated cell lines, thereby facilitating an effective treatment of a variety of cancer diseases in which the expression of p21 Ras is implied as a causative or related agent. As explained in the following, this objective is best achieved through the utilisation of an extremely high affinity chemistry termed LNA (Locked Nucleic Acid).

The present invention is directed to oligomeric compounds, particularly LNA antisense oligonucleotides, which are targeted to a nucleic acid encoding p21 Ras and which modulate the expression of the p21 Ras. This modulation was a particularly potent down regulation of p21 Ras protein as well as inhibition of tumor growth. The LNA-containing oligomeric compounds can be as short as a 10-mers and certainly highly active as a 15mer, 16-mers or 17-mers, which is considerably shorter than the reported antisense compounds targeting p21 Ras. These 15 - 17mer oligomeric compounds have an IC_{50} for mRNA knock-down in the sub-nanomolar range. Thus, the invention enables a considerable shortening of the usual length of an antisense oligomer (from 20-25 mers to, e.g., 8-17 mers) without compromising the affinity required for pharmacological activity. Furthermore, it is anticipated that shorter oligomeric compounds have higher cell permeability than longer oligomeric compounds. For at least these reasons, the present invention is a considerable contribution to the art.

SUMMARY OF THE INVENTION

The present invention is directed to oligomeric compounds, particularly LNA antisense oligonucleotides, which are targeted to a nucleic acid encoding the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras, and which modulate the expression of the

ras. Pharmaceutical and other compositions comprising the oligomeric compounds of the invention are also provided.

The invention provides for a compound consisting of a sequence of total of between 10-30 nucleobases, wherein said compound comprises a subsequence of at least 8 contiguous nucleobases, wherein said subsequence corresponds to a contiguous sequence which is present in the nucleic acids which encode at least two, such as at least three, or three, non-identical mammalian p21 ras family members, wherein said subsequence may comprise no more than one mismatch when compared to each of the corresponding sequences present in the nucleic acids which encode said at least two, such as at least three or three, non-identical mammalian p21 ras family members.

The compound may further comprise a 5' flanking nucleobase sequence, or a 3' flanking sequence, or both a 5' and a 3' flanking sequence which is/are contiguous to said subsequence, wherein said flanking sequence or sequences consist of a total of between 2 and 22 nucleobase units, which when combined with said sub-sequence, the combined contiguous nucleobase sequence, *i.e.* consisting of said subsequence and said flanking sequence or sequences, is at least at least 80% homologous to the corresponding sequences of each of said polynucleotides which encode said mammalian p21 ras family members.

The invention also provides for a method for the identification of at least one compound capable of down-regulation, preferably simultaneous down-regulation, of at least two, such as at least three, or three, non-identical mammalian p21 Ras proteins in a human or mammalian cell, comprising the sequential steps of:

A: Compare the polynucleotide sequences which, in a human or mammalian cell, encode said at least two, such as at least three, or three, non-identical mammalian p21 Ras proteins.

B: from the comparison in step A, identify a subsequence of at least 8 consecutive nucleotides which is present in each of the polynucleotide sequences encoding the at least two, such as at least three, or three, non-identical mammalian p21 Ras proteins, wherein said at least 8 consecutive nucleotides may comprise no more than 1 mismatch when compared to each of the corresponding nucleic acids which encode said non-identical mammalian p21 Ras proteins;

C: Identify a complementary nucleobase sequence which consists of a total of 10-30 nucleobases, wherein the complementary nucleobase sequence comprises corresponds to the subsequence identified in step B.

The invention provides compounds consisting of from 10-50 nucleobases (herein nucleotides and nucleotide analogues are referred collectively as nucleobases), such as between 10-30 nucleobases, wherein said compound comprises a subsequence of at least 8 nucleotides, said subsequence being located within (*i.e.* corresponding to) a sequence selected from those listed in Tables 1 and 2.

The invention provides a compound consisting of from 10-50 nucleobases such as nucleotides, such as between 10-30 nucleobases such as nucleotides, wherein said compound comprises a subsequence of at least 8 nucleobases such as nucleotides, said subsequence being located within a sequence selected from one or more of the group consisting of: SEQ IDs NOs 4-9, SEQ IDs NOs 12-14 and/or SEQ ID NOs 15-47.

The invention provides methods for the preparation of compounds which consist of 10-50 nucleobases such as nucleotides, such as between 10-30 nucleobases such as nucleotides, or analogues thereof, comprising a subsequence of at least 8 nucleobases such as nucleotides or analogues thereof, said compounds being capable of modulating expression of at least two non-identical *ras* proteins in a human or animal cell. In this embodiment, the subsequences may be selected from those listed in tables 1 and 2, and SEQ IDs NOs 4-9, SEQ IDs NOs 12-14 and/or SEQ ID NOs 15-47.

Further provided are methods of modulating the expression of *ras* in cells or tissues comprising contacting said cells or tissues with one or more of the oligomeric compounds or compositions of the invention.

Also disclosed are methods of treating an animal or a human, suspected of having or being prone to a disease or condition, associated with expression of *ras* by administering a therapeutically or prophylactically effective amount of one or more of the oligomeric compounds or compositions of the invention.

Further, methods of using oligomeric compounds for the inhibition of expression of *ras* and for treatment of diseases associated with *ras* activity are provided. Examples of such diseases are different types of cancer, such as for instance lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows p21 ras protein expression in 15PC3 and T24 cells transfected with 5 and 10 nM various compound (lane 1: 0 nM; lanes 2 and 3: SEQ ID NO: 4A (5 and 10 nM, respectively); lanes 4 and 5: SEQ ID NO: 5A (5 and 10 nM, respectively); lanes 6 and 7: SEQ ID NO: 6A (5 and 10 nM, respectively); lanes 8 and 9: SEQ ID NO: 7A (5 and 10 nM, respectively); lanes 10 and 11: SEQ ID NO: 8A (5 and 10 nM, respectively); lanes 12 and 13: SEQ ID NO: 9A (5 and 10 nM, respectively)). Ponceau stain indicates equal load of total protein.

Figure 1B shows p21 ras protein expression in T24 cells transfected with 1, 2 and 3 nM compound (left panel: lanes 1, 11 and 12: 0 nM; lanes 2-4: SEQ ID NO: 4A (1, 2 and 3 nM, respectively); lanes 5-7: SEQ ID NO: 5A (1, 2 and 3 nM, respectively); lanes 8-10: SEQ ID NO: 6A (1, 2 and 3 nM, respectively) – right panel: lanes 1, 8 and 12: 0 nM; lanes 2-4: SEQ ID NO: 7A (1, 2 and 3 nM, respectively); lanes 5-7: SEQ ID NO: 8A (1, 2 and 3 nM, respectively); lanes 9-11: SEQ ID NO: 9A (1, 2 and 3 nM, respectively)). Ponceau stain indicates equal load of total protein.

Figure 1C shows p21 ras protein expression in mouse fibroblast cells transfected with 2 and 4 nM compound (upper panel: lanes 1 and 8: 0 nM; lanes 2 and 3: SEQ ID NO: 7A (2 and 4 nM, respectively); lanes 4 and 5: SEQ ID NO: 8A (2 and 4 nM, respectively); lanes 6 and 7: SEQ ID NO: 9A (2 and 4 nM, respectively); lane 9: SEQ ID NO: 11 (control; 4 nM) – lower panel: lanes 1 and 8: 0 nM; lanes 2 and 3: SEQ ID NO: 4A (2 and 4 nM, respectively); lanes 4 and 5: SEQ ID NO: 5A (2 and 4 nM, respectively); lanes 6 and 7: SEQ ID NO: 6A (2 and 4 nM, respectively); lane 9: SEQ ID NO: 11 (control; 4 nM)). Ponceau stain indicates equal load of total protein.

Figure 2A shows growth inhibition of MiaPaCa II cells transfected with SEQ ID NO: 7A at 0.5 (upper right), 1 (lower left) and 2 nM (lower right). No growth inhibition is observed using the control compound SEQ ID NO: 11A at 2 nM (upper left).

Figure 2B shows growth inhibition of T24 cells transfected with SEQ ID NO: 7A at 0.5 (upper right), 1 (lower left) and 2 nM (lower right). No growth inhibition is observed using the control compound SEQ ID NO: 11A at 2 nM (upper left).

Figure 2C shows growth inhibition of mouse fibroblast cells transfected with SEQ ID NO: 7A at 0.5 (upper right), 1 (lower left) and 2 nM (lower right). No growth inhibition is observed using the control compound SEQ ID NO: 11A at 2 nM (upper left).

Figure 3A shows inhibition of growth in vivo upon treatment with SEQ ID NO: 7A (3142) in mice bearing MiaPaCa II xenograft tumours compared to the control compound SEQ ID NO: 10A (3215).

Figure 3B shows inhibition of growth in vivo upon treatment with SEQ ID NO: 5A (3140) in mice bearing MiaPaCa II xenograft tumours compared to the control compound SEQ ID NO: 10A (3215).

Figure 3C shows mouse body temperature during treatment with SEQ ID NO: 5A (3140), SEQ ID NO: 7A (3142) and the control compound SEQ ID NO: 11A (2744).

Figure 3D shows mouse Body weight 3 days and 6 days after start treatment with the indicated dosage of Pan Ras SEQ ID NO: 7A (3142), mismatch control SEQ ID NO: 10A (3215) and saline treated nude mice (n=5 per group).

Figure 4 shows position of the targeted sequences in the Ras family. Compared are the sequences of H-Ras, K-Ras and N-Ras and within the sequences the targeted regions are indicated. Mismatches with the LNA oligonucleotides are indicated.

Figure 5 shows effect of SEQ ID NO: 7A on cell growth in the indicated cell lines as measured with the MTT assay.

Figure 6. Appearance of MiaPaca T24 and Fibroblast cells 3 days in culture after transfection with lipofectamin 2000 only (control; upper panels), transfection using lipofectamin 2000 with 5 nM mismatch SEQ ID NO: 10A (mismatch LNA; middle panels) or Pan Ras SEQ ID NO: 7A (Pan Ras LNA; lower panels). Westernblot showing knockdown of Pan Ras levels in control treated and 1-5 nM (SEQ ID NO: 7A) transfected fibroblasts is shown in the lower panel.

Figure 7. Appearance Fibroblast cells in culture 3 days after nucleofection with 5 nM mismatch SEQ ID NO: 10A (right panel) and Pan Ras SEQ ID NO: 7A (left panel).

Figure 8. Western blot showing Pan Ras knockdown 1 and 2 days after nucleofection of control (C = SEQ ID NO: 10A; lanes 1 and 3) and Pan Ras SEQ ID NO: 7A (lanes 2 and 4).

Figure 9A. Western blots showing the effect of Pan Ras knockdown using the indicated amounts of SEQ ID NO: 7A (lanes 2, 3 and 4 (1, 2 and 4 nM, respectively)) on ERK phosphorylation in 15PC3 cells after 15 minutes of insulin (100nM) treatment.

Figure 9B. Western blots showing the effect of Pan Ras knockdown in mouse fibroblasts transfected with SEQ ID NO: 7A (lanes 2, 5, 8 and 11) on Erk phosphorylation compared to mismatch controls (SEQ ID NO: 10A (lanes 3, 6, 9 and 12) or 0 nM (lanes 1, 4, 7 and 10)).

Figure 9C. Western Blots showing the effect of Pan Ras knockdown using SEQ ID NO: 7A (lanes 2, 3, 4 and 5; 0.5, 1, 2 and 3 nM, respectively) on Bcl-2 and P53 levels in T24 cells.

Figure 10. Quantification of Pan Ras protein levels was determined by Western-immuno blot in tumor lysates from xenografts after the treatment with Pan Ras SEQ ID NO: 7A at a dose of 2 mg/kg.

Figure 11. Quantification of Pan Ras protein levels in liver samples after the treatment with Pan Ras SEQ ID NO: 7A at a 2 mg/kg dose, as determined on Western-immuno blot.

Figure 12. ASAT levels in serum after 8 days of treatment with SEQ ID NO: 7A (bars 2, 3 and 4; 0.5, 1 and 2 mg/kg, respectively), saline (bar 1) and a mismatch control SEQ ID NO: 10A (bar 2; 2 mg/kg). The ASAT levels are indicated as Units/liter at 37°C (n=5 per group).

DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras. The modulation is ultimately a change in the amount of *ras* produced. In one preferred embodiment this is accomplished by providing antisense compounds, which hybridise with nucleic acids encoding Ha-ras, Ki-ras and N-ras. The modulation is preferably an inhibition of the expression of Ha-ras, Ki-ras and N-ras, which leads to a decrease in the number of functional proteins produced.

Preferred subsequences are shown in Table 1:

The compound of the invention may comprise one of the sequences from SET 1, such as two of the sequences from SET 1, including all three sequences from SET 1.

The compound of the invention may comprise at least one of the sequences from SET 2, such as two of the sequences from SET 2, including all three sequences from SET 2.

The compound of the invention may comprise at least one of the sequences from SET 3, including 2, 3, 4, 5, 6, 7 or all 8 sequences from SET 3.

The compound of the invention may comprise at least one of the sequences from SET 3, including 2, 3, 4, 5 or all 6 sequences from SET 4.

The compound of the invention may comprise one or both of the sequences from SET 2.

The compound of the invention may comprise at least one of the sequences from SET 6, including 2, 3, 4, 5, 6, 7, 8, 9 or all 10 sequences from SET 1.

The compound may further comprise a 5' flanking nucleobase sequence, or a 3' flanking sequence, or both a 5' and 3' flanking sequence which is/are contiguous to said subsequence.

SET1:	SET 3	SET 5
AGCTG*AT	(*) (G) (A) (A) TCCTCTAT	C*CATGTA
TTCTG*AT	(*) (G) (A) (A) ATCCTCTA (T)	GATGTA*T (G) (G) (T) (C)
AT*AGCTG	(*) (G) (A) AATCCTCT (A) (T)	SEQ ID NOS: 36 & 37.
SEQ ID	(*) GAATCCTC (T) (A) (T)	
NOS: 15,	(A) *GAATCCT (C) (T) (A) (T)	
16 & 17.	(T) A*GAATCC (T) (C) (T) (A) (T)	
	(G) TA*GAATC (C) (T) (C) (T) (A) (T)	
	(*) GTA*GAAT (C) (C) (T) (C) (T) (A) (T)	
	SEQ ID NOS: 21, 22, 23, 24, 25,	
	26, 27 & 28.	
SET2:	SET 4	SET 6
TC*ACAAA	(C) (T) TACTC*TC	(G) (G) (C) (A) AA*ACACA
TC*TATTC	(T) GTACTC*T	(G) (G) (C) AAA*ACAC (A)
TATTC*TC	TGTACTC*	(G) (G) CAAA*ACA (C) (A)
SEQ ID	CTGTACTC	(G) GCAAA*AC (A) (C) (A)
NOS: 18,	*CTGTACT (C)	GGCAAA*A (C) (A) (C) (A)
19 & 20.	(G) C*CTGTAC	TGGCAAA* (A) (C) (A) (C) (A)
	GC*CTGTA	ATGGCAAA (*) (A) (C) (A) (C) (A)
	SEQ ID NOS: 29, 30, 21, 32, 33,	*ATGGCAA (A) (*) (A) (C) (A) (C) (A)
	34 & 35.	T*ATGGCA (A) (A) (*) (A) (C) (A) (C) (A)
		TT*ATGGC (A) (A) (A) (*) (A) (C) (A) (C) (T)
		SEQ ID NOS: 38, 39, 40, 41, 42, 43,
		44, 45, 46 & 47

Table 1. "*" designates a nucleotide of choice (e.g. A, T, C, G) and "()" designates an optional nucleotide. The subsequences are complementary sequences to those identified from the alignment of three members of the human pRas family as shown in Figure 4. The above sequences are listed as SEQ ID NOS: 15-47, along with preferred pairs of nucleotides of choice (using official IUPAC-IUB single letter codes). Preferably the oligomeric compound of the invention comprises at least one of the above sequences.

The flanking sequence or sequences may consist of a total of between 2 and 22 nucleobase units, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleobases, or such as between 4 to 12 nucleobases or such as between 2 and 10 nucleobases, such as between 5 to 10 nucleobases, or between 5 and 8 nucleobases, such as between 7 to 9 nucleobases.

In one embodiment said flanking sequence comprises of at least 2 nucleobase units which are 5' to said sub-sequence .

In one embodiment said flanking sequence comprises between 1 and 6 nucleobase units which are 5' to said sub-sequence .

In one embodiment said flanking sequence comprises of at least 2 nucleobase units which are 3' to said sub-sequence .

In one embodiment said flanking sequence comprises between 1 and 6 nucleobase units which are 3' to said sub-sequence

It is preferred that the sequences of each of the flanking sequences, independantly form a contiguous sequence.

The combined contiguous nucleobase sequence, *i.e.* consisting of said subsequence and, if present, said flanking sequence or sequences, is at least at least 80% homologous to the corresponding sequences of each of said polynucleotides, such as the target sequences as referred to herein, which encode said mammalian p21 ras family members, such as at least 81%, such as at least 82%, such as at least 83%, such as at least 84%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94% or at least 95% homologous to the corresponding sequences of each of said polynucleotides.

In one embodiment, the 3' flanking sequence and/or 5' flanking sequence may, independently, comprise or consist of between 1 and 10 nucleobases, such as 2, 3, 4, 5, 6, 7, 8, or 9 nucleobases, such as between 2 and 6 nucleobases, such as 3 or 4 nucleobases, which may be, in one embodiment nucleotide analogues, such as LNA nucleobases, or in another embodiment a combination of nucleotides and nucleotide analogues.

Preferably the compound of the invention modulates the expression of a G-protein, preferably p21ras, such as mammalian p21ras, most preferably human p21 ras, such as human Ha-ras, Ki-ras and/pr N-ras

Preferably the compound of the invention modulates the expression of ras selected from one or more of the following: Ha-ras, Ki-ras and/or N-ras.

Preferably the compound of the invention is sufficiently complementary to hybridise with the nucleic acids encoding at least two members, such as at least three, or three members, of the p21 ras family and inhibits the expression of the at least two members, such as at least three members, such as three members of the p21 ras family.

The at least two, such as at least three or three members of the p21 ras family are all from the same mammalian species, such as human, or mouse or rat, preferably human.

It is highly preferable that the compound of the invention is an RNA antagonist, such as an antisense oligonucleotide.

It will be recognised that the compound of the invention which consists of a contiguous sequence of nucleobases (*i.e.* a nucleobase sequence), may comprise further non-nucleobase components, such as the conjugates herein defined.

Preferably the compound according to the invention is an antisense oligonucleotide.

In one embodiment, alternatively the oligomeric compound according to the invention may be an RNA antagonist selected from the group consisting of: an oligozyme, a siRNA, an siLNA, an aptamer, a decoy, a ribozyme.

Preferred compounds from SET 1:

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 15, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 16, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 17, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 12 or equivalent nucleobase sequence, such as SEQ ID NO: 12A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 4 or equivalent nucleobase sequence, such as SEQ ID NO: 4A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 71 or equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 72 or equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 73 or equivalent nucleobase sequence.

Preferred compounds from SET 2

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 18, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 19, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 20 or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide consisting or comprising of SEQ ID NO: 5 or an equivalent nucleobase sequence, such as SEQ ID No 5A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 74, or an equivalent nucleobase sequence

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 75, or an equivalent nucleobase sequence

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 76, or an equivalent nucleobase sequence

Preferred compounds from SET 3

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 21, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 22, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 23, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 24, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 25, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 26 , or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 27, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 28, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 6, or an equivalent nucleobase sequence, such as SEQ ID NO: 6A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 77, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 78, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 79, or an equivalent nucleobase sequence.

Preferred compounds from SET 4

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 29, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 30, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 31, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 32, , or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 33, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 34, , or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 35, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 7, or an equivalent nucleobase sequence, such as SEQ ID NO 7A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 13, or an equivalent nucleobase sequence, such as SEQ ID NO 13A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 80, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 81, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 82, or an equivalent nucleobase sequence.

Preferred compounds from SET 5

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 36, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide comprising of SEQ ID NO: 37, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 8, or an equivalent nucleobase sequence, such as SEQ ID No 8A

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 83, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 84, or an equivalent nucleobase sequence.

Preferred compounds from SET 6

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 38, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 39, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 40, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 41, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 42, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 43, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 44, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 45, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 46, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 47, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 9, or an equivalent nucleobase sequence, such as SEQ ID NO: 9A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 14, or an equivalent nucleobase sequence, such as SEQ ID NO: 14A.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 85, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 86, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 87, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 88, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 89, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 90, or an equivalent nucleobase sequence.

In one embodiment the present invention provides for an oligonucleotide compound, such as an antisense oligonucleotide, consisting or comprising of SEQ ID NO: 91, or an equivalent nucleobase sequence.

Particularly preferred compounds are antisense oligonucleotides comprising from about 10 to about 30 nucleobases, or from 12 to 25 nucleobases and most preferably are antisense compounds comprising 13-18 nucleobases such as 14, 15, 16 or 17 nucleobases. In one embodiment it is preferred that the compound of the invention comprises less than 20 nucleobases.

Other preferred oligonucleotides include sequences of 14, 15 and 16 continuous nucleobases selected from a sequence from the group consisting of: SEQ ID No 4, 5, 6, 7, 8 and 9.

In one embodiment, the compound of the invention consists of a nucleobase sequence with is 100% complementary to a corresponding region of a nucleic acid target sequence, such as a mRNA sequence, of at least one of the mammalian p21 family member.

The compound of the invention, preferably does not comprise more than four, such as not more than three, such as not more than two, such as not more than one mismatch with the corresponding nucleic acid sequences which encode the at least two, such as at least three, or three mammalian p21 ras family members.

In one embodiment, the compound of the invention is 100% complementary to a corresponding region of a nucleic acid target sequence, such as a mRNA sequence corresponding to either Ha-ras, Ki-ras or N-ras, but may also comprise between one and three, or one and four, mismatches to the complement of the a nucleic acid target sequence, such as a mRNA sequence of the other family members, such as one or two of the other family members (Ha-ras, Ki-ras or N-ras respectfully), such as one mismatch, two mismatches, three mismatches or four mismatches. In another embodiment, the compound comprises between one and four mismatches such as one mismatch, two mismatches, three mismatches or four mismatches to the complement of all three family members Ha-ras, Ki-ras or N-ras.

When the subsequence consists of 8 or 9 nucleobases, it may comprise only one mismatch with the corresponding nucleic acid sequences which encode the at least two, such as at least three, or three mammalian p21 ras family members. However, for longer subsequences of at least 10, such as at least 11 nucleobases, such as at least 12, at least 13, at least 14 or at least 15 nucleobases, additional mismatches may be introduced, such as a total of one, two, three or four mismatches with the corresponding nucleic acid sequences which encode the at least two, such as at least three, or three mammalian p21 ras family members, may be introduced into the subsequence. However, in regards to longer subsequences of at least 10 nucleobases, as listed above, the subsequence must comprise at least a core contiguous sequence of at least 8 nucleobases, wherein within the core contiguous sequence, at most, only one mismatch with the corresponding nucleic acid sequences which encode the at least two, such as at least three, or three mammalian p21 ras family members is allowed.

In an embodiment, the compound of the invention comprises 1 mismatch against one of the nucleic acid target sequences, such as a mRNA sequence, of Ha-ras, Ki-ras or N-ras, and 2 mismatches against one of the nucleic acid target sequences, such as a mRNA sequence of, Ha-ras, Ki-ras or N-ras, and is 100% complementary against the nucleic acid target sequence, such as a mRNA sequence of, one of Ha-ras, Ki-ras or N-ras.

In one embodiment, the compound of the invention comprises 1 mismatch against two of the nucleic acid target sequences, such as a mRNA sequence of Ha-ras, Ki-ras or N-ras, and is 100% complementary against one of the nucleic acid target sequence, such as a mRNA sequence, of Ha-ras, Ki-ras or N-ras.

In one embodiment, the compound of the invention comprises 1 mismatch against the nucleic acid target sequence, such as a mRNA sequence, of H-ras, and has two mismatches against the nucleic acid target sequence, such as a mRNA sequence, of K-ras, and is preferably 100% complementary against the nucleic acid target sequence, such as a mRNA sequence, of N-ras.

In one embodiment, the compound has two mismatches against the nucleic acid target sequence, such as a mRNA sequence, of K-ras, and has 1 mismatch against the nucleic acid target sequence, such as a mRNA sequence, of N-ras, and is preferably 100% complementary against the nucleic acid target sequence, such as a mRNA sequence, of H-ras.

In one embodiment, the compound of the invention has 1 mismatch against the nucleic acid target sequence, such as a mRNA sequence, of H-ras and has 1 mismatch against the nucleic acid target sequence, such as a mRNA sequence, of K-ras, and is preferably 100% complementary against the nucleic acid target sequence, such as a mRNA sequence, of N-ras.

In an embodiment, the compound is 100% complementary to either the nucleic acid target sequence, such as a mRNA sequence, of Ha-ras, Ki-ras or N-ras and at least 75%, 80%, 85%, 86%, $86\frac{2}{3}\%$, 87%, 87.5%, 88%, 89%, 90%, 91%, 92%, 93%, $93\frac{1}{3}\%$, 93.75%, 94%, 95%, 96% or at least 97% complementary to the the nucleic acid target sequences, such as a mRNA sequences, of the other family members. For example, sequence ID NO 4, 5, 6, 8 and 9 are 100% complementary to one family member and are 87-94% complementary to the two other members.

In one embodiment, the compound is at least 90%, 91%, 92%, 93%, $93\frac{1}{3}\%$, 93.75%, 94%, 95%, 96% or at least 97% complementary to the nucleic acid target sequence, such as a mRNA sequence, of all three family members Ha-ras, Ki-ras or N-ras.

In one embodiment, the compound is $93\frac{1}{3}\%$, or 93.75% complementary to the nucleic acid target sequence, such as an mRNA sequence, of one or more of Ha-ras, Ki-ras or N-ras.

In one embodiment, the compound is $86\frac{2}{3}\%$ or 87.5% complementary to the nucleic acid target sequence, such as an mRNA sequence, of one or more of Ha-ras, Ki-ras or N-ras.

The compounds of the invention, which modulate expression of the target, may be identified through experimentation or through rational design based on sequence information on the target and know-how on how best to design an oligomeric compound against a desired target. The sequences of these compounds are preferred embodiments of the invention. Likewise, the sequence motifs in the target to which these preferred oligomeric compounds are complementary (referred to as "hot spots") are preferred sites for targeting.

In one specific embodiment, the compound according to the invention is a double stranded oligonucleotide, wherein each strand comprises (or consists of) a total of 10-30 nucleotides and/or nucleotide analogues. It should be understood that the one strand of the double-stranded complex (oligonucleotide) corresponds to the oligonucleotide compound defined herein, and that the other strand is an oligonucleotide having a complementary sequence. However, in an alternative embodiment, the compound of the invention is not double stranded.

In one embodiment, the compound of the invention does not comprise RNA.

In one embodiment, the compound of the invention may comprise both a polynucleotide region, *i.e.* a nucleobase region, and a further non-nucleobase region. When referring to the compound of the invention consisting of a nucleobase sequence, the compound may comprise non-nucleobase components, such as a conjugate component.

Alternatively, the compound of the invention may consist entirely of a nucleobase region.

In one embodiment the nucleobase portion and/or subsequence is selected from at least 9, least 10, least 11, least 12, least 13, least 14 and least 15 consecutive nucleotides or nucleotide analogues, which preferably are complementary to the target nucleic acid(s), although, as described above, may comprise one or two mismatches, with either one, two or

three of the target nucleic acids – and may therefore have differing degrees of complementarity against different target nucleic acids.

In one embodiment, the compound according to the invention consists of no more than 22 nucleobases, such as no more than 20 nucleobases, such as no more than 18 nucleobases, such as 15, 16 or 17 nucleobases, optionally conjugated with one or more non-nucleobase entity.

In one embodiment the nucleobase portion is selected from, or comprises, one or more of the following sequences: SEQ ID NO: 15-47 (or a sequence comprising equivalent nucleobases).

The one preferred embodiment, the compound may target a target nucleic acid which is an RNA transcript(s) of the gene(s) encoding the target proteins, such as mRNA or premRNA, and may be in the form of a compound selected from the group consisting of; antisense inhibitors, antisense oligonucleotides, siRNA, miRNA, ribozymes and oligozymes.

Referring to the principles by which the compound, such as the LNA oligonucleotide, can elicit its therapeutic action, the target of the present invention may be the mRNA or the protein derived from (i.e. corresponding to) the the Ha- ras, Ki-ras and N-ras genes, In the most preferred embodiment the oligomeric compound is designed as an antisense inhibitor directed against the Ha-ras, Ki-ras and N-ras pre-mRNA or Ha-ras, Ki-ras and N-ras mRNA. The oligonucleotides may hybridize to any site along the Ha-ras, Ki-ras and N-ras pre-mRNA or mRNA such as sites in the 5' untranslated leader, exons, introns and 3' untranslated tail. However, if preferred that the oligonucleotides hybridise to the mature mRNA form of the target nucleic acid.

In a preferred embodiment, the compound hybridizes to a portion of the human Ha-ras, Ki-ras and N-ras pre-mRNA or mRNA that comprises the translation-initiation site. More preferably, the Ha-ras, Ki-ras and N-ras oligonucleotide comprises a CAT sequence, which is complementary to the AUG initiation sequence of the Ha-ras, Ki-ras and N-ras pre-mRNA or RNA. In another embodiment, the Ha-ras, Ki-ras and N-ras oligonucleotide hybridizes to a portion of the splice donor site of the human Ha-ras, Ki-ras and N-ras pre-mRNA. In yet another embodiment, Ha-ras, Ki-ras and N-ras oligonucleotide hybridizes to a portion of the splice acceptor site of the human Ha-ras, Ki-ras and N-ras pre-mRNA. In another embodiment, the Ha-ras, Ki-ras and N-ras oligonucleotide hybridizes to portions of the human Ha-ras, Ki-ras and N-ras pre-mRNA or mRNA involved in polyadenylation, transport or degradation.

In many cases the identification of an oligomeric compound, such as an LNA oligonucleotide, effective in modulating ras activity *in vivo* or clinically is based on sequence information on the target gene. However, one of ordinary skill in the art will appreciate that such oligomeric compounds can also be identified by empirical testing. As such p21 Ras oligomeric compounds having, for example, less sequence homology, greater or fewer modified nucleotides, or longer or shorter lengths, compared to those of the preferred embodiments, but which nevertheless demonstrate responses in clinical treatments, are also within the scope of the invention. The Examples provide suitable methods for performing empirical testing.

In one embodiment, the compound may target the protein encoding by the target nucleic acid and may be in the form of an oligomer compound selected from the group consisting of; aptamer, spiegelmer and decoy.

In one embodiment, the compound may target the target gene DNA, and may take the form of, for example, "Chimeraplasts" and "TFOs".

Preferred compounds of the invention are shown in table 2 and 3.

It will be recognised that when referring to a preferred nucleotide sequence motif or nucleotide sequence, which consists of only nucleotides, the compounds of the invention which are defined by that sequence may comprise a corresponding nucleotide analogues in place of one or more of the nucleotides present in said sequence, such as LNA nucleobases or other nucleotide analogues which raise the T_m of the oligonucleotide/target duplex.

When designed as an antisense inhibitor, for example, the oligonucleotides of the invention bind to the target nucleic acid and modulate the expression of its cognate protein. Preferably, such modulation produces an inhibition of expression of at least 10% or 20% compared to the normal expression level, more preferably at least a 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% inhibition compared to the normal expression level. In the same of a different embodiment, the inhibition of expression is less than 100%, such as less than 98% inhibition, less than 95% inhibition, less than 90% inhibition, less than 80% inhibition, such as less than 70% inhibition. In the same of a different embodiment, the inhibition of expression is sufficient to cause the death of cancerous cells, whilst allowing non-cancerous cells to survive. Modulation of expression level is determined by measuring protein levels, e.g. by the methods herein described such as SDS-PAGE followed by western blotting using suitable antibodies raised against the target protein. Alternatively, modulation of expression levels can be determined by measuring levels of mRNA, *eg.* by one of the methods herein described, such as northern blotting or quantitative RT-PCR.

Compounds according to the invention, are, in one embodiment, those consisting or comprising a sequence selected from SEQ ID NOS: 4, 5, 6, 7, 8, 9, 12, 13 and/or 14, such as SEQ ID NOS: 71-91, wherein, in one embodiment the nucleotides present in the compound may be substituted with a corresponding nucleotide analogue and, wherein said compound may comprise one, two or three mismatches against said selected sequence.

Preferred compounds according to the invention are those consisting or comprising of SEQ ID NOS: 4, 5, 6, 7, 8, 9, 12, 13 and/or 14 wherein they contain at least one nucleic acid analogue, wherein in one embodiment, the LNA nucleobases may be substituted with an alternative corresponding nucleotide analogue, and wherein said compound may comprise one, two, or three mismatches against said selected sequence. Nucleotide analogues which increase the T_m of the oligonucleotide/target nucleic acid target, as compared to the equivalent nucleotide are preferred.

Further preferred aspect of the invention is directed to compounds consisting or comprising of SEQ ID NOs: 3A, 4A, 5A, 6A, 7A, 8A, 9A, 12A, 13A and/or 14A,

Preferably, the compound according to the invention comprises at least one nucleotide analogue, such as Locked Nucleic Acid (LNA) unit, such as 4, 5, 6, 7, 8, 9, or 10 nucleotide analogues, such as Locked Nucleic Acid (LNA) units, preferably between 4 to 9 nucleotide analogues, such as LNA units, such as 6-9 nucleotide analogues, such as LNA units, most preferably 6, 7 or 8 nucleotide analogues, such as LNA units.

The term LNA is used as herein defined. Preferably the LNA units comprise at least one beta-D-oxy-LNA unit(s) such as 4, 5, 6, 7, 8, 9, or 10 beta-D-oxy-LNA units. The compound of the invention, such as the antisense oligonucleotide, may comprise more than one type of LNA unit. Suitably, the compound may comprise both beta-D-oxy-LNA, and one or more of the following LNA units: thio-LNA, amino-LNA, oxy-LNA, ena-LNA and/or alpha-LNA in either the D-beta or L-alpha configurations or combinations thereof.

Preferably, the compound, such as an antisense oligonucleotide, may comprise both nucleotide analogues, such as LNA units, and DNA units. Preferably the combined total of nucleobases, such as, LNA and DNA units, is between 14-20, such as between 15-18, more preferably 16 or 17 nucleobase units. Preferably the ratio of nucleotide analogues to DNA present in the oligomeric compound of the invention is between 0.5 and 1, more preferably between 0.6 and 0.9, such as between 0.7 and 0.8.

Preferably, the compound of the invention, such as an antisense oligonucleotide, consists of a total of 12-25 nucleotides and/or nucleotide analogues, wherein said compound comprises a

subsequence of at least 8 nucleotides or nucleotide analogues, said subsequence being located within (*i.e.* corresponding to) a sequence selected from the group consisting of SEQ ID NOS: 4, 5, 6, 7, 8, 9, 12, 13 and 14, such as a subsequence selected from any one of the group consisting of SEQ ID NOs 15-47.

In one aspect of the invention, the nucleotides (and/or nucleotide analogues) are linked to each other by means of a phosphorothioate group. An interesting embodiment of the invention is directed to compounds of SEQ NOS: 4, 5, 6, 7, 8, 9, 12, 13 and/or 14, wherein each linkage group within each compound is a phosphorothioate group. Such modifications are denoted by the subscript S.

In further embodiments, the compound of the invention, such as the antisense oligonucleotide of the invention may comprises or consist of 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleobases.

Preferably the compound according to the invention, such as an antisense oligonucleotide, comprises or consists of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 nucleotide analogues, such as LNA units, in particular 4, 5, 6, 7, 8, 9 or 10 nucleotide analogues, such as LNA units, such as between 1 and 10 nucleotide analogues, such as LNA units such as between 2 and 8 nucleotide analogues such as LNA units.

It is preferable that said subsequence or combined nucleobase sequence comprises a continuous sequence of at least 7 nucleobase residues, such as at least 8 or at least 9 nucleobase residues, including 7, 8 or 9 nucleobases, which, when in formed in a duplex with the complementary target RNA (such as a target nucleic acid) corresponding to each of said polynucleotides which encode said mammalian p21 ras family members, are capable of recruiting RNaseH, such as DNA nucleotides.

EP 1 222 309 provides in vivo methods for determining RNaseH activity, which may be used to determine the ability to recruit RNaseH. A compound is deemed capable of recruiting RNase H if, when provided with the complementary RNA target, it has an initial rate, as measured in pmol/l/min, of at least 1 %, such as at least 5%, such as at least 10% or less than 20% of the equivalent DNA only oligonucleotide, with no 2' substitutions, with phosphorothioate linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309. .

A compound is deemed essentially incapable of recruiting RNaseH if, when provided with the complementary RNA target, and RNaseH, the RNaseH initial rate, as measured in pmol/l/min, is less than 1%, such as less than 5%, such as less than 10% or less than 20% of the initial rate determined using the equivalent DNA only oligonucleotide, with no 2' substitutions, with phosphiothiote linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309.

However, it is also recognised that antisense oligonucleotides may function via non RNaseH mediated degradation of target mRNA, such as by steric hindrance of translation.

The compound of the invention may comprise a nucleobase sequence which comprises both nucleotides and nucleotide analogues, and may be in the form of a gapmer, a headmer or a mixmer.

A headmer is defined by a contiguous stretch of nucleotide analogues at the 5'-end followed by a contiguous stretch of DNA or modified nucleobases units recognizable and cleavable by the RNaseH towards the 3'-end (such as at least 7 such nucleobases), and a tailmer is defined by a contiguous stretch of DNA or modified monomers recognizable and cleavable by the RNaseH at the 5'-end (such as at least 7 such nucleobases), followed by a contiguous stretch of nucleotide analogues towards the 3'-end. Other chimeras according to the invention, called mixmers consisting of an alternate composition of DNA or modified monomers recognizable and cleavable by RNaseH and nucleotide analogues. Some nucleotide analogues may also be able to mediate RNaseH binding and cleavage. Since α -L-LNA recruits RNaseH activity to a certain extent, smaller gaps of DNA or modified monomers recognizable and cleavable by the RNaseH for the gapmer construct might be required, and more flexibility in the mixmer construction might be introduced.

Preferably, the compound of the invention is an antisense oligonucleotide which is a gapmer.

Preferably the gapmer comprises a polynucleotide sequence of formula (5' to 3'), A-B-C (and optionally D), wherein; A (5' region) consists or comprises of at least one nucleotide analogue, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, preferably between nucleotide analogues, such as 2-5 LNA units, most preferably 4 nucleotide analogues, such as LNA units and; B (central domain), preferably immediately 3' (*i.e.* contiguous) to A, consists or comprises at least one DNA sugar unit, such as 1-12 DNA units, preferably between 4-12 DNA units, more preferably between 6-10 DNA units, such as between 7-9 DNA units, most preferably 8 DNA units, and; C(3' region) preferably immediately 3' to B, consists or comprises of at least one nucleotide analogues, such as at least one LNA unit, such as between 1-6 nucleotide analogues, such as LNA units, preferably

between 2-5 nucleotide analogues, such as LNA units, most preferably 4 nucleotide analogues, such as LNA units. Preferred gapmer designs are disclosed in WO2004/046160.

Preferred gapmer designs include, when:

- A Consists of 3 or 4 consecutive nucleotide analogues
- B Consists of 8 or 9 consecutive DNA nucleotides or equivalent nucleobases which are capable of recruiting RNaseH
- C Consists of 3 or 4 consecutive nucleotide analogues
- D Consists, where present, of one DNA nucleotide.

Or when

- A Consists of 3 consecutive nucleotide analogues
- B Consists of 9 consecutive DNA nucleotides or equivalent nucleobases which are capable of recruiting RNaseH
- C Consists of 3 consecutive nucleotide analogues
- D Consists, where present, of one DNA nucleotide.

Or when

- A Consists of 4 consecutive nucleotide analogues
- B Consists of 8 consecutive DNA nucleotides or equivalent nucleobases which are capable of recruiting RNaseH
- C Consists of 4 consecutive nucleotide analogues
- D Consists, where present, of one DNA nucleotide.

The DNA nucleotides in the central domain (B) may be substituted with one or more, or even all the DNA nucleotides may be substituted with a nucleobase, including nucleotide analogues which are capable or recruiting RNase H.

In a gapmer oligonucleotide, it is highly preferable that any mismatches are not within the central domain (B) above, are at least within a minimum stretch of 7 continuous nucleobases of the central domain, such as 7, 8 or 9 continuous nucleobases, which preferably comprises or consists of DNA units.

In a gapmer oligonucleotide, it is preferred that any mismatches are located towards the 5' or 3' termini of the gapmer. Therefore, it is preferred that in a gapmer oligonucleotide which comprises mismatches with the target mRNA, that such mismatches are located either in 5'

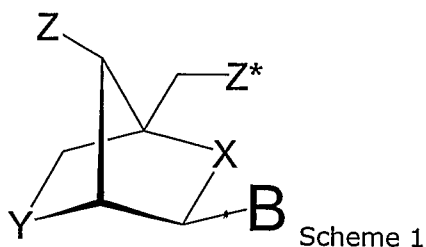
(A) and/or 3' (C) regions, and/or said mismatches are between the 5' or 3' nucleotide unit of said gapmer oligonucleotide and target molecule.

Preferably, the gapmer, of formula A-B-C, further comprises a further region, D, which consists or comprises, preferably consists, of one or more DNA sugar residue terminal of the 3' region (C) of the oligomeric compound, such as between one and three DNA sugar residues, including between 1 and 2 DNA sugar residues, most preferably 1 DNA sugar residue.

Preferably, within the compound according to the invention, such as an antisense oligonucleotide, which comprises LNA, all LNA C residues are 5'methyl-Cytosine.

Preferably the LNA units of the compound, such as an antisense oligonucleotide, of the invention are selected from one or more of the following: thio-LNA, amino-LNA, oxy-LNA, ena-LNA and/or alpha-LNA in either the D-beta or L-alpha configurations or combinations thereof. Beta-D-oxy-LNA is a preferred LNA for use in the oligomeric compounds of the invention. Thio-LNA may also be preferred for use in the oligomeric compounds of the invention. Amino-LNA may also be preferred for use in the oligomeric compounds of the invention. Oxy-LNA may also be preferred for use in the oligomeric compounds of the invention. Ena-LNA may also be preferred for use in the oligomeric compounds of the invention. Alpha-LNA may also be preferred for use in the oligomeric compounds of the invention.

The Locked Nucleic Acid (LNA) used in the compound, such as an antisense oligonucleotide, of the invention has the structure of the general formula



X and Y are independently selected among the groups -O-, -S-, -N(H)-, N(R)-, -CH₂- or -CH- (if part of a double bond), -CH₂-O-, -CH₂-S-, -CH₂-N(H)-, -CH₂-N(R)-, -CH₂-CH₂- or -CH₂-CH- (if part of a double bond), -CH=CH-, where R is selected from hydrogen and C₁₋₄-alkyl ; Z and Z* are independently selected among an internucleotide linkage, a terminal group or a

protecting group; B constitutes a natural or non-natural nucleobase; and the asymmetric groups may be found in either orientation.

Phosphorothioate linkages may be preferred, particularly in the gaps (B) of gapmer oligonucleotides. The regions A and B may comprise other linkages, or mixtures of different linkages – for example both phosphate and phosphorothioate linkages, or just phosphate linkages, or other linkages as disclosed herein.

The term "thio-LNA" comprises a locked nucleobase in which at least one of X or Y in Scheme 1 is selected from S or $-\text{CH}_2-\text{S}-$. Thio-LNA can be in both beta-D and alpha-L-configuration.

The term "amino-LNA" comprises a locked nucleobase in which at least one of X or Y in Scheme 1 $-\text{N}(\text{H})-$, $\text{N}(\text{R})-$, $\text{CH}_2-\text{N}(\text{H})-$, $-\text{CH}_2-\text{N}(\text{R})-$ where R is selected from hydrogen and C_{1-4} -alkyl. Amino-LNA can be in both beta-D and alpha-L-configuration.

The term "oxy-LNA" comprises a locked nucleotide in which at least one of X or Y in Scheme 2 represents $-\text{O}-$ or $-\text{CH}_2-\text{O}-$. Oxy-LNA can be in both beta-D and alpha-L-configuration.

The term "ena-LNA" comprises a locked nucleotide in which Y in Scheme 1 is $-\text{CH}_2-\text{O}-$ (where the (wherein the oxygen atom of $-\text{CH}_2-\text{O}-$ is attached to the 2-position relative to the nucleobase B).

The term "alpha-L-LNA" comprises a locked nucleotide represented as shown in Scheme 2 (structure to the right).

The term "LNA derivatives" comprises all locked nucleotide in Scheme 1 as well as beta-D-methylene LNA, e.g. thio-LNA, amino-LNA, alpha-L-oxy-LNA and ena-LNA, except beta-D-oxy-LNA.

The term 'locked nucleotide' refers to a 'locked nucleobase', and is not used in the same context as the term 'nucleotide' as defined herein.

In Scheme 1, the 4 chiral centers are shown in a fixed configuration. However, the configurations in Scheme 1 are not necessarily fixed. Also comprised in this invention are compounds of the general Scheme 1 in which the chiral centers are found in different configurations, such as those represented in Schemes 2 or 3. Thus, the intention in the illustration of Scheme 1 is not to limit the configuration of the chiral centre. Each chiral

center in Scheme 1 can exist in either R or S configuration. The definition of R (rectus) and S (sinister) are described in the IUPAC 1974 Recommendations, Section E, Fundamental Stereochemistry: The rules can be found in Pure Appl. Chem. 45, 13-30, (1976) and in "Nomenclature of organic Chemistry" pergamon, New York, 1979.

Z and Z* are independently selected among an internucleoside linkage, a terminal group or a protecting group

The internucleoside linkage may be selected from the group consisting of: $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-S-P(O)_2-O-$, $-S-P(O,S)-O-$, $-S-P(S)_2-O-$, $-O-P(O)_2-S-$, $-O-P(O,S)-S-$, $-S-P(O)_2-S-$, $-O-PO(R^H)-O-$, $O-PO(OCH_3)-O-$, $-O-PO(NR^H)-O-$, $-O-PO(OCH_2CH_2S-R)-O-$, $-O-PO(BH_3)-O-$, $-O-PO(NHR^H)-O-$, $-O-P(O)_2-NR^H-$, $-NR^H-P(O)_2-O-$, $-NR^H-CO-O-$, $-NR^H-CO-NR^H-$, and/or the internucleoside linkage may be selected from the group consisting of: $-O-CO-O-$, $-O-CO-NR^H-$, $-NR^H-CO-CH_2-$, $-O-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-$, $-CO-NR^H-CH_2-$, $-CH_2-NR^H-CO-$, $-O-CH_2-CH_2-S-$, $-S-CH_2-CH_2-O-$, $-S-CH_2-CH_2-S-$, $-CH_2-SO_2-CH_2-$, $-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-CO-$, $-CH_2-NCH_3-O-CH_2-$, where R^H is selected from hydrogen and C_{1-4} -alkyl,

Suitable sulphur (S) containing internucleoside linkages as provided above may be preferred.

The terminal groups are selected independently among from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, C_{1-6} -alkylthio, amino, Prot-N(R^H)-, Act-N(R^H)-, mono- or di(C_{1-6} -alkyl)amino, optionally substituted C_{1-6} -alkoxy, optionally substituted C_{1-6} -alkyl, optionally substituted C_{2-6} -alkenyl, optionally substituted C_{2-6} -alkenyloxy, optionally substituted C_{2-6} -alkynyl, optionally substituted C_{2-6} -alkynyloxy, monophosphate, monothiophosphate, diphosphate, dithiophosphate triphosphate, trithiophosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphonyl, hydroxymethyl, Prot-O-CH₂-, Act-O-CH₂-, aminomethyl, Prot-N(R^H)-CH₂-, Act-N(R^H)-CH₂-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH(R^H), respectively, Act is an activation group for -OH, -SH, and -NH(R^H), respectively, and R^H is selected from hydrogen and C_{1-6} -alkyl;

The protection groups of hydroxy substituents comprises substituted trityl, such as 4,4'-dimethoxytrityloxy (DMT), 4-monomethoxytrityloxy (MMT), and trityloxy, optionally substituted 9-(9-phenyl)xanthyloxy (pixyl), optionally substituted methoxytetrahydropyranyloxy (mthp), silyloxy such as trimethylsilyloxy (TMS), triisopropylsilyloxy (TIPS), *tert*-butyldimethylsilyloxy (TBDMS), triethylsilyloxy, and phenyldimethylsilyloxy, *tert*-butylethers,

acetals (including two hydroxy groups), acyloxy such as acetyl or halogen substituted acetyls, *e.g.* chloroacetyloxy or fluoroacetyloxy, isobutyryloxy, pivaloyloxy, benzoyloxy and substituted benzoyls, methoxymethyloxy (MOM), benzyl ethers or substituted benzyl ethers such as 2,6-dichlorobenzoyloxy (2,6-Cl₂Bzl). Alternatively when Z or Z* is hydroxyl they may be protected by attachment to a solid support optionally through a linker.

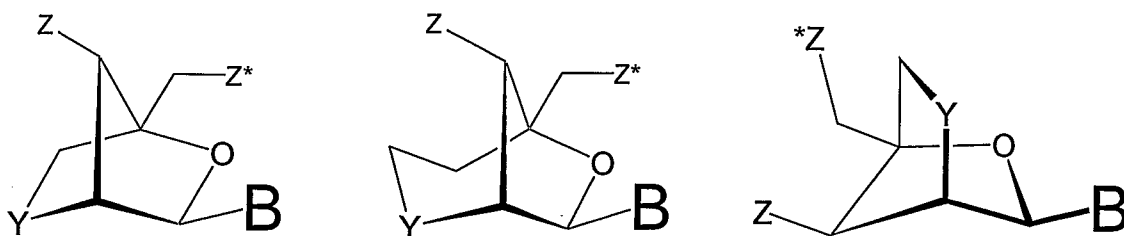
When Z or Z* is amino groups illustrative examples of the amino protection protections are fluorenylmethoxycarbonylamino (Fmoc), *tert*-butyloxycarbonylamino (BOC), trifluoroacetylamino, allyloxycarbonylamino (alloc, AOC), Z benzyloxycarbonylamino (Cbz), substituted benzyloxycarbonylamino such as 2-chloro benzyloxycarbonylamino (2-ClZ), monomethoxytritylamino (MMT), dimethoxytritylamino (DMT), phthaloylamino, and 9-(9-phenyl)xanthenylamino (pixyl).

In the embodiment above, Act designates an activation group for -OH, -SH, and -NH(R^H), respectively. Such activation groups are, *e.g.*, selected from optionally substituted O-phosphoramidite, optionally substituted O-phosphotriester, optionally substituted O-phosphordiester, optionally substituted H-phosphonate, and optionally substituted O-phosphonate.

In the present context, the term "phosphoramidite" means a group of the formula -P(OR^x)-N(R^y)₂, wherein R^x designates an optionally substituted alkyl group, *e.g.* methyl, 2-cyanoethyl, or benzyl, and each of R^y designate optionally substituted alkyl groups, *e.g.* ethyl or isopropyl, or the group -N(R^y)₂ forms a morpholino group (-N(CH₂CH₂)₂O). R^x preferably designates 2-cyanoethyl and the two R^y are preferably identical and designate isopropyl. Thus, an especially relevant phosphoramidite is N,N-diisopropyl-O-(2-cyanoethyl)-phosphoramidite.

B constitutes a natural or non-natural nucleobase and selected among adenine, cytosine, 5-methylcytosine, isocytosine, pseudoisocytosine, guanine, thymine, uracil, 5-bromouracil, 5-propynyluracil, 5-propynyl-6-fluoroluracil, 5-methylthiazoleuracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, 7-propyne-7-deazaadenine, 7-propyne-7-deazaguanine, and 2-chloro-6-aminopurine. In some embodiment, the Locked Nucleic Acid (LNA) used in the oligomeric compound of the invention, such as an antisense oligonucleotide, comprises the nucleobases which are modified nucleobases selected from the group consisting of 5-methylcytosine, isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, 2-chloro-6-aminopurine.

Preferably, the Locked Nucleic Acid (LNA) used in the oligomeric compound, such as an antisense oligonucleotide, of the invention comprises at least one nucleotide comprises a Locked Nucleic Acid (LNA) unit according any of the formulas



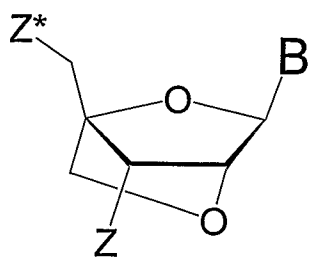
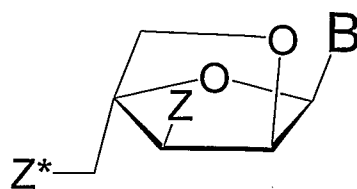
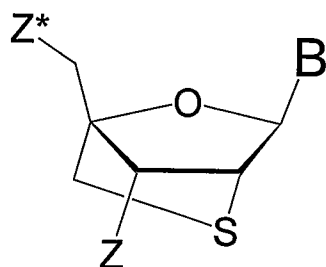
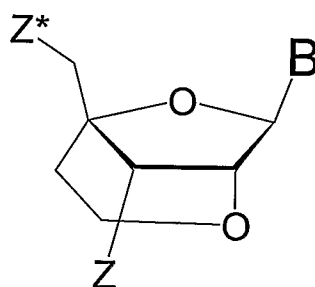
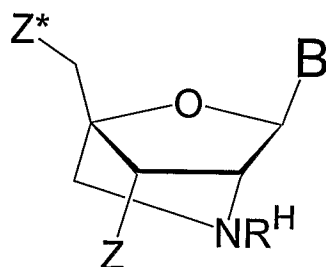
Scheme 2

wherein Y is -O-, -S-, -NH-, or N(R^H); Z and Z* are independently selected among an internucleoside linkage, a terminal group or a protecting group; and B constitutes a natural or non-natural nucleobase.

LNA monomers and their preparation are described in WO 99/14226 and subsequent applications, WO 00/56746, WO 00/56748, WO 00/66604, WO 00/125248, WO 02/28875, WO 2002/094250 and WO 2003/006475. One particular example of a thymidine LNA monomer is the (1S,3R, 4R, 7S)-7-hydroxy-1-hydroxymethyl-5-methyl-3-(thymine-1yl)-2,5-dioxabicyclo[2:2:1]heptane.

Specifically preferred LNA units are shown in Scheme 3 where B and Z* and Z are as previously defined, and wherein R^H is selected from hydrogen and C₁₋₄-alkyl.

In one embodiment, the nucleobase sequence of the compound of the invention consists or comprises of a sequence which is, or corresponds to, a sequence selected from the group consisting of: SEQ ID NOS: 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 88, 89, 90 and 91, or a contiguous sequence of at least 12, 13, 14, or 15 consecutive nucleobases present in said sequence, wherein the nucleotides or nucleobases present in the compound may be substituted with a corresponding nucleobase such as a nucleotide or nucleotide analogue and wherein said compound may comprise one, two, or three mismatches against said selected sequence.

 β -D-oxy-LNA α -L-Oxy-LNA β -D-thio-LNA β -D-ENA β -D-amino-LNA**Scheme 3**

It will be understood by the skilled person, that in one embodiment when referring to specific gapmer oligonucleotide sequences, such as those provided herein (e.g. SEQ ID NOS: 60, 61, 62, 63, 64, 65, 68, 69 and 70), when the linkages are phosphorothioate linkages, alternative linkages, such as those disclosed herein may be used, for example phosphate linkages may be used, particularly for linkages between nucleotide analogues, such as LNA, units.

Likewise, when referring to specific gapmer oligonucleotide sequences, such as those provided herein (e.g. SEQ ID NOS: 60, 61, 62, 63, 64, 65, 68, 69 and 70), when the C residues are annotated as 5'methyl modified cytosine, in one embodiment, one or more of the Cs present in the oligonucleotide may be unmodified C residues.

In one embodiment the nucleobase sequence consists or comprises of a sequence which is, or corresponds to, a sequence selected from the group consisting of: SEQ ID NOS: 4, 5, 6, 7, 8, 9, 12, 13 and 14, or a contiguous sequence of at least 12, 13, 14, 15, or 16 consecutive nucleobases present in said sequence, wherein the nucleotides present in the compound may be substituted with a corresponding nucleotide analogue and wherein said compound may comprise one, two, or three mismatches against said selected sequence.

The compound according claim 41, where the nucleobase sequence consists or comprises of a sequence selected from the group consisting of SEQ ID NOS: 60, 61, 62, 63, 64, 65, 68, 69 and 70, or a contiguous sequence of at least 12, 13, 14, 15, 16, or 17 consecutive nucleobases present in said sequence, wherein the LNA nucleobases may optionally be substituted with an alternative corresponding nucleotide analogue, and wherein said compound may optionally comprise one, two, or three mismatches against said selected sequence, and optionally, linkage groups other than phosphorothioate may be used.

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 4A.
(SEQ ID NO 60).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 5A.
(SEQ ID NO 61).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 6A.
(SEQ ID NO 62).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 7A.
(SEQ ID NO 63).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 8A.
(SEQ ID NO 64).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 9A. (SEQ ID NO 65).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 12A. (SEQ ID NO 68).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 13A. (SEQ ID NO 69).

Preferably the compound according to the invention consists or comprises of SEQ ID NO: 14A. (SEQ ID NO 70).

Method of identification and preparation of compounds of the invention:

The same features of the compound of the invention, as disclosed herein may be utilised in a method for identification of at least one compound capable of down-regulation, preferably simultaneous down-regulation, of at least two non-identical mammalian p21 Ras proteins in a human or mammalian cell. Typically, such a method comprises the sequential steps of:

- A Compare the polynucleotide sequences which, in a human or mammalian cell, encode said at least two, such as at least three or three on-identical mammalian p21 Ras proteins, such as the nucleic acid as referred to herein, including the target nucleic acid and natural variants thereof.
- B From the comparison in step A, identify a subsequence, as according to the subsequence as referred to herein in relation to the compound according to the invention,
- C Identify a complementary nucleobase sequence which consists of a total of 10-30 nucleobases, wherein the complementary nucleobase sequence comprises corresponds to the subsequence identified in step B.

Step B or C may, in one preferred embodiment, further comprise an additional step, wherein said additional step comprises the identification of either a 3' or a 5' flanking, or both a 5' and a 3' flanking sequence as referred to herein in relation to the compound according to the invention.

The compound, polynucleotide sequences, the mammalian p21 Ras protein, the target nucleic acids, the subsequence, the complementary nucleobase sequence, the flanking sequence(s), as referred to in the method of identifying/or preparing at least one compound, may therefore be as defined by the compound of the invention.

It is preferable that the comparison performed in step A is in the form of a sequence alignment. Preferably the comparison performed in step A is in the form of a sequence alignment. BLAST ALIGN and ClustalW algorithms using standard settings may be used for this purpose. ClustalW alignment is available at <http://www.ebi.ac.uk/clustalw/>. Other forms of sequence alignment include BLAST searches, which may be accessed using the NCBI web site. The comparison may also allow the preparation of a consensus sequence, which can be used to identify suitable sites from which to identify a corresponding nucleobase sequence, such as the subsequence and, optionally the flanking sequence(s), as according to the compound of the invention.

In one embodiment, the compound of the invention which is capable of targeting at least three, such as three members of the p21 ras family, may be identified by comparing only two nucleic acid sequences (polynucleotide sequences), for example by preparing or utilising a consensus sequence to two of the nucleic acids.

In one embodiment, due to the high degree of conservation of equivalent p21 ras nucleic acid sequences between some mammalian species, that the comparison is made between p21 nucleic acid sequences from different mammalian species, such as human, mouse and rat. However, it is preferable that the comparison is made from the nucleic acid sequences from the same species, preferably human. In one embodiment, the sequences from suitable animal models may also be compared during step A (or even step B), thereby allowing the selection of subsequence and optionally flanking sequences which, when combined, form a nucleobase sequence which will hybridize to the equivalent p21 ras targets in both humans and the model species. Suitable animal model species include mouse and rat.

The skilled person will appreciate that preferred oligonucleotides are those that hybridize to a portion of the Ha-ras, Ki-ras and N-ras pre-mRNA or mRNA whose sequence does not commonly occur in transcripts from unrelated genes so as to maintain treatment specificity. The method of identifying or preparing a compound according to the invention, may therefore comprise a further step, performed during or subsequent to step B and/or step C, which further step comprises comparing the subsequence or combined (contiguous) nucleobase sequence identified, to at least one non p21 ras genetic sequence present in the same mammal source (such as human), such a step could, for example, be performed using a BLAST search against the appropriate genome sequence, or experimentally, e.g. by determining the T_m of the oligonucleotide to the additional target nucleic acid – such as using the methods herein disclosed.

The oligomeric compound of the invention are designed to be sufficiently complementary to the target to provide the desired clinical response e.g. the oligomeric compound must bind

with sufficient strength and specificity to its target to give the desired effect. In one specific embodiment, said compound modulating p21 Ras may be designed so as to also modulate other specific nucleic acids which do not encode p21 Ras. This can, in one embodiment be determined or predicted by determining the T_m of the oligonucleotide to the additional target nucleic acid – such as using the methods herein disclosed.

It is preferred that the oligomeric compound according to the invention is designed so that intra- and intermolecular oligonucleotide hybridisation is avoided.

In one embodiment, step C further comprises selecting DNA nucleotides, or equivalent nucleobases which are capable of recruiting RNaseH for use in the region of the compound which corresponds to said sub-sequence.

In one embodiment, which may be the same or different as the above embodiment, step C further comprises selecting at least one nucleotide analogue for use the region of the compound which corresponds to said flanking region.

The invention also provides for a method for the preparation of a compound capable of down regulation, preferably concurrent or simultaneous down regulation, of at least two non-identical Ras proteins in a human or mammalian cell, comprising the sequential steps of A to C according to the above method, and a further step D, which comprises preparing the compound and a further optional step E, which comprises testing said compound prepared in step D to determine efficacy of concurrent or simultaneous down regulation of at least two non-identical Ras proteins in a human or mammalian cell.

The invention also provides a compound or compounds prepared by the above method of preparation of a compound.

Preferably, the complementary oligonucleotide compound prepared according to the above method is as defined in accordance to the compound according to the invention, such as antisense oligonucleotide according to the invention, e.g. the preferred form is an antisense oligonucleotide which comprises nucleotide analogues, wherein the preferred nucleotide analogues are LNA units, and the preferred form is a gapmer.

Within the context of the above method, it is recognised that numerous methods of identifying subsequences can be used, e.g. through experimentation or through rational design methods, such as preparation of a sequence alignment or a consensus sequence.

Preferably the comparison is between the following human or mammalian polynucleotide sequences: i) Ha-ras, Ki-ras and N-ras; ii) Ki-ras and N-ras; iii) Ki-ras and Ha-ras; iv) Ha-ras and N-ras. Preferred sequences of Ha-ras, Ki-ras and N-ras are herein disclosed as SEQ ID NOS: 1, 2 and 3, and preferred sequences include natural allelic variants thereof. It will be apparent that the sequences used for comparison may not be full length sequences, *i.e.* may not encode for the full mature polypeptide sequence, but may be fragments of such sequences, particularly of highly conserved regions which are conserved between different members of the p21ras family. It is also envisaged that the sequences used may, in one embodiment, comprise a limited number of substitutions from those sequences usually found in nature – for example they may comprise specific substitutions found in the polynucleotides in cancerous cells, or naturally occurring allelic variants of said sequences

The following embodiments apply to both the compound of the invention and the method of the invention:

In one embodiment, the at least two, such as at least three non-identical proteins (such as SEQ ID Nos 48-50, which disclose the human Ha-ran, K-ras and N-ras amino acid sequences) have at least 80% homology at the amino acid sequence level, such as at least 85% homology at the amino acid level. In the same embodiment, they preferably have at least 85% homology to SEQ ID NO 92.

Amino acid and polynucleotide homology may be determined using ClustalW algorithm using standard settings: see <http://www.ebi.ac.uk/emboss/align/index.html>, Method : EMBOSS::water (local): Gap Open = 10.0, Gap extend = 0.5, using Blosum 62 (protein), or DNAsfull for nucleotide sequences.

Such an alignment/homology determination may, in one embodiment, such as in the method for the identification or preparation of a compound, may not include the entire length of the sequences, but may be a local alignment method. Such methods are known in the art to be very useful for scanning databases or other circumstances to find matches between small regions of sequences. Such an alignment could therefore be between fragments of polypeptides of the target proteins or the nucleic acids which encode such polypeptides.

Members of the mammalian p21 ras family may be determined by amino acid homology to the p21 reference sequence (SEQ ID NO 92). Suitable members of the p21 ras family have at least 85%, such as at least 90% at the amino acid level when aligned to the consensus sequence (SEQ ID No 92) using the above defined ClustalW algorithm.

Alternatively, members of the mammalian p21 ras family may be determined by simply determining the proportion of amino acids they have in common to the consensus sequence (SEQ ID No 92). For example, human N-RAS has 172 out of 189 (91%), HaRAS: 177/189 (93%), Ki-RAS: 174/189 (92%). Therefore, in a preferred aspect, members of the mammalian p21 ras family are human proteins, which either have the suitable level of homology (see above paragraph) to SEQ ID No 92, or, alternatively, when aligned to SEQ ID No 92 (for example using a BLAST alignment), they have at least 85%, such as at least 90% or 91% of the amino acids present in SEQ ID No 92.

In one embodiment, the at least two, such as at least three or three (non-identical) members of the mammalian p21 ras family do not have more than 94%, such as do not have more than 95%, such as more than 96%, such as more than 97%, such as more than 98% or more than 99% homology with each other at the amino acid sequence level.

Preferably, the compound prepared in step c) modulates the expression of p21ras.

Preferably, the compound prepared in step c) modulates the expression of Ha-ras, Ki-ras and N-ras.

Conjugates

In one embodiment of the invention the oligomeric compound is linked to ligands/conjugates, which may be used, *e.g.* to increase the cellular uptake of antisense oligonucleotides. This conjugation can take place at the terminal positions 5'/3'-OH but the ligands may also take place at the sugars and/or the bases. In particular, the growth factor to which the antisense oligonucleotide may be conjugated, may comprise transferrin or folate. Transferrin-polylysine-oligonucleotide complexes or folate-polylysine-oligonucleotide complexes may be prepared for uptake by cells expressing high levels of transferrin or folate receptor. Other examples of conjugates/ligands are cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end-capping" with one or more nuclease-resistant linkage groups such as phosphoromonothioate, and the like.

The invention also provides for a conjugate comprising the compound according to the invention as herein described, and at least one non-nucleotide or non-polynucleotide moiety covalently attached to said compound. Therefore, in one embodiment where the compound of the invention consists of a specified nucleic acid, as herein disclosed, the compound may

also comprise at least one non-nucleotide or non-polynucleotide moiety (e.g. not comprising one or more nucleotides or nucleotide analogues) covalently attached to said compound.

Pharmaceutical Compositions:

The invention also provides for a pharmaceutical composition comprising a compound or a conjugate as herein described or a conjugate, and a pharmaceutically acceptable diluent, carrier or adjuvant.

The LNA containing oligomeric compounds of the present invention can be utilized for, for example, as research reagents for diagnostics, therapeutics and prophylaxis. In research, the antisense oligonucleotides may be used to specifically inhibit the synthesis of ras genes in cells and experimental animals thereby facilitating functional analysis of the target or an appraisal of its usefulness as a target for therapeutic intervention. In diagnostics the antisense oligonucleotides may be used to detect and quantitate ras 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 ras is treated by administering antisense compounds in accordance with this invention. Further provided are methods of treating an animal particular mouse and rat and treating a human, suspected of having or being prone to a disease or condition, associated with expression of ras by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

Preferably the pharmaceutical composition of the invention further comprises a pharmaceutically acceptable carrier.

Preferably, the compound of the invention is included in a unit formulation such as in a pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount without causing serious side effects in the treated patient.. However, in some forms of therapy, serious side effects may be acceptable in terms of ensuring a positive outcome to the therapeutic treatment.

The dosage of the pharmaceutical composition is dependent on severity and responsiveness of the disease state to be treated, and the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the

body of the patient. Optimum dosages may vary depending on the relative potency of individual oligonucleotides. Generally it can be estimated based on EC_{50} s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μ g to 1 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 10 years or by continuous infusion for hours up to several months. The repetition rates for dosing can be estimated based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state.

The formulated drug may comprise pharmaceutically acceptable binding agents and adjuvants. Capsules, tablets and pills etc. may contain for example the following compounds: microcrystalline cellulose, gum or gelatin as binders; starch or lactose as excipients; stearates as lubricants; various sweetening or flavouring agents. For capsules the dosage unit may contain a liquid carrier like fatty oils. Likewise coatings of sugar or enteric agents may be part of the dosage unit. The oligonucleotide formulations may also be emulsions of the active pharmaceutical ingredients and a lipid forming a micellular emulsion.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be (a) oral (b) pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, (c) topical including epidermal, transdermal, ophthalmic and to mucous membranes including vaginal and rectal delivery; or (d) parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. In one embodiment the active oligo is administered IV, IP, orally, topically or as a bolus injection or administered directly in to the target organ.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Compositions and formulations for oral administration include but is not restricted to powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents

and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Delivery of drug to tumour tissue may be enhanced by carrier-mediated delivery including, but not limited to, cationic liposomes, cyclodextrins, porphyrin derivatives, branched chain dendrimers, polyethylenimine polymers, nanoparticles and microspheres (Dass CR. J Pharm Pharmacol 2002; 54(1):3-27).

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels and suppositories. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances, which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

For parenteral, subcutaneous, intradermal or topical administration the formulation may include a sterile diluent, buffers, regulators of tonicity and antibacterials. The active compound may be prepared with carriers that protect against degradation or immediate elimination from the body, including implants or microcapsules with controlled release properties. For intravenous administration the preferred carriers are physiological saline or phosphate buffered saline.

An oligonucleotide of the invention may be mixed with any material that do not impair the desired action, or with material that supplement the desired action. These could include other drugs including other nucleoside compounds.

Optionally, the pharmaceutical according to the invention comprises therapeutic agents, such as further antisense compounds, chemotherapeutic compounds, anti-inflammatory compounds, antiviral compounds and/or immuno-modulating compounds. Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, antiviral drugs, and immuno-modulating drugs may also be combined in compositions of the invention.

Two or more combined compounds may be used together or sequentially, *i.e.* the compound according to the invention may be used prior to, during or subsequent to one or more of the other therapeutic agents referred to herein.

Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, ibuprofen, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

Preferably, the pharmaceutical composition according to the invention further comprises at least one chemotherapeutic agent. Said chemotherapeutic agent is preferably selected from the group consisting of adrenocorticosteroids, such as prednisone, dexamethasone or decadron; altretamine (hexalen, hexamethylmelamine (HMM)); amifostine (ethyol); aminoglutethimide (cytadren); amsacrine (M-AMSA); anastrozole (arimidex); androgens, such as testosterone; asparaginase (elspar); bacillus calmette-gurin; bicalutamide (casodex); bleomycin (blenoxane); busulfan (myleran); carboplatin (paraplatin); carmustine (BCNU, BiCNU); chlorambucil (leukeran); chlorodeoxyadenosine (2-CDA, cladribine, leustatin); cisplatin (platinol); cytosine arabinoside (cytarabine); dacarbazine (DTIC); dactinomycin (actinomycin-D, cosmegen); daunorubicin (cerubidine); docetaxel (taxotere); doxorubicin (adriomycin); epirubicin; estramustine (emcyt); estrogens, such as diethylstilbestrol (DES); etoposide (VP-16, VePesid, etopophos); fludarabine (fludara); flutamide (eulexin); 5-FUDR (floxuridine); 5-fluorouracil (5-FU); gemcitabine (gemzar); goserelin (zodalex); herceptin (trastuzumab); hydroxyurea (hydrea); idarubicin (idamycin); ifosfamide; IL-2 (proleukin, aldesleukin); interferon alpha (intron A, roferon A); irinotecan (camptosar); leuprolide (lupron); levamisole (ergamisole); lomustine (CCNU); mechlorathamine (mustargen, nitrogen mustard); melphalan (alkeran); mercaptopurine (purinethol, 6-MP); methotrexate (mexate); mitomycin-C (mutamucin); mitoxantrone (novantrone); octreotide (sandostatin); pentostatin (2-deoxycoformycin, nipent); plicamycin (mithramycin, mithracin); prorocarbazine (matulane); streptozocin; tamoxifen (nolvadex); taxol (paclitaxel); teniposide (vumon, VM-26); thiotepa; topotecan (hycamtin); tretinoin (vesanoid, all-trans retinoic acid); vinblastine (valban); vincristine (oncovin) and vinorelbine (navelbine).

In a certain embodiments, the present invention provides pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other

chemotherapeutic agents which function by a non-antisense mechanism. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g. mithramycin and oligonucleotide), sequentially (e.g. mithramycin and oligonucleotide for a period of time followed by another agent and oligonucleotide), or in combination with one or more other such chemotherapeutic agents or in combination with radiotherapy. All chemotherapeutic agents known to a person skilled in the art are here incorporated as combination treatments with compound according to the invention.

In another embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Two or more combined compounds may be used together or sequentially. *i.e.* the compound according to the invention may be used prior to, during or subsequent to one or more of the other therapeutic agents referred to herein.

The pharmaceutical composition of the invention may constitute a pro-drug. Therefore, in one embodiment of the invention the compound of the invention may be in the form of a pro-drug. Oligonucleotides are by virtue negatively charged ions. Due to the lipophilic nature of cell membranes the cellular uptake of oligonucleotides are reduced compared to neutral or lipophilic equivalents. This polarity "hindrance" can be avoided by using the pro-drug approach (see e.g. Crooke, R. M. (1998) in Crooke, S. T. *Antisense research and Application*. Springer-Verlag, Berlin, Germany, vol. 131, pp. 103-140). In this approach the oligonucleotides are prepared in a protected manner so that the oligo is neutral when it is administered. These protection groups are designed in such a way that so they can be removed then the oligo is taken up by the cells. Examples of such protection groups are S-acetylthioethyl (SATE) or S-pivaloylthioethyl (*t*-butyl-SATE). These protection groups are nuclease resistant and are selectively removed intracellularly.

Preferably the pharmaceutical composition of the invention further comprises anti-inflammatory compounds and/or antiviral compounds.

The invention described herein encompasses a method of preventing or treating cancer comprising a therapeutically effective amount of the compound of the invention, or a compound prepared according to the method of the invention, which is preferably a p21 Ras modulating oligomeric compound, to a mammal, such as a human, in need of such therapy.

The cancer, which may be treated by the pharmaceutical composition according to the invention, may be selected from the group consisting of: non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukemia (e.g., acute leukemia such as acute lymphocytic leukemia, acute

myelocytic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma), colon carcinoma, rectal carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, cervical cancer, testicular cancer, lung carcinoma, bladder carcinoma, melanoma, head and neck cancer, brain cancer, cancers of unknown primary site, neoplasms, cancers of the peripheral nervous system, cancers of the central nervous system, tumors (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, seminoma, embryonal carcinoma, Wilms' tumor, small cell lung carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, and retinoblastoma), heavy chain disease, metastases, and any disease or disorder characterized by uncontrolled or abnormal cell growth.

Suitably, the compositions of the invention are considered useful in treating one or more of the cancers selected from: The compositions of the invention are considered as particularly useful for treating one or more of the cancers selected from: Hodgkin's lymphoma, leukaemia such as acute lymphocytic leukaemia, colon carcinoma, rectal carcinoma, brain cancer, neural blastomas, lung cancer, pancreatic cancer, melanoma, acute myelogenous leukaemia, liver cancer, thyroid cancer, kidney cancer, urinary tract cancer and bladder cancer.

The compositions of the invention are considered as particularly useful for treating one or more of the cancers selected from: Hodgkin's lymphoma, leukaemia such as acute lymphocytic leukaemia, colon carcinoma, brain cancer, neural blastomas.

In one embodiment, for example for the treatment of brain cancer, it is preferred that phosphorothioate linkages are not used in the compound according to the invention.

In one embodiment, the term 'treatment' as used herein refers to both treatment of an existing disease (e.g. a cancer as herein referred to), or prevention of a disease, *i.e.* prophylaxis.

The invention also provides for the use of the compound or conjugate of the invention as described for the manufacture of a medicament for the treatment of cancer. Preferably said medicament is for the treatment of cancer which is in the form of i) a solid tumor and/or a carcinoma, and/or ii) a sarcoma, and/or iii) glioma.

Preferably said carcinoma as referred to in the use(s) according to the invention, such as use for the manufacture of a medicament and/ or methods according to the invention, such as methods for treating cancer, is selected from the group consisting of malignant melanoma, basal cell carcinoma, ovarian carcinoma, breast carcinoma, non-small cell lung cancer, renal cell carcinoma, bladder carcinoma, recurrent superficial bladder cancer, stomach carcinoma, prostatic carcinoma, pancreatic carcinoma, lung carcinoma, cervical carcinoma, cervical dysplasia, laryngeal papillomatosis, colon carcinoma, colorectal carcinoma and carcinoid tumors. More preferably, said carcinoma is selected from the group consisting of malignant melanoma, non-small cell lung cancer, breast carcinoma, colon carcinoma and renal cell carcinoma. Preferably, said carcinoma is a malignant melanoma, preferably selected from the group consisting of superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral melanoma, amelanotic melanoma and desmoplastic melanoma.

Preferably said sarcoma as referred to in the use(s), such as use for the manufacture of a medicament and/or methods according to the invention, such as methods for treating cancer, is selected from the group consisting of osteosarcoma, Ewing's sarcoma, chondrosarcoma, malignant fibrous histiocyoma, fibrosarcoma and Kaposi's sarcoma.

The invention also provides for a method for treating cancer, said method comprising administering a compound according to the invention as herein described, and/or a conjugate according to the invention, and/or a pharmaceutical composition according to the invention to a patient in need thereof.

Preferably, the cancer referred to in the methods of treatment according to the invention and/or uses for the manufacture of a medicament, are selected from the group consisting of cancer diseases is a lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries cancer.

The invention also provides for a method of inhibiting the expression of p21 Ras, in cells or tissues comprising contacting said cells or tissues with the compound according to the invention as herein described so that expression of p21 Ras is inhibited.

The invention also provides for a method of modulating expression of a gene involved in a cancer disease comprising contacting the gene or RNA from the gene with a compound of the invention as herein described, whereby gene expression is modulated.

The invention also provides for a method of treating a mammal suffering from or susceptible from a cancer disease, comprising administering to the mammal an therapeutically effective amount of an oligonucleotide targeted to p21 ras that comprises one or more LNA units and/or with a compound of the invention as herein described.

The invention also provides for a method of modulating the red blood cell proliferation, cellular proliferation, ion metabolism, glucose and energy metabolism, pH regulation or matrix metabolism comprising contacting a cell with the compound of the invention, such as an antisense oligonucleotide of the invention, so that the cell is modulated.

The invention provides for the use of the compounds per se or as conjugate as herein defined for the manufacture of a medicament for the treatment of cancer, and/or for reducing inflammation associated with the cancers herein disclosed, preferably lung cancer.

In one embodiment, the invention provides a composition wherein said composition comprises at least two non-identical compounds according to the invention, such as three non-identical compounds according to the invention. The composition may be used in place of the compound of the invention in the above methods, such as theraprutic methods according to the invention. For example a oligonucleotide which is 100% complementary to Ha-ras, may be combined in a pharmaceutical composition, with an oligonucleotide which is 100% complementary to Ki-ras, and/or an oligonucleoitde which is 100% complementary to N-ras, such oligonucleotides are referred to herein.

In one aspect each compound of the invention selected for said composition is 100% complementary to one member of the p21 ras family, such as either Ha-ras, Ki-ras and N-ras, so that the composition comprises at least two compounds which are 100% complementary to at least two, preferably three, separate members of the p21 ras family. Examples of such compositions include compositions which comprise: Either SEQ ID No 4/4A or SEQ ID No 9/9A, which are 100% complementary to N-ras; and either SEQ ID No 5/5A or SEQ ID No 6/6A, or SEQ ID No 8/8A, which are 100% complementary to Ki-ras; and SEQ ID No 13/13A, which is 100 complementary to Ha-ras. Suitably the composition comprises the two or three non-identical compounds in therapeutically effective amounts.

It is preferred that the mammal is a human being, and the mammalian pRas, such as the target proteins, are human pRas.

Definitions

The term "nucleotide" is used in its normal meaning known in the art. Specific embodiments include, when the nucleotide is a DNA unit, *i.e.* a 2-deoxyribose unit or RNA unit, which is bonded through its number one carbon atom to one of the nitrogenous bases adenine (A), cytosine (C), thymine (T) or guanine (G), uracil (U) (RNA) and which is bonded through its number five carbon atom to an internucleoside phosphate group, or to a terminal group.

When used herein, the term "nucleotide analogue" refers to a non-naturally occurring nucleotide wherein either the ribose unit is different from 2-deoxyribose or RNA and/or the nitrogenous base is different from A, C, T and G and/or the internucleotide phosphate linkage group is different. In one embodiment, not-naturally occurring refers to that, at the time of the present invention it was not publicly known that the nucleotide analogues were found in the mammalian body. In the same or different embodiment, not naturally-occurring refers to that the mammalian body does not comprise the biochemical pathway necessary for synthesis of the nucleotide analogue *in vivo*, although may comprise the nucleotide analogue.

The terms "located within" and "corresponding to"/ "corresponds to" refer to the comparison between the nucleobase sequence of the compound of the invention, or subsequence, flanking sequence, or combined nucleobase sequence, and the equivalent nucleotide sequence of i) the reverse complement of the nucleic acid target, such as the mRNA which encodes the p21 ras target protein, and/or ii) the sequence of nucleotides provided in the group consisting of SEQ ID NOS: 4-9, 11-14 and/or 15-47 (*i.e.* preferred subsequences and/or oligonucleotides), or in one embodiment the reverse complements thereof. Nucleotide analogues are compared directly to their equivalent or corresponding nucleotides.

The terms "corresponding nucleotide analogue" and "corresponding nucleotide" are intended to indicate that the nucleobase in the nucleotide analogue and the nucleotide are identical. For example, when the 2-deoxyribose unit of the nucleotide is linked to an adenine, the "corresponding nucleotide analogue" contains a pentose unit (different from 2-deoxyribose) linked to an adenine.

The term nucleobase is used as a collective term which encompasses both nucleotides and nucleotide analogues. A nucleobase sequence is a sequence which comprises at least two

nucleotides or nucleotide analogues. In one embodiment the nucleobase sequence may comprise of only nucleotides, such as DNA units, in an alternative embodiment, the nucleobase sequence may comprise of only nucleotide analogues, such as LNA units.

The term "nucleic acid" is defined as a molecule formed by covalent linkage of two or more nucleotides.

The terms "nucleic acid" and "polynucleotide" are used interchangeable herein.

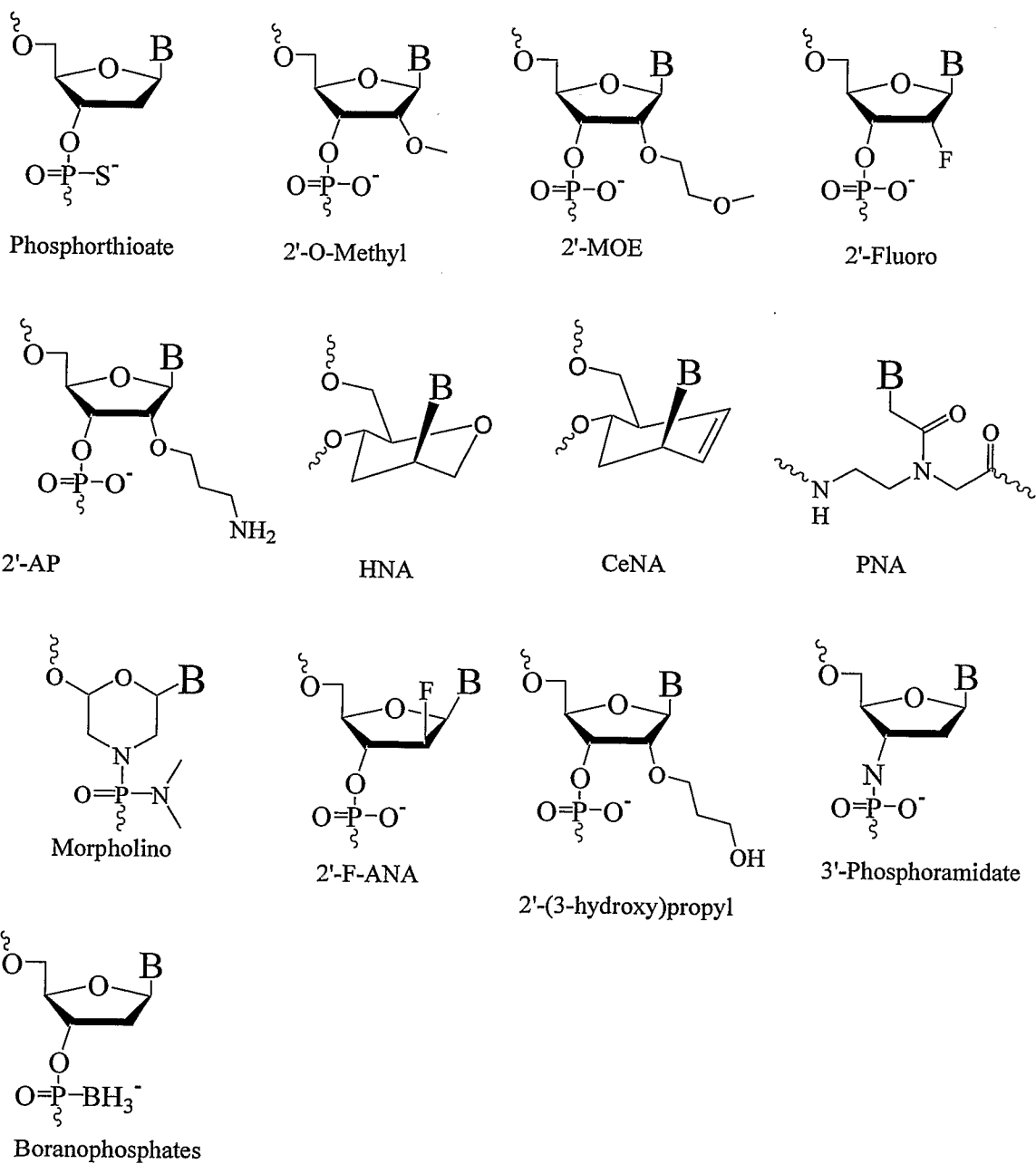
The term "target nucleic acid", as used herein refers to the DNA encoding a p21 ras protein, preferably selected from Ha-ras, Ki-ras and N-ras (such as SEQ IDs No 1-3), as well as the RNA species which are prepared from said DNA, such as Ha-ras, Ki-ras and/or N-ras pre-mRNA and mRNA, preferably derived from SEQ ID No 1-3, or allelic variants thereof.. The mRNA is the preferred form of the target nucleic acid, particularly when utilising antisense oligonucleotides. In one embodiment the target nucleic acid may be a cDNA prepared from said RNA. At least one target nucleic acid refers to, in one embodiment, the target nucleic acid from Ha-ras, Ki-ras and N-ras, or, Ha-ras and Ki-ras, or Ki-ras and N-ras, or Ha-ras, or, Ki-ras, or N-ras. It is preferred that the target nucleic acid is a mammalian, such as a human nucleic acid. Suitably, the target nucleic acid(s) corresponds to the sequence to the nucleic acid which encodes the mammalian p21ras family member(s), taking into account any allelic variations which may exist within the mammalian population (such as the individual mammalian species).

Examples of nucleotide analogues and nucleic acid analogues are described in e.g. Freier & Altmann (Nucl. Acid Res., 1997, 25, 4429-4443) and Uhlmann (Curr. Opinion in Drug & Development (2000, 3(2): 293-213). Scheme 4 illustrates selected examples of nucleotide analogues suitable for making nucleic acids.

The term "compound" when used in the context of a "compound of the invention", includes the terms "oligomeric compound", "oligonucleotide", "antisense oligonucleotide", and "oligo". The compound of the invention preferably comprises an antisense oligonucleotides, preferably consists of an antisense oligonucleotide, but may however also consist of one or more other non-nucleobase portions.

It is preferred that the compound according to the invention is a linear molecule or is synthesised as a linear molecule.

The term "oligonucleotide" refers, in the context of the present invention, to an oligomer (also called oligo) which comprises of monomer units of nucleobases. Suitable and preferred nucleotides and nucleotide analogues are as described herein. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly or with specific improved functions. Fully or partly modified or substituted oligonucleotides are often preferred over native forms because of several desirable properties of such oligonucleotides such as for instance, the ability to penetrate a cell membrane, good resistance to extra- and intracellular nucleases, high affinity and specificity for the nucleic acid target. The LNA analogue is particularly preferred exhibiting the above-mentioned properties.



Scheme 4

By the term "unit" is understood a monomer.

The term "LNA" refers to a nucleotide containing one bicyclic nucleotide analogue, also referred to as a LNA monomer or LNA unit, or an in the context of an "LNA oligonucleotides" or "LNA antisense oligonucleotide", an oligonucleotide containing one or more bicyclic nucleoside analogues (LNA units). Relevant LNA monomers are those defined in Scheme 1, 2

and 3 above. One particular example of a thymidine LNA monomer is the (1S, 3R, 4R, 7S)-7-hydroxy-1-hydroxymethyl-5-methyl-3-(thymine-1-yl)-2,5-dioxabicyclo[2.2.1]heptane.

The term "at least one" comprises the integers larger than or equal to 1, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and so forth.

In one embodiment, such as when referring to the nucleic acid or protein targets of the compounds of the invention, the term "at least one" includes the terms "at least two" and "at least three" and "at least four", likewise the term "at least two" may comprise the terms "at least three" and "at least four".

The term "linkage group" is intended to mean a group capable of covalently coupling together two nucleotides, two nucleotide analogues, and a nucleotide and a nucleotide analogue, etc. Specific and preferred examples include phosphate groups and phosphorothioate groups.

In the present context the term "conjugate" is intended to indicate a heterogeneous molecule formed by the covalent attachment of a compound as described herein (*i.e.* a compound comprising a sequence of nucleotides analogues) to one or more non-nucleotide/non-nucleotide-analogue, or non-polynucleotide moieties. Examples of non-nucleotide or non-polynucleotide moieties include macromolecular agents such as proteins, fatty acid chains, sugar residues, glycoproteins, polymers, or combinations thereof. Typically proteins may be antibodies for a target protein. Typical polymers may be polyethylene glycol. When the compound of the invention consists of a nucleobase sequence, it may, in one embodiment further comprise a non-nucleobase portion, such as the above conjugates.

As used herein, the term "pharmaceutically acceptable salts" refers to salts that retain the desired biological activity of the herein identified compounds and exhibit minimal undesired toxicological effects. Non-limiting examples of such salts can be formed with organic amino acid and base addition salts formed with metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, sodium, potassium, and the like, or with a cation formed from ammonia, *N,N*-dibenzylethylene-diamine, *D*-glucosamine, tetraethylammonium, or ethylenediamine; or (c) combinations of (a) and (b); *e.g.*, a zinc tannate salt or the like.

The term "carcinoma" is intended to indicate a malignant tumor of epithelial origin. Epithelial tissue covers or lines the body surfaces inside and outside the body. Examples of epithelial tissue are the skin and the mucosa and serosa that line the body cavities and internal organs,

such as intestines, urinary bladder, uterus, etc. Epithelial tissue may also extend into deeper tissue layers to form glands, such as mucus-secreting glands.

The term "sarcoma" is intended to indicate a malignant tumor growing from connective tissue, such as cartilage, fat, muscles, tendons and bones.

The term "glioma", when used herein, is intended to cover a malignant tumor originating from glial cells

The term "solid tumor", when used herein, is an abnormal mass of tissue that may be benign or malignant (cancerous). Examples of solid tumors are carcinomas, sarcomas and lymphomas.

In the present context, the term "C₁₋₄-alkyl" is intended to mean a linear or branched saturated hydrocarbon chain wherein the chain has from one to four carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl and tert-butyl.

As used herein, the term "gene" means the gene including exons, introns, non-coding 5' and 3' regions and regulatory elements and all currently known variants thereof and any further variants, which may be elucidated.

In one embodiment, the term "oligomeric compound" refers to an oligonucleotide which can induce a desired therapeutic effect in humans through for example binding by hydrogen bonding to a target nucleic acid. It is also envisaged that the oligomeric compounds disclosed herein may have non-therapeutic applications, such as diagnostic applications.

As used herein, the terms "RNA antagonist" refers to an oligonucleotide which targets any form of RNA (including pre-mRNA, mRNA, miRNA, siRNA etc)

As used herein, the term "mRNA" means the presently known mRNA transcript(s) of a targeted gene, and any further transcripts, which may be identified.

As used herein, the term "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

As used herein, the term "targeting" an antisense compound to a particular target nucleic acid means providing the antisense oligonucleotide to the cell, animal or human in such a way that the antisense compound are able to bind to and modulate the function of its intended target.

As used herein, "hybridisation" means hydrogen bonding, which may be Watson-Crick, Hoogsteen, reversed Hoogsteen hydrogen bonding, etc. between complementary nucleotide bases. Watson and Crick showed approximately fifty years ago that deoxyribo nucleic acid (DNA) is composed of two strands which are held together in a helical configuration by hydrogen bonds formed between opposing complementary nucleobases in the two strands. The four nucleobases, commonly found in DNA are guanine (G), adenine (A), thymine (T) and cytosine (C) of which the G nucleobase pairs with C, and the A nucleobase pairs with T. In RNA the nucleobase thymine is replaced by the nucleobase uracil (U), which similarly to the T nucleobase pairs with A. The chemical groups in the nucleobases that participate in standard duplex formation constitute the Watson-Crick face. Hoogsteen showed a couple of years later that the purine nucleobases (G and A) in addition to their Watson-Crick face have a Hoogsteen face that can be recognised from the outside of a duplex, and used to bind pyrimidine oligonucleotides via hydrogen bonding, thereby forming a triple helix structure.

It is highly preferred that the compounds of the invention are capable of hybridizing to the target nucleic acid, such as the mRNA.

In a preferred embodiment, the oligonucleotides are capable of hybridising against the target nucleic acid(s), such as the corresponding p21 mRNA(s), to form a duplex with a T_m of at least 37°C, such as at least 40°C, at least 50°C, at least 55°C, or at least 60°C. In one aspect the T_m is between 37°C and 80°C, such as between 50 and 70°C.

Measurement of T_m

A 3 µM solution of the compound in 10 mM sodium phosphate/100 mM NaCl/ 0.1 nM EDTA, pH 7.0 is mixed with its complement DNA or RNA oligonucleotide at 3 µM concentration in 10 mM sodium phosphate/100 mM NaCl/ 0.1 nM EDTA, pH 7.0 at 90 °C for a minute and allowed to cool down to room temperature. The melting curve of the duplex is then determined by measuring the absorbance at 260 nm with a heating rate of 1 °C/min. in the range of 25 to 95 °C. The T_m is measured as the maximum of the first derivative of the melting curve.

In the context of the present invention "complementary" refers to the capacity for precise pairing between two nucleotides sequences with one another. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the corresponding position of a DNA or RNA molecule, then the oligonucleotide and the DNA

or RNA are considered to be complementary to each other at that position. The DNA or RNA and the oligonucleotide are considered complementary to each other when a sufficient number of nucleotides in the oligonucleotide can form hydrogen bonds with corresponding nucleotides in the target DNA or RNA to enable the formation of a stable complex. To be stable *in vitro* or *in vivo* the sequence of an antisense compound need not be 100% complementary to its target nucleic acid, *i.e.* they may comprise one or more nucleotides or nucleotide analogues which do not pair with the corresponding nucleotide in the target DNA or RNA, these are referred to herein as "mismatches". The terms "complementary" and "hybridisable" thus imply that the compound of the invention binds sufficiently strongly and specifically to the target molecule to provide the desired interference with the normal function of the target(s). Suitably, the sequence of an antisense compound may comprise one mismatch or two mismatches or three mismatches or four mismatches compared to the target DNA or RNA.

The term "variant" as used in herein in the context of a polypeptide (sequence), such as a pRas polypeptide, including H-ras, Ki-ras and N-ras (e.g. SEQ ID NO: 48, SEQ ID NO: 49 and SEQ ID NO: 50) refers to a polypeptide which is prepared from the original (parent) polypeptide, or using the sequence information from the polypeptide, by insertion, deletion or substitution of one or more amino acids in said sequence, *i.e.* at least one amino acids, but preferably less than 50 amino acids, such as less than 40, less than 30, less than 20, or less than 10 amino acids, such as 1 amino acid, 1-2 amino acids, 1-3 amino acids, 1-4 amino acids, 1-5 amino acids.

The term "homologue" as used herein in the context of a polypeptide sequence, such as a pRas polypeptide, including H-ras, Ki-ras and N-ras (e.g. SEQ ID NO: 48, SEQ ID NO: 49 and SEQ ID NO: 50) refers to a polypeptide which is at least 70% homologous, such as at least 80% homologous, such as at least 85% homologous, or at least 90% homologous, such as at least 95%, 96%, 97%, 98% or 99% homologous to said polypeptide sequence. Homology between two polypeptide sequences may be determined using ClustalW alignment algorithm using standard settings, as referred to herein.

The term "fragment" as used herein in the context of a polypeptide sequence, such as a pRas polypeptide, including H-ras, Ki-ras and N-ras (e.g. SEQ ID NO: 48, SEQ ID NO: 49 and SEQ ID NO: 50) refers to a polypeptide which consists of only a part of the polypeptide sequence. A fragment may therefore comprise at least 5% such as at least 10% of said polypeptide sequence, including at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of said polypeptide sequence. In one embodiment, it is envisaged that a fragment could be as small

as a polypeptide sequence of 5-10 amino acids, such as a conserved sequence motif which is common between the respective members of the p21 ras family.

Antisense drugs

In one embodiment of the invention the oligomeric compounds are suitable antisense drugs. The design of a potent and safe antisense drug requires the fine-tuning of diverse parameters such as affinity/specificity, stability in biological fluids, cellular uptake, mode of action, pharmacokinetic properties and toxicity.

Benefits of using LNA

LNA with an oxymethylene 2'-O, 4'-C linkage (β -D-oxy-LNA), exhibits unprecedented binding properties towards DNA and RNA target sequences. Likewise LNA derivatives, such as amino-, thio- and α -L-oxy-LNA display unprecedented affinities towards complementary RNA and DNA and in the case of thio-LNA the affinity towards RNA is even better than with the β -D-oxy-LNA.

In addition to these remarkable hybridization properties, LNA monomers can be mixed and act cooperatively with DNA and RNA monomers, and with other nucleic acid analogues, such as 2'-O-alkyl modified RNA monomers. As such, the oligonucleotides of the present invention can be composed entirely of β -D-oxy-LNA monomers or it may be composed of β -D-oxy-LNA in any combination with DNA, RNA or contemporary nucleic acid analogues which includes LNA derivatives such as for instance amino-, thio- and α -L-oxy-LNA. The unprecedented binding affinity of LNA towards DNA or RNA target sequences and its ability to mix freely with DNA, RNA and a range of contemporary nucleic acid analogues has a range of important consequences according to the invention for the development of effective and safe antisense compounds.

Firstly, in one embodiment of the invention it enables a considerable shortening of the usual length of an antisense oligo (from 20-25 mers to, *e.g.*, 12-15 or 12-16mers) without compromising the affinity required for pharmacological activity. One embodiment of the invention is to, due to the sequence of the humane genome is available and the annotation of its genes rapidly progressing, identify the shortest possible, sufficiently complementary to the target mRNAs. Comparison against the human genome sequence can also be used to reduce the probability of, or ensure that, the antisense oligo does not bind to non-target mRNAs.

The use of LNA to may reduce the size of oligos significantly eases the process and prize of manufacture thus providing the basis for antisense therapy to become a commercially competitive treatment offer for a diversity of diseases.

The unprecedented affinity of LNA can be used to substantially enhance the ability of an antisense oligo to hybridize to its target mRNA *in-vivo* thus significantly reducing the time and effort required for identifying an active compound as compared to the situation with other chemistries.

The unprecedented affinity of LNA is used to enhance the potency of antisense oligonucleotides thus enabling the development of compounds with more favourable therapeutic windows than those currently in clinical trials.

Typically, the LNA oligonucleotides of the invention will contain other residues than β -D-oxy-LNA such as native DNA monomers, RNA monomers, N3'-P5' phosphoroamidates, 2'-F, 2'-O-Me, 2'-O-methoxyethyl (MOE), 2'-O-(3-aminopropyl) (AP), hexitol nucleic acid (HNA), 2'-F-arabino nucleic acid (2'-F-ANA) and D-cyclohexenyl nucleoside (CeNA). Also, the β -D-oxy-LNA-modified oligonucleotide may also contain other LNA units in addition to or in place of an oxy-LNA group. In particular, preferred additional LNA units include thio-LNA or amino-LNA monomers in either the D- β or L- α configurations or combinations thereof or ena-LNA. In general, an LNA-modified oligonucleotide will contain at least about 5, 10, 15 or 20 percent LNA units, based on total nucleotides of the oligonucleotide, such as at least about 20, 25, 30, 40, 50, 60, 70, 80 or 90 percent LNA units, based on total bases of the oligonucleotide.

Stability in biological fluids: One embodiment of the invention includes the incorporation of LNA monomers into a standard DNA or RNA oligonucleotide to increase the stability of the resulting oligomeric compound in biological fluids e.g. through the increase of resistance towards nucleases (endonucleases and exonucleases). The extent of stability will depend on the number of LNA monomers used, their position in the oligonucleotide and the type of LNA monomer used. Compared to DNA and phosphorothioates the following order of ability to stabilize an oligonucleotide against nucleolytic degradation can be established: DNA << phosphorothioates ~ oxy-LNA < α -L-LNA < amino-LNA < thio-LNA.

Given the fact that LNA is compatible with standard DNA synthesis and mixes freely with many contemporary nucleic acid analogues nuclease resistance of LNA- oligomeric compounds can be further enhanced according to the invention by either incorporating other analogues that display increased nuclease stability or by exploiting nuclease-resistant internucleotide linkages e.g. phosphoromonothioate, phosphorodithioate, and methylphosphonate linkages, etc.

The clinical effectiveness of antisense oligonucleotides depends to a significant extent on their pharmacokinetics e.g. absorption, distribution, cellular uptake, metabolism and excretion. In turn these parameters are guided significantly by the underlying chemistry and the size and three-dimensional structure of the oligonucleotide.

As mentioned earlier, LNA according to the invention is not a single, but several related chemistries, which exhibit stunning affinity towards complementary DNA and RNA. Thus, the LNA family of chemistries are uniquely suited of development oligos according to the invention with tailored pharmacokinetic properties exploiting either the high affinity of LNA to modulate the size of the active compounds or exploiting different LNA chemistries to modulate the exact molecular composition of the active compounds. In the latter case, the use of for instance amino-LNA rather than oxy-LNA will change the overall charge of the oligo and affect uptake and distribution behavior. Likewise the use of thio-LNA instead of oxy-LNA will increase the lipophilicity of the oligonucleotide and thus influence its ability to pass through lipophilic barriers such as for instance the cell membrane.

Modulating the pharmacokinetic properties of an LNA oligonucleotide according to the invention may further be achieved through attachment of a variety of different moieties. For instance, the ability of oligonucleotides to pass the cell membrane may be enhanced by attaching for instance lipid moieties such as a cholesterol moiety, a thioether, an aliphatic chain, a phospholipid or a polyamine to the oligonucleotide. Likewise, uptake of LNA oligonucleotides into cells may be enhanced by conjugating moieties to the oligonucleotide that interacts with molecules in the membrane, which mediates transport into the cytoplasm.

The pharmacodynamic properties can, according to the invention be enhanced with groups that improve oligomer uptake, enhance biostability such as enhance oligomer resistance to degradation, and/or increase the specificity and affinity of oligonucleotides hybridisation characteristics with target sequence e.g. a mRNA sequence.

As mentioned earlier, the LNA family of chemistries provide unprecedented affinity, very high bio-stability and the ability to modulate the exact molecular composition of the oligonucleotide. The use of LNA oligonucleotides may also provide benefit in terms of reduced toxicity, improved safety and better efficacy, as compared to, for example, the phosphorothioate class of oligonucleotides, which have been found to elicit a number of adverse effects such as complement activation, prolonged PTT (partial thromboplastin time), thrombocytopenia, hepatotoxicity (elevation of liver enzymes), cardiotoxicity, splenomegaly and hyperplasia of reticuloendothelial cells.

The LNA nucleotide analogue building blocks can be prepared following published procedures listed in the examples,

The LNA oligonucleotides can be prepared as described in the Examples and in WO 99/14226, WO 00/56746, WO 00/56748, WO 00/66604, WO 00/125248, WO 02/28875, WO 2002/094250 and WO 03/006475. Oligo- and polynucleotides of the invention may be produced using the polymerisation techniques of nucleic acid chemistry well known to a person of ordinary skill in the art of organic chemistry. Generally, standard oligomerisation cycles of the phosphoramidite approach (S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, 49, 6123; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223) is used, but e.g. H-phosphonate chemistry, phosphotriester chemistry can also be used.

For some monomers of the invention longer coupling time, and/or repeated couplings with fresh reagents, and/or use of more concentrated coupling reagents may be necessary or beneficial.

The phosphoramidites employed coupled with satisfactory >95% step-wise coupling yields. Thiolation of the phosphate is performed by exchanging the normal, e.g. iodine/pyridine/H₂O, oxidation used for synthesis of phosphodiester oligomers with an oxidation using Beaucage's reagent (commercially available) other sulfurisation reagents are also comprised. The phosphorothioate LNA oligomers were efficiently synthesised with stepwise coupling yields $\geq$ 98%.

The β -D-amino-LNA, β -D-thio-LNA oligonucleotides, α -L-LNA and β -D-methylamino-LNA oligonucleotides were also efficiently synthesised with step-wise coupling yields $\geq$ 98% using the phosphoramidite procedures.

Purification of LNA oligomeric compounds was done using disposable reversed phase purification cartridges and/or reversed phase HPLC and/or precipitation from ethanol or butanol. Capillary gel electrophoresis, reversed phase HPLC, MALDI-MS, and ESI-MS was used to verify the purity of the synthesized oligonucleotides. Furthermore, solid support materials having immobilised thereto an optionally nucleobase protected and optionally 5'-OH protected LNA are especially interesting as material for the synthesis of LNA containing oligomeric compounds where an LNA monomer is included in at the 3' end. In this instance, the solid support material is preferable CPG, e.g. a readily (commercially) available CPG material or polystyrene onto which a 3'-functionalised, optionally nucleobase protected and

optionally 5'-OH protected LNA is linked using the conditions stated by the supplier for that particular material.

Because the Ras family is involved in tumor formation both within the cancer cells and in the tumor microenvironment for many cancer types, it appears that these cancer cells have become increasingly dependent and maybe even addicted to their activated or high Ras levels. Therefore, although not wishing to be bound to a specific theory, it appears that the knockdown of Ras may hurt a cancer cell more as compared to a normal cell, provided that the knockdown is not complete. Our results herein disclosed indicate that this can be exploited for the treatment of cancer. However, it must be noted that there is a certain group of tumors that may not respond, or may not respond fully, to Ras inhibition treatments. Recent studies demonstrate that during the latter stages of tumor maintenance tumors can become independent of Ras signaling, and thus their addiction to Ras will be diminished, due to activation and autonomy of the PI3K-AKT pathway Lim and Conter (2005), Cancer Cell (: 381-392. For these tumors combinatorial therapeutic approaches are recommended.

EXAMPLES

Example 1: Monomer synthesis

The LNA monomer building blocks and derivatives thereof were prepared following published procedures and references cited therein, see:

WO 03/095467 A1

D. S. Pedersen, C. Rosenbohm, T. Koch (2002) Preparation of LNA Phosphoramidites, Synthesis 6, 802-808.

M. D. Sørensen, L. Kværnø, T. Bryld, A. E. Håkansson, B. Verbeure, G. Gaubert, P. Herdewijn, J. Wengel (2002) α -L-ribo-configured Locked Nucleic Acid (α -l-LNA): Synthesis and Properties, J. Am. Chem. Soc., 124, 2164-2176.

S. K. Singh, R. Kumar, J. Wengel (1998) Synthesis of Novel Bicyclo[2.2.1] Ribonucleosides: 2'-Amino- and 2'-Thio-LNA Monomeric Nucleosides, J. Org. Chem. 1998, 63, 6078-6079.

C. Rosenbohm, S. M. Christensen, M. D. Sørensen, D. S. Pedersen, L. E. Larsen, J. Wengel, T. Koch (2003) Synthesis of 2'-amino-LNA: a new strategy, *Org. Biomol. Chem.* 1, 655-663.

D. S. Pedersen, T. Koch (2004) Analogues of LNA (Locked Nucleic Acid). Synthesis of the 2'-Thio-LNA Thymine and 5-Methyl Cytosine Phosphoramidites, *Synthesis* 4, 578-582.

Example 2: Oligonucleotide synthesis

Oligonucleotides were synthesized using the phosphoramidite approach on an Expedite 8900/MOSS synthesizer (Multiple Oligonucleotide Synthesis System) at 1 μ mol or 15 μ mol scale. For larger scale synthesis an Äkta Oligo Pilot was used. At the end of the synthesis (DMT-on), the oligonucleotides were cleaved from the solid support using aqueous ammonia for 1-2 h at room temperature, and further deprotected for 4 h at 65°C. The oligonucleotides were purified by reverse phase HPLC (RP-HPLC). After the removal of the DMT-group, the oligonucleotides were characterized by AE-HPLC, RP-HPLC, and CGE and the molecular mass was further confirmed by ESI-MS. See below for more details.

Preparation of the LNA-solid support:

Preparation of the LNA succinyl hemiester

5'-O-Dmt-3'-hydroxy-LNA monomer (500 mg), succinic anhydride (1.2 eq.) and DMAP (1.2 eq.) were dissolved in DCM (35 mL). The reaction was stirred at room temperature overnight. After extractions with NaH_2PO_4 0.1 M pH 5.5 (2x) and brine (1x), the organic layer was further dried with anhydrous Na_2SO_4 filtered and evaporated. The hemiester derivative was obtained in 95% yield and was used without any further purification.

Preparation of the LNA-support

The above prepared hemiester derivative (90 μ mol) was dissolved in a minimum amount of DMF, DIEA and pyBOP (90 μ mol) were added and mixed together for 1 min. This pre-activated mixture was combined with LCAA-CPG (500 Å, 80-120 mesh size, 300 mg) in a manual synthesizer and stirred. After 1.5 h at room temperature, the support was filtered off and washed with DMF, DCM and MeOH. After drying, the loading was determined to be 57 μ mol/g (see Tom Brown, Dorcas J.S. Brown. Modern machine-aided methods of oligodeoxyribonucleotide synthesis. In: F. Eckstein, editor. *Oligonucleotides and Analogues A Practical Approach*. Oxford: IRL Press, 1991: 13-14).

Elongation of the oligonucleotide

The coupling of phosphoramidites (A(bz), G(ibu), 5-methyl-C(bz)) or T- β -cyanoethyl-phosphoramidite) 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 acetonitrile (0.25 M) as activator. The thiolation is carried out by using xanthane chloride (0.01 M in acetonitrile:pyridine 10%). The rest of the reagents are the ones typically used for oligonucleotide synthesis. The protocol provided by the supplier was conveniently optimised.

Purification by RP-HPLC:

Column: Xterra RP₁₈
Flow rate: 3 mL/min
Buffers: 0.1 M ammonium acetate pH 8 and acetonitrile

Abbreviations

DMT: Dimethoxytrityl
DCI: 4,5-Dicyanoimidazole
DMAP: 4-Dimethylaminopyridine
DCM: Dichloromethane
DMF: Dimethylformamide
THF: Tetrahydrofurane
DIEA: *N,N*-diisopropylethylamine
PyBOP: Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
Bz: Benzoyl
Ibu: Isobutyryl

Example 3: Stability of LNA compounds in human or rat plasma

Oligo stability was tested in plasma from humans or rats (it could also be mouse, monkey or dog plasma). In 45 μ l plasma 5 μ l oligo is added (a final concentration of 20 μ M). The oligos are incubated in plasma for times ranging from 0 h- 96 h at 37 °C (the plasma is tested for nuclease activity up to 96 h and shows no difference in nuclease cleavage-pattern). At the indicated time the sample were snap frozen in liquid nitrogen. 2 μ l (equals 40 pmol) oligo in plasma was diluted by adding 15 μ l of water and 3 μ l 6x loading dye (Invitrogen). As marker a 10 bp ladder (Invitrogen 10821-015) is used. To 1 μ l ladder 1 μ l 6x loading and 4 μ l water was added. The samples were mixed, heated to 65 °C for 10 min and loaded to a

prerun gel (16% acrylamide, 7 M UREA, 1x TBE, prerun at 50 Watt for 1h) and run at 50-60 Watt for 2 ½ h. Subsequently the gel was stained with 1x SyBR gold (molecular probes) in 1x TBE for 15 min. The bands were visualised using a phosphoimager from Biorad.

Example 4: In vitro model: Cell culture

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. Target can be expressed endogenously or by transient or stable transfection of a nucleic acid encoding said nucleic acid. The expression level of target nucleic acid can be routinely determined using, for example, Northern blot analysis, Real-Time PCR, Ribonuclease protection assays. The following cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen.

Cells were cultured in the appropriate medium as described below and maintained at 37°C at 95-98% humidity and 5% CO₂. Cells were routinely passaged 2-3 times weekly.

T24: The human bladder cancer cell line T24 was cultured in McCoy's 5a modified medium (Sigma) + 10% fetal bovine serum (FBS) + Glutamax I + pen/strep.

MiaPaca II: The human pancreas cancer cell line MiaPaCa II was cultured in DMEM (Sigma) + 10% fetal bovine serum (FBS) + Glutamax I + pen/strep.

15PC3: The human prostate cancer cell line 15PC3 was cultured in DMEM (Sigma) + 10% fetal bovine serum (FBS) + Glutamax I + gentamicin

A549: The human non-small cell lung cancer cell line A549 was purchased from ATCC, Manassas and was cultured in DMEM + 10% FBS + Glutamax I + gentamicin

Mouse fibroblast: Mouse fibroblasts from ear tissue of female NMRI nu/nu mice were immortalised with SV40 virus and maintained in DMEM + 10% FBS + pen/strep.

HUVEC-C: human umbilical vein endothelial cells were purchased from ATCC and propagated according to the manufacturers instructions.

HMVEC-d: (DMVEC's- dermal human microvascular endothelial cells) were purchased from Clonetics and cultured as described by manufacturer.

HMVEC: human microvascular endothelial cells were purchased from Clonetics and cultured as stated by manufacturer.

Human embryonic lung fibroblasts: were purchased from ATCC and cultured as described by manufacturer.

HMEC-1: Human mammary epithelial cells were purchased from Clonetics and maintained as recommended by the manufacturer

Example 5: In vitro model: Treatment with antisense oligonucleotide

The cells were treated with oligonucleotide using the cationic liposome formulation LipofectAMINE 2000 (Gibco) as transfection vehicle. Cells were seeded in 6-well cell culture plates (NUNC) and treated when 80-90% confluent. Oligo concentrations used ranged from 1 nM to 10 nM final concentration. Formulation of oligo-lipid complexes were carried out essentially as described by the manufacturer using serum-free OptiMEM (Gibco) and a final lipid concentration of 10 µg/mL LipofectAMINE 2000. Cells were incubated at 37°C for 4 hours and treatment was stopped by removal of oligo-containing culture medium. Cells were washed and serum-containing media was added. After oligo treatment cells were allowed to recover for 20 hours before they were photographed to assess growth inhibition (see Figures 2A, 2B and 2C) or harvested for RNA analysis. For protein analysis cells were allowed to recover for 44 hours.

Example 6: In vitro model: Extraction of RNA and cDNA synthesis

Total RNA Isolation

Total RNA was isolated either using RNeasy mini kit (Qiagen cat. no. 74104) or using the Trizol reagent (Life technologies cat. no. 15596). For RNA isolation from cell lines, RNeasy is the preferred method and for tissue samples Trizol is the preferred method.

Total RNA was isolated from cell lines using the Qiagen RNA OPF Robot – BIO Robot 3000 according to the protocol provided by the manufacturer. Tissue samples were homogenised using an Ultra Turrax T8 homogeniser (IKA Analysen technik) and total RNA was isolated using the Trizol reagent protocol provided by the manufacturer.

First strand synthesis

First strand synthesis was performed using OmniScript Reverse Transcriptase kit (cat# 205113, Qiagen) according to the manufacturers instructions.

For each sample 0.5 µg total RNA was adjusted to 12 µl each with RNase free H₂O and mixed with 2 µl poly (dT)₁₂₋₁₈ (2.5 µg/mL) (Life Technologies, GibcoBRL, Roskilde, DK), 2 µl dNTP mix (5 mM each dNTP), 2 µl 10x Buffer RT, 1 µl RNAGuard™Rnase INHIBITOR (33.3U/mL), (cat# 27-0816-01, Amersham Pharmacia Biotech, Hørsholm, DK) and 1 µl OmniScript Reverse Transcriptase (4 U/µl) followed by incubation at 37°C for 60 minutes and heat inactivation of the enzyme at 93°C for 5 minutes.

Example 7: In vitro model: Analysis of Oligonucleotide Inhibition of p21 Ras Expression by Real-time PCR

Antisense modulation of p21 Ras expression can be assayed in a variety of ways known in the art. For example, p21 Ras mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR. Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or mRNA.

Methods of RNA isolation and RNA analysis such as Northern blot analysis is routine in the art and is taught in, for example, Current Protocols in Molecular Biology, John Wiley and Sons.

Real-time quantitative (PCR) can be conveniently accomplished using the commercially available iQ Multi-Color Real Time PCR Detection System, available from BioRAD.

Real-time Quantitative PCR Analysis of p21 Ras mRNA Levels

Quantitation of mRNA levels was determined by real-time quantitative PCR using the iQ Multi-Color Real Time PCR Detection System (BioRAD) according to the manufacturers instructions.

Real-time Quantitative PCR is a technique well known in the art and is taught in for example Heid et al. Real time quantitative PCR, Genome Research (1996), 6: 986-994.

Platinum Quantitative PCR SuperMix UDG 2x PCR master mix was obtained from Invitrogen cat# 11730. Primers and TaqMan® probes were obtained from MWG-Biotech AG, Ebersberg, Germany.

Probes and primers to human Ha-ras were designed to hybridise to a human Ha-ras sequence, using published sequence information (GenBank accession number NM_005343, incorporated herein as SEQ ID NO: 1). Probes and primers for Ki-ras and N-ras can easily be designed by a person skilled in the art using the sequences NM_033360 incorporated herein as SEQ ID NO: 2 and NM_002524 incorporated herein as SEQ ID NO: 3.

For human Ha-ras the PCR primers were:

forward primer: 5' gccggatgcaggaaggag 3' (SEQ ID NO: 51) (final concentration in the assay; 0.3 μ M reverse primer: 5' gctccagcagcccttcctt 3' (SEQ ID NO: 52) (final concentration in the assay; 0.3 μ M)) and the PCR probe was: 5' FAM- cgtccttccttccttccttccttcgtctg -TAMRA 3' (SEQ ID NO: 53, final concentration in the assay; 0.1 μ M)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA quantity was used as an endogenous control for normalizing any variance in sample preparation.

The sample content of human GAPDH mRNA was quantified using the human GAPDH ABI Prism Pre-Developed TaqMan Assay Reagent (Applied Biosystems cat. no. 4310884E) according to the manufacturers instructions.

For quantification of mouse GAPDH mRNA the following primers and probes were designed: Sense primer 5'aaggctgtgggcaagggtcatc 3' (SEQ ID NO: 54, 0.3 μ M final concentration),

antisense primer 5' gtcagatccacgacggacacatt (SEQ ID NO: 55, 0.6 μ M final concentration),

TaqMan probe 5' FAM-gaagctcactggcatggcatggccttcctgttc-TAMRA 3' (SEQ ID NO: 56, 0.2 μ M final concentration).

Real time PCR

The cDNA from the first strand synthesis performed as described in example 8 was diluted 2-20 times, and analyzed by real time quantitative PCR. The primers and probe were mixed with 2 x Platinum Quantitative PCR SuperMix UDG (cat. # 11730, Invitrogen) and added to 3.3 μ l cDNA to a final volume of 25 μ l. Each sample was analysed in triplicates. Assaying 2 fold dilutions of a cDNA that had been prepared on material purified from a cell line expressing the RNA of interest generated standard curves for the assays. Sterile H₂O was used instead of cDNA for the no template control. PCR program: 50° C for 2 minutes, 95° C for 10 minutes followed by 40 cycles of 95° C, 15 seconds, 60° C, 1 minutes. Relative

quantities of target mRNA sequence were determined from the calculated Threshold cycle using the iCycler iQ Real-time Detection System software.

Example 8: in vitro analysis: Northern Blot Analysis of Ha-ras mRNA Levels

Northern blot analysis was carried out by procedures well known in the art essentially as described in Current Protocols in Molecular Biology, John Wiley & Sons.

Expression levels of Ha-ras RNA was determined by Northern blot analysis using a protocol as described in Ten Asbroek et al.(2000), Polymorphisms in the large subunit of human RNA polymerase II as target for allele-specific inhibition (see Figures 1A, 1B and 1C). Nucleic Acid Research 28: 1133-1138. Hybridisation probes were generated by RT-PCR and subsequent cloning into pGEM-T Easy vector (Promega). The Ha-ras probe consisted of the sequence from position 1657-3485 (exon sequences only) of SEQ ID NO: 1.

Example 9: In vitro analysis: Western blot analysis of p21 Ras protein levels

Protein levels of ras can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA, RIA (Radio Immuno Assay) or fluorescence-activated cell sorting (FACS). Antibodies directed to ras can be identified and obtained from a variety of sources, such as Upstate Biotechnologies (Lake Placid, USA), Novus Biologicals (Littleton, Colorado), Santa Cruz Biotechnology (Santa Cruz, California) or can be prepared via conventional antibody generation methods.

Western blot analysis was carried out by procedures well known in the art as described for instance in Current Protocols in Molecular Biology, John Wiley & Sons using p21 Ras mouse monoclonal antibody, ABCAM ab 13050 in a 1:1000 dilution (see Figures 1A, 1B and 1C). This down regulation of total ras protein was shown to have an extended duration of action with ras protein selectively reduced for upto 48h (Figure 8) following introduction of Seq ID No 7A to cells.

The membranes in both figures were stained in Ponceau stain to assess equal load and transfer of protein.

Example 10: In vitro analysis: Antisense Inhibition of Human Ha-ras, Ki-ras and N-ras Expression by oligonucleotide compound

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Ha-ras, Ki-ras and N-ras RNA using published sequences (GenBank accession number NM_005343 incorporated herein as SEQ ID NO: 1, NM_033360 incorporated herein as SEQ ID NO: 2, NM_002524 incorporated herein as SEQ ID NO: 3). The compounds with 16 or 17 nucleotides in length are shown in Table 2 having SEQ ID NO and number and specific designs A, B and C. "Target site" indicates the first nucleotide number on the particular target sequence to which the oligonucleotide binds. SEQ ID NO: 10 and SEQ ID NO: 11 are mismatched control oligonucleotides

Table 2 Oligonucleotide compounds of the invention

The compounds were evaluated for their potential to knockdown Ha-ras, Ki-ras and N-ras protein in T24 cells. The data are presented as percentage down-regulation relative to mock transfected cells. Note that all LNA C are 5'-Methyl-Cytosine.

SEQ ID NO	Length	Oligo Sequence	Target site Ha-ras	Target site Ki-ras	Target site N-ras
SEQ ID NO: 4	17	TTCTGGATTAGCTGGAT	249(c) 94%	242(c) 88%	314(c) 100%
SEQ ID NO: 5	17	TCATATTCGTCCACAAA	270(c) 94%	263(c) 100%	335(c) 88%
SEQ ID NO: 6	17	CTGTAGGAATCCTCTAT	294(c) 94%	287(c) 100%	359(c) 94%
SEQ ID NO: 7	17	CATGGCACTGTACTCCT	373(c) 94%	366(c) 94%	438(c) 94%
SEQ ID NO: 8	16	CTCATGTACTGGTCCC	391(c) 94%	384(c) 100.0%	456(c) 88%
SEQ ID NO: 9	17	TTGATGGCAAATACACA	426(c) 94%	419(c) 94%	491(c) 100%
SEQ ID NO: 10	17	ACCTATGTCTACGCTGC (control)			
SEQ ID NO: 11	16	TCTGTAATAGCCCCC (control)			
SEQ ID NO: 12	16	TTCTGGATTAGCTGGA	250(c) 93.75%	243(c) 87.5%	315(c) 100%
SEQ ID NO: 13	16	CATGGCACTGTACTCC	374(c) 100%	367(c) 87.5%	439(c) 93.75%

SEQ ID NO: 14	16	TTGATGGCAAATACAC	427(c) 93.75%	420(c) 93.75%	492(c) 100%
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Table 3 Example of design of oligonucleotide compounds

SEQ ID NO	Specific design of Oligonucleotide compound
	Capital bold letters: β -D-oxy-LNA (however, α -L-oxy-LNA in SEQ ID NOS: 57 and 58) Small letters: DNA sugar (deoxynucleotide) s = phosphorothioate backbone o = -O-P(O) ₂ -O-
SEQ ID NO: 4A (SEQ ID NO 60)	5'- T_sT_s^mC_sT_sg_sg_sa_st_sa_sg_s^mC_st_sG_sG_sA_st-3'
SEQ ID NO: 5A (SEQ ID NO 61)	5'- T_s^mC_sA_sT_sa_st_st_sc_sg_st_sc_sa_s^mC_sA_sA_sa -3'
SEQ ID NO: 6A (SEQ ID NO 62)	5'- C_sT_sG_sT_sa_sg_sg_sa_st_sc_sc_st_s^mC_sT_sA_st -3'
SEQ ID NO: 7A (SEQ ID NO 63)	5'- ^m C_sA_sT_sG_sg_sc_sa_sc_st_sg_st_sa_sc_sT_s^mC_s^mC_st -3'
SEQ ID NO: 8A (SEQ ID NO 64)	5'- ^m C_sT_s^mC_sA_st_sg_st_sa_sc_st_sg_sg_sT_s^mC_s^mC_sc -3'
SEQ ID NO: 9A (SEQ ID NO 65)	5'- T_sT_sG_sA_st_sg_sg_sc_sa_sa_st_sa_s^mC_sA_s^mC_sa -3'
SEQ ID NO: 10A (SEQ ID NO 66)	5'- A_s^mC_s^mC_sT_sa_st_sg_st_sc_st_sa_sc_sg_s^mC_sT_sG_sc -3' (control)
SEQ ID NO: 11A (SEQ ID NO 67)	5'- T_s^mC_sT_sg_st_sa_sa_st_sa_sg_s^mC_s^mC_s^mC_s^mC_sc -3' (control)
SEQ ID NO: 12A (SEQ ID NO 68)	5'- T_sT_s^mC_sT_sg_sg_sa_st_sa_sg_s^mC_sT_sG_sG_sa-3'
SEQ ID NO: 13A (SEQ ID NO 69)	5'- ^m C_sA_sT_sG_sg_sc_sa_sc_st_sg_st_sa_sC_sT_s^mC_sc -3'
SEQ ID NO: 14A (SEQ ID NO 70)	5'- T_sT_sG_sA_st_sg_sg_sc_sa_sa_st_sA_s^mC_sA_sc -3'
SEQ ID NO: 57	5'- T_s^mC_s^mC_sg_st_s^mC_sa_st_s^mC_sg_s^mC_st_s^mC_s^mC_sT_sc -3' (control)
SEQ ID NO: 58	5'- T_s^mC_sT_sg_st_sa_sa_st_sa_sg_s^mC_s^mC_s^mC_s^mC_sc -3' (control)
SEQ ID NO: 59	5'- T_s^mC_s^mC_sg_st_s^mC_sa_st_s^mC_sg_s^mC_st_s^mC_s^mC_sT_sc -3'

As showed in Figure 1B, SEQ ID NOS: 4A, 5A, 6A, 7A, 8A and 9A demonstrated at least 60% inhibition of Ha-ras expression at 3 nM in these experiments and are therefore preferred. It is expected that SEQ ID NOS: 12A, 13A, and 14A will exhibit similar properties.

A compound of particular interest is SEQ ID NO: 4A. Another compound of particular interest is SEQ ID NO: 5A. Another compound of particular interest is SEQ ID NO: 6A. Another compound of particular interest is SEQ ID NO: 7A. Another compound of particular interest is SEQ ID NO: 8A. Another compound of particular interest is SEQ ID NO: 9A. Another compound of particular interest is SEQ ID NO: 12A. Another compound of particular interest is SEQ ID NO: 13A.

Another compound of particular interest is SEQ ID NO: 14A.

Other suitable sequences of the compound of the invention

15mers

TCTGGATTAGCTGGA (SEQ ID NO 71)
TTCTGGATTAGCTGG (SEQ ID NO 72)
CTGGATTAGCTGGAT (SEQ ID NO 73)
TCATATTCGTCCACA (SEQ ID NO 74)
ATATTCGTCCACAAA (SEQ ID NO 75)
CATATTCGTCCACAA (SEQ ID NO 76)
CTGTAGGAATCCTCT (SEQ ID NO 77)
GTAGGAATCCTCTAT (SEQ ID NO 78)
TGTAGGAATCCTCTA (SEQ ID NO 79)
CATGGCACTGTACTC (SEQ ID NO 80)
TGGCACTGTACTCCT (SEQ ID NO 81)
ATGGCACTGTACTCC (SEQ ID NO 82)
CTCATGTACTGGTC (SEQ ID NO 83)
CATGTACTGGTCCC (SEQ ID NO 84)
TTGATGGCAAATACA (SEQ ID NO 85)
GATGGCAAATACACA (SEQ ID NO 86)
TGATGGCAAATACAC (SEQ ID NO 87)

16mers

TCATATTCGTCCACAA (SEQ ID NO 88)
CATATTCGTCCACAAA (SEQ ID NO 89)
TGTAGGAATCCTCTAT (SEQ ID NO 90)
CTGTAGGAATCCTCTA (SEQ ID NO 91)

LNA oligonucleotides are prepared from the above sequences using the methods described herein, and using the gapmer design criteria, for example as shown by the bold residues above (nucleotide analogue nucleobases such as LNA nucleobases) and the non bold (DNA or other nucleobases which are capable of recruiting RNaseH).

Example 11: Apoptosis induction by LNA antisense compounds targeting HA-ras

Measurement of apoptosis using BD™ cytometric bead array (CBA) (cat 557816).

Cells were transfected using lipofectamine 2000 as described (see Example 5). 24 h following transfection, the cells from the supernatant was spun down and the adherent cells were trypsinised and spun down. The cell pellet was resuspended/washed in PBS and counted to bring cell concentration to 2×10^6 cells/mL lysis buffer containing protease inhibitors. The procedure was proceeded as described by manufacturer with the following modifications. When cells were lysed, they were lysed for 40 min and vortexed with a 10 min interval. 1×10^5 cells were incubated with Caspase 3 beads, mixed briefly and incubated for 1 h at room temperature, before they were analysed by flow cytometri. The data were analysed using the BD™ CBA software, transferred to Excel where all data were related to mock (which is set to one).

Furthermore, an oligonucleotide directed against H-Ras or its mismatch control was tested (in two different designs (α -L-LNA versus oxy-LNA; SEQ ID NOS: 57, 58 and 59) and SEQ ID NO: 11A in an in vitro caspase 3 assay (CBA). The matched and the mismatched oxy LNA induced apoptosis to similar extend (when compared to mock) as the matched α -L-LNA, whereas the mismatched α -L-LNA oligo did not induce apoptosis noteworthy. The data presented here clearly demonstrate that downregulation of H-Ras by antisense inhibition induced apoptosis (Caspase 3).

Example 12: Antisense oligonucleotide inhibition of Ha-ras in proliferating cancer cells

Cells were seeded to a density of 12000 cells per well in white 96 well plate (Nunc 136101) in DMEM the day prior to transfection. The next day cells were washed once in prewarmed OptiMEM followed by addition of 72 μ L OptiMEM containing 5 μ g/mL Lipofectamine2000 (Invitrogen). Cells were incubated for 7 min before adding 18 μ L oligonucleotides diluted in OptiMEM. The final oligonucleotide concentration ranged from 5 nM to 100 nM. After 4 h of treatment, cells were washed in OptiMEM and 100 μ L serum containing DMEM was added.

Following oligo treatment cells were allowed to recover for the period indicated, viable cells were measured by adding 20 μ l the tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) (CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega). Viable cells were measured at 490 nm in a Powerwave (Biotek Instruments). Growth rate (Δ OD/h) were plotted against oligo concentration.

Example 13: Measurement of Ploidy (cell cycle) and DNA degradation (apoptosis) of cells following treatment with oligomeric compounds targeting Ha-ras

The late stage in the apoptotic cascade leads to large numbers of small fragments of DNA which can be analysed by propidium iodide staining of the cells. Furthermore, propidium iodide staining can be used to assess ploidy in treated cells. To assess ploidy/ apoptosis of cells treated with oligomeric compound directed against Ha-ras, cells were washed in PBA and fixed for 1 h in 70% EtOH at 4°C. After treatment with 50 μ g/mL RNase (Sigma) for 20 min at room temperature cells were washed with PBS and incubated with 40 μ g/mL propidium iodide (Sigma or BD) for 30 min. All samples were analysed using fluorescence activated cell sorter (FACSCalibur, Becton Dickinson) and Cell Quest software. In the DNA histogram the hypodiploid or the sub-G1 peak represented the apoptotic cells.

Example 14: Measurement of changes in the mitochondrial membrane potential of cells following treatment with oligomeric compounds targeting p21 Ras

To measure changes in the mitochondrial membrane potential the MitoSensor[™] reagent method (Becton Dickinson, Cat # K2017-1) was used. MitoSensor[™] reagent is taken up by healthy cells, in which it forms aggregates that emit red fluorescence. Upon apoptosis the mitochondrial membrane potential changes and does not allow the reagent to aggregate within the mitochondria and therefore it remains in the cytoplasm in its monomeric form where it emits green fluorescence. Cells treated with oligomeric compounds directed against p21 Ras were washed and incubated in MitoSensor Reagent diluted in Incubation buffer as described by manufacturer. Changes in membrane potential following oligo treatment was detected by fluorescence activated cell sorter (FACSCalibur, Becton Dickinson) and by the use of Cell Quest software.

Example 15: Inhibition of capillary formation of Endothelial cells following antisense oligo treatment

Endothelial monolayer cells (e.g. HUVEC) were incubated with antisense oligos directed against p21 Ras. Tube formation was analysed by either of the two following methods.

The first method was the BD BioCoat angiogenesis tube formation system. Cells were transfected with oligos as described (Example 5). Transfected cells were seeded at 2×10^4 cells /96 well onto matrigel polymerized BD Biocoat angiogenesis plates. The plates were incubated for the hours/days indicated with or without PMA (5- 50 nM), VEGF (20-200 ng/mL), Suramin or vehicle. The plates were stained with Cacein AM as stated by the manufacturer and images were taken. Total tube length was measured using MetaMorph.

Alternatively, cells were seeded in rat tail type I collagen (3 mg/mL, Becton Dickinson) in 0.1 volumen of 10 x DMEM, neutralised with sterile 1 M NaOH and kept on ice or in matrigel. Cells were added to the collagen suspension at a final concentration of 1×10^6 cells/mL collagen. The cell-collagen mixture was added to 6-well or 35 mm plates and placed in a humidified incubator at 37°C. When geled 3 mL of culture medium plus an extra 10% FBS were added and cells were allow to form capillary-like vascular tubes over the period indicated in the presence or absence of PMA (16nM), VEGF (50 ng/mL). Tube formation was quantified following cryostat sectioning of the gels and examination of sections by phase-contrast microscopy.

Example 16: Biodistribution of oligonucleotides in mice

Female NMRI athymic nude mice of 6 weeks old were purchased from M&B, Denmark and allowed to acclimatize for at least one week before entering experiments. Human cancer cells typically 10^6 cells suspended in 300 µl matrigel (BD Bioscience) were subcutaneously injected into the flanks of 7-8 week old NMRI athymic female nude mice. When tumour growth was evident, tritium labelled oligonucleotides were administrated at 5 mg/kg/day for 14 days using ALZET osmotic pumps implanted subcutaneously. The oligonucleotides were tritium labelled as described by Graham MJ et al. (J Pharmacol Exp Ther 1998; 286(1): 447-458). Oligonucleotides were quantitated by scintillation counting of tissue extracts from all major organs (liver, kidney, spleen, heart, stomach, lungs, small intestine, large intestine, lymph nodes, skin, muscle, fat, bone, bone marrow) and subcutaneous transplanted human tumour tissue.

Example 17: *In vivo* tumor growth inhibition

Eight to ten week old NMRI nu/nu mice (Charles River, the Netherlands) were injected subcutaneously in the flank with 10^6 MiaPaca II cells in 300 µl Matrigel (Collaborative Biomedical products, Bedford, Ma, USA). The cells were injected within one hour after harvesting by trypsin treatment. Before injection the cells were washed with cold PBS, counted with a haemocytometer and subsequently mixed with the Matrigel on ice. One week after tumor cell injection, when tumor take was positive, an osmotic mini pump (Alzet model

1002, Alzet corp., Palo Alto, Ca, USA) was implanted dorsally according to the instructions of the manufacturer. The osmotic minipumps were incubated in PBS 20 hours at 37°C prior to implantation to start up the pump. The osmotic minipumps were filled with oligonucleotides (0.5; 1 or 2 mg/kg/day) or 0.9% saline. Tumor growth was monitored daily following the implantation of the osmotic mini pump. Tumor volume was measured and calculated as described previously (Meyer, et al. Int J. Cancer, 43: 851-856, 1989.) (See Figures 3A and 3B) All mice were implanted with IPTT-200 temperature transponder chips (BMDS inc., Seaford, Delaware, USA) to allow temperature measurements and identification of the mice using a DAS 5002 scanner (BMDS inc.) during treatment.

Example 18: RNA antagonists targeting p21 Ras show low toxicity levels in mice

The levels of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase in the serum were determined, in order to study the possible effects of this 8-day treatment in the nude mice. Serum samples were taken from each mouse after the 8-day experiment. The mice did not seem externally to be sick, and no big changes in behavior were observed. During treatment the body temperature of the mice was also monitored using IPTT-200 temperature transponders (Figure 3C) and the mice were weighed (Figure 3D). The body temperature did not change significantly during the treatment, not even at high dose 2 mg/Kg/day, which is an indication that no major toxicity effects are occurring.

The following experiments were carried out to further illustrate the efficacy of the RNA antagonist compounds in the modulation of p21 RAS expression and inhibition of tumor growth.

Example 19: Identification of Target Sequences

The target sequences present in Ha-ras, Ki-ras and N-Ras were identified by alignment of the three sequences. The sequence alignment was performed on SEQ ID NOS: 1, 2 and 3 using the ClustalW algorithm on standard settings. Suitable target sequences were identified by selecting regions which showed no more than 2 mismatches over 16 or 17 consecutive nucleotide residues in all of the following (As shown in Table 2 this corresponds to at least 87% or 88% homology/identity respectfully). The alignment obtained and selected target sites are shown in Figure 4.

Example 20: Synthesis of LNA

Six oligonucleotides were prepared against the six target sites identified in Example 19. The SEQ IN NOS: 4A, 5A, 6A, 7A, 8A and 9A (Table 3) were synthesized by Santaris Pharma AS

(Hørsholm, Denmark) as described in Hansen *et al.* (2003) *Nucleosides Nucleotides Nucleic Acids* 22, 1607-1609, Fluiter *et al.* (2003) *Nucleic Acids Res.* 31:953-962, and Fluiter *et al.* (2005) *ChemBioChem* 6: 1104-1109. The β -d-LNA (LNA) monomers were obtained from Exiqon A/S (Denmark). In all oligonucleotides 5-methyl-C was used. All syntheses were carried out using the phosphoramidite approach on an Expedite 8900 MOSS synthesizer (Multiple Oligonucleotide Synthesis System, ABI Applied Biosystems, Foster city, CA, USA) at a 1 μ mol scale. The oligonucleotides were purified by reverse phase HPLC (RP-HPLC). After the removal of the DMT-group, the oligonucleotides were characterized by AE-HPLC, and the molecular mass was further confirmed by ESI-MS and Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) on a Biflex III MALDI (Brucker instruments, Leipzig, Germany). The oligonucleotide target sequences are depicted in Figure 4. Melting temperatures were measured using complementary DNA as opposite strand as per Fluiter *et al.*, 2005. Tritium labeling of oligonucleotides was performed using the heat exchange method described by Graham *et al.* (1993) *Nucleic Acids Research* 21, 3737-3743.

Example 21: Cell lines

The cell lines used for the following experiments were: 15PC3 and MiaPaCa-2. All cell lines were maintained by serial passage in Dulbecco's modified Eagle's medium (DMEM). Cells were grown at 37°C and 5% CO₂. Media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin. All cell lines are available from the European Collection of Cell Cultures (<http://www.ecacc.org.uk/>).

Example 22: Transfections

The oligonucleotide transfections were performed in 6 well culture plates with Lipofectamine 2000 (Invitrogen) as liposomal transfection agent according to Example 5. Fluorescently (FAM) labeled LNA oligonucleotides were used to determine the transfection efficiency. Nucleofection was performed using the nucleofector and the human dermal fibroblast nucleofector kit (Amaxa biosystems, Gaithersburg, MD, USA). For fluorescence microscopy experiments, cells were plated on glass coverslips in a 6-well culture plate, and transfected with FAM-labeled LNA oligonucleotides. Cells were fixed on the glass in PBS containing 4% para-formaldehyde and embedded in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence microscopy was done with a Vanox microscope (Olympus) and appropriate filters. MTT assays were performed using standard protocols. For the ERK phosphorylation studies cells were serum starved o/n and treated with 1 μ g/mL insulin (Sigma-Aldrich), or 10 ng/mL EGF (Sigma-Aldrich) or 100nM PMA (Sigma-Aldrich) for 5 minutes.

Example 23: Western blotting

Western-immuno blots were done after denaturing acryl amide electrophoresis under standard conditions using the mini-protean system(Biorad). Pan Ras was detected using the P21 Ras mouse monoclonal (ab13050, Abcam, Cambridge, UK). Also other antibodies against ERK, P-ERK, and BCL-2 were purchased from Abcam. P53 antibody was obtained from SantaCruz biotechnology (SantaCruz, CA, USA). Ponceau staining of the blots was used as loading control. Immunodetection of proteins was done using the Lumi-Light enhanced chemi-luminescence kit (Roche) and the LAS-3000 darkbox imaging system (FujiFilm, Tokyo, Japan).

Example 24: In vivo Model

Eight to ten week old athymic nude NMRI nu/nu mice (Charles River, the Netherlands) were injected subcutaneously in the flank with 10^6 15PC3 cells in 300 μ l Matrigel (Collaborative Biomedical products, Bedford, Ma, USA). The cells were injected within one hour after harvesting by trypsin treatment. One week after tumor cell injection, when tumor take was positive, an osmotic mini pump (Alzet model 1002, Durect Co., Cupertino, Ca, USA) was implanted dorsally according to the instructions of the manufacturer. The osmotic minipumps were incubated in PBS 20 hours at 37°C prior to implantation to start up the pump, in order to quickly reach a steady delivery rate after implantation. In vitro testing showed that the Alzet 1002 minipumps reached a steady pumping rate within 24 hours. The osmotic minipumps were filled with oligonucleotides dissolved in 0.9% saline solution (using the dosages as indicated in the figure references) or 0.9% saline. For each treatment five mice per group were used. Tumor growth was monitored daily following the implantation of the osmotic mini pump. Tumor volume was measured as described in Fluiter *et al.*(2002) Cancer Research 62, 2024-2028.

Example 25: General in vivo toxicity assays

Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase levels in serum were determined using standard diagnostic procedures with the H747 (Hitachi/Roche) with the appropriate kits (Roche Diagnostics). Body temperature was monitored daily for each mouse using IPTT-200 transponder chips and a DAS 5002 chip reader (Biomedic Data Systems, Seaford, Delaware, USA).

Example 26:

In Example 19 and 20, a series of LNA containing RNA antagonists that target multiple ras genes (Pan Ras antagonists) were designed. The LNA oligonucleotides were designed to be complimentary to the most homologous domains in the Ras sequences (Figure 4). Six LNA sequences were designed, being very homologous to the human Ras sequences and mouse

Ras sequences. For each LNA oligonucleotide we used a 16 or 17 mer design of (starting from 5' end) 4 LNA, a center of 9 DNA, 3LNA and one DNA. The LNA modifications in the oligonucleotides raises the T_m over 75°C as reported previously (Fluiter, 2005).

All oligonucleotides showed IC₅₀ values in the low nanomolar range with respect to protein down-regulation and inhibition of cell growth (Figure 5) when transfected into T24 cells.

Since all oligonucleotides had similar IC₅₀ values we proceeded to further test one SEQ ID NO: 7A which is highly homologous with both human and mouse Ras sequences.

Example 27:

Transfection with LNA Pan Ras oligonucleotide SEQ ID NO: 7A resulted in a strong inhibition of cell growth in 3 different cancer cell lines 15PC3 and MiaPaCa and Fibroblast with activating Ras-mutations. However, WT-Ras mouse fibroblasts did not respond as strongly as the cancer cells (Figure 5). In contrast to the cancer cells most fibroblasts did not show changes in their morphology although there was clear knockdown of their Pan-Ras levels. Cancer cells rounded and detached from their plates within 48 hours after transfection (Figure 6) while extended culture of fibroblasts treated with Seq ID No 7A caused no detrimental effects (Figure 7).

We routinely used lipofectamine 2000 as transfection agent for all cell types resulting in over 95% transfection efficacy for most cell types. To exclude that the lack of effect in fibroblasts is due to the transfection procedure the Amaxa nucleofection system was also tested to transfect LNA into the fibroblasts. Using this technique we achieved almost 100% transfection efficacy with total nuclear localization of a FAM-labeled LNA, as judged through the fluorescence microscope. Ras knockdown of up to 80% was achieved at 2 days post nucleofection. Despite this knockdown of Ras, fibroblasts did not change their morphology nor did widespread cell death occur.

Example 28:

In order to study whether Pan Ras knockdown had effects of down stream pathways, we tested the phosphorylation of ERK after a brief stimulation with insulin. 15PC3 prostate cancer cells were not able to phosphorylate ERK in response to insulin after knockdown of Ras using SEQ ID NO: 7A, indicating this pathway was blocked after Ras knockdown (Figure 9A). Knockdown of Pan Ras resulted furthermore in reduction of Bcl-2 levels and increase of P53 levels (Figure 9C) as reported previously by Halaschek-Wiener et al., (2004). In mouse fibroblasts transfection with SEQ ID NO: 7A resulted in effective inhibition of Erk phosphorylation (Figure 9B). Stimulation with insulin or EGF of fibroblasts after transfection

with SEQ ID NO: 7A did not result in Erk phosphorylation in contrast to cells transfected with the control SEQ ID NO: 10A (Figure 9B). Stimulation with PMA which bypasses Ras signaling was used as a positive control for ERK phosphorylation and was not affected as much by Ras targeting LNA. This demonstrates that the SEQ ID NO: 7A specifically inhibits the Ras-ERK pathway both in cancer cells and in fibroblasts. However, as stated before the fibroblasts, unlike the cancer cells did not seem to suffer from Ras knockdown.

Example 29:

In view of the lack of toxicity in fibroblasts we tested whether the Pan Ras knockdown strategy was tolerated in vivo. Nude mice bearing pancreas tumor xenografts (MiaPaCa-2) were treated with Pan Ras targeting LNA and a control LNA (SEQ ID NO: 10A). The Pan Ras LNA oligonucleotide SEQ ID NO: 7A is completely homologous with murine N-ras but has at least one mismatch to all other variants of both the human and mouse Ras sequences. Using osmotic mini-pumps the Pan Ras LNA was administered for two weeks in three dosages and during this time the health of the mice was monitored through measuring the body temperature, weight and observing overall appearance. The Pan Ras LNA effectively inhibited tumor growth with an approximate 3 fold reduction in tumor burden at 2mg/kg. At one quarter of this dose there was still a significant reduction in tumor load (Figure 3A). Ras protein content in these tumors was significantly reduced (Figure 10), while the protein knockdown in the liver was even more substantial (Figure 11). In light of this striking difference in sensitivity, it was important to note that no adverse effects were seen in health parameters as measured in Example 18. After treatment, the ASAT and ALAT levels in the blood serum was checked to assess liver damage. We only observed only a mild increase in serum ASAT in the mice treated with 1 and 2 mg/kg dosage (Figure 12) while overall appearance and histology of the liver was not changed (not shown) indicating that there was no substantial damage after treatment with Pan Ras LNA.

The present invention has been described with specificity in accordance with certain of its preferred embodiments. Therefore, the following examples serve only to illustrate the invention and are not intended to limit the same.

CLAIMS

1. A compound consisting of a contiguous sequence of a total of between 10-30 nucleobases, wherein said compound comprises a subsequence of at least 8 contiguous nucleobases, wherein said subsequence corresponds to a contiguous sequence which is present in the nucleic acids which encode at least three non-identical mammalian p21 ras family members, wherein said subsequence may comprise no more than one mismatch when compared to each of the corresponding sequences present in the nucleic acids which encode said at least three non-identical mammalian p21 ras family members.
2. The compound according to claim 1, wherein said subsequence corresponds to a sequence which is present in the nucleic acids which encode three p21 ras family members, wherein said subsequence may comprise no more than one mismatch when compared to the corresponding nucleic acids which encode said three mammalian p21 ras family members.
3. The compound according to claim 1, wherein said compound comprises a 5' and/or a 3' flanking nucleobase sequence, which is/are contiguous to said subsequence, wherein said flanking sequence or sequences consist of a total of between 2 and 22 nucleobase units, which when combined with said sub-sequence, the combined contiguous nucleobase sequence is at least at least 80% homologous to the corresponding sequences of each of said nucleic acids which encode said mammalian p21 ras family members.
4. The compound according to any one of claims 1 to 3, wherein said subsequence or combined nucleobase sequence comprises a contiguous sequence of at least 7 nucleobase residues which, when formed in a duplex with the complementary target RNA corresponding to each of said nucleic acids which encode said mammalian p21 ras family members, are capable of recruiting RNaseH.
5. The compound according to claim 4, wherein said subsequence or combined nucleobase sequence comprises of a contiguous sequence of at least 8 or 9 nucleobase residues which, when formed in a duplex with the complementary target RNA corresponding to each of said nucleic acids which encode said mammalian p21 ras family members, are capable of recruiting RNaseH..
6. The compound according to any one of the preceding claims wherein said subsequence is at least 9 or at least 10 nucleobases in length, such as at least 11 nucleobases or at least 12 nucleobases in length.

7. The compound according to any one of the preceding claims, wherein said members of the p21 ras family share at least 85% sequence homology with SEQ ID NO 92 (Pan-Ras consensus sequence).
8. The compound according to any one of the preceding claims, wherein said members of the p21 ras family share no more than 95% sequence homology at the amino acid level.
9. The compound according to any one of the preceding claims, wherein the p21 ras family members are selected from the group consisting of: Ha-ras, Ki-ras and N-ras.
10. The compound according to any one of the preceding claims, where in the p21 ras family members are selected from the group consisting of: Ha-ras and Ki-ras; Ha-ras and N-ras; Ki-ras and N-ras, and; Ha-ras, Ki-ras and N-ras.
11. The compound according to any one of the preceding claims, wherein said nucleic acids which encode each member of the p21 ras family are naturally occurring in a mammalian species, such as a human.
12. The compound according to any one of the preceding claims, wherein said said compound consists of no more than 22 nucleobases, such as no more than 20 nucleobases, such as no more than 18 nucleobases, optionally conjugated with one or more non-nucleobase compounds.
13. The compound according to claims 12 wherein said compound consists of either 15, 16 or 17 nucleobases, optionally conjugated with one or more non-nucleobase compounds.
14. The compound according to any one of the preceding claims wherein said compound comprises of no more than 4 mismatches with each of the corresponding nucleic acids which encode each of said mammalian p21 ras family members.
15. The compound according to claim any one of the preceding claims, wherein said subsequence or said combined contiguous nucleobase sequence corresponds to a sequence present in a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 4, 5, 6, 7, 8, 9, 12, 13 and 14.
16. The compound according to claim 15, wherein said subsequence corresponds to a sequence present in a nucleic acid sequence selected from the group consisting of SEQ ID NOS: 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, and 47.

17. The compound according to any one of the preceding claims which is an antisense oligonucleotide.
18. The compound according to claim 17, wherein the antisense oligonucleotide consists of a combined total of between 12 and 25 nucleobases, wherein the nucleobase sequence of said oligonucleotide is at least 80% homologous to a corresponding region of each of said nucleic acids which encode said mammalian p21 ras family members.
19. The compound according to any one of the preceding claims, wherein said compound, said subsequence, said combined contiguous nucleobase sequence and/or said flanking sequence or sequences, comprise at least one nucleotide analogue.
20. The compound according to claim 19, wherein said compound, said subsequence, said combined contiguous nucleobase sequence and/or said flanking sequence or sequences comprise a total of between 2 and 10 nucleotide analogues, such as between 5 and 8 nucleotide analogues.
21. The compound according to any one of the preceding claims, wherein the antisense oligonucleotide is a gapmer, a headmer, a tailmer or a mixmer, which comprises nucleobases which are both nucleotides and nucleotide analogues.
22. The compound according to claim 21, wherein said compound, said sub-sequence, or said combined contiguous nucleobase sequence is a gapmer of formula, in 5' to 3' direction, A-B-C, and optionally of formula A-B-C-D, wherein:
- A consists or comprises of at least one nucleotide analogue, such as between 1-6 nucleotide analogues, preferably between 2-5 nucleotide analogues, preferably 3 or 4 nucleotide analogues, most preferably 3 or 4 consecutive nucleotide analogues and;
 - B consists or comprises at least five consecutive nucleobases which are capable of recruiting RNaseH, such as between 1 and 12, or between 6-10, or between 7-9, such as 8 consecutive nucleobases which are capable of recruiting RNaseH, and;
 - C consists or comprises of at least one nucleotide analogue, such as between 1-6 nucleotide analogues, preferably between 2-5 nucleotide analogues, preferably 3 or 4 nucleotide analogues, most preferably 3 or 4 consecutive nucleotide analogues and;
 - D consists or comprises, preferably consists, of one or more DNA nucleotide, such as between 1-3 or 1-2 DNA nucleotides.
23. The compound according to claim 22, wherein:
- A Consists of 3 or 4 consecutive nucleotide analogues;

- B Consists of 8 or 9 consecutive DNA nucleotides or equivalent nucleobases which are capable of recruiting RNaseH;
 - C Consists of 3 or 4 consecutive nucleotide analogues;
 - D Consists, where present, of one DNA nucleotide.
24. The compound according to claim 22, wherein:
- A Consists of 3 consecutive nucleotide analogues;
 - B Consists of 9 consecutive DNA nucleotides or equivalent nucleobases which are capable of recruiting RNaseH;
 - C Consists of 3 consecutive nucleotide analogues;
 - D Consists, where present, of one DNA nucleotide.
25. A compound according to claim 22, wherein:
- A Consists of 4 consecutive nucleotide analogues;
 - B Consists of 8 consecutive DNA nucleotides or equivalent nucleobases which are capable of recruiting RNaseH;
 - C Consists of 4 consecutive nucleotide analogues;
 - D Consists, where present, of one DNA nucleotide.
26. The compound according to claims 22-25, wherein regions A and C correspond to said 5' and said 3' flanking regions, and region B corresponds to said sub-sequence.
27. The compound according to anyone of claims 22 - 26, wherein B comprises or consists of DNA nucleobases.
28. The compound according to any one of claims 19 - 27, wherein at least one nucleotide analogue is a Locked Nucleic Acid (LNA) unit.
29. The compound according to claim 28, which comprise between 1 and 10 LNA units such as between 2 and 8 nucleotide LNA units.
30. The compound according to claim 29 where all the nucleotide analogues present in said compound are LNA units.
31. The compound according to any one of the claims 28-30, wherein the LNAs are independently selected from oxy-LNA, thio-LNA, and amino-LNA, in either of the D- β and L- α configurations or combinations thereof.
32. The compound according to claim 31, wherein the LNAs are all β -D-oxy-LNA.

33. The compound according to any one of the preceding claims, wherein at least one of the nucleobases present in the nucleotides or nucleotide analogues is a modified nucleobase selected from the group consisting of 5-methylcytosine, isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, and 2-chloro-6-aminopurine.
34. The compound according to any one of the preceding claims, wherein said compound hybridises with a corresponding Ha-ras, Ki-ras and N-ras mRNA with a T_m of at least 50°C.
35. The compound according to any one of the preceding claims, wherein said compound hybridises with a corresponding Ha-ras, Ki-ras and N-ras mRNA with a T_m of no greater than 80°C.
36. The compound according to any one of the preceding claims, wherein the nucleobase sequence is at least 80% complementary to a corresponding sequence found within each of the nucleic acids which encodes said p21 ras family members.
37. The compound according to any one of the preceding claims wherein the Ha-ras nucleic acid sequence is, or is present in, SEQ ID No 1 or allelic variants thereof.
38. The compound according to any one of the preceding claims wherein the Ki-ras nucleic acid sequence is, or is present in, SEQ ID No 2 or allelic variants thereof.
39. The compound according to any one of the preceding claims wherein the N-ras nucleic acid sequence is, or is present in, SEQ ID No 3 or allelic variants thereof.
40. The compound according to any one of the preceding claims, where the nucleobase sequence consists or comprises of a sequence which is, or corresponds to, a sequence selected from the group consisting of: SEQ ID NOS: 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 88, 89, 90 and 91, wherein the nucleotides present in the compound may be substituted with a corresponding nucleotide analogue and wherein said compound may comprise one, two, or three mismatches against said selected sequence.
41. The compound according to any one of the preceding claims, where the nucleobase sequence consists or comprises of a sequence which is, or corresponds to, a sequence selected from the group consisting of: SEQ ID NOS: 4, 5, 6, 7, 8, 9, 12, 13 and 14, wherein the nucleotides present in the compound may be substituted with a corresponding nucleotide analogue and wherein said compound may comprise one, two, or three mismatches against said selected sequence.

42. The compound according claim 41, where the nucleobase sequence consists or comprises of a sequence selected from the group consisting of SEQ ID NOS: 60, 61, 62, 63, 64, 65, 68, 69 and 70, wherein the LNA nucleobases may optionally be substituted with an alternative corresponding nucleotide analogue, and wherein said compound may optionally comprise one, two, or three mismatches against said selected sequence, and optionally, linkage groups other than phosphorothioate may be used.

43. The compound according to claim 42 which consists of SEQ ID NOS: 60, 61, 62, 63, 64, 65, 68, 69 and 70,

44. A method for the identification of at least one compound capable of down-regulation, of at least three non-identical mammalian p21 Ras proteins in a human or mammalian cell, comprising the sequential steps of:

- A Compare the nucleic acid sequences which, in a human or mammalian cell, encode at least three non-identical mammalian p21 Ras proteins.
- B from the comparison in step A, identify a subsequence of at least 8 consecutive nucleotides which is present in each of the nucleic acid sequences encoding the at least three non-identical mammalian p21 Ras proteins, wherein said at least 8 consecutive nucleotides may comprise no more than 1 mismatch when compared to each of the corresponding nucleic acids which encode said non-identical mammalian p21 Ras proteins;
- C Identify a complementary nucleobase sequence which consists of a total of 10-30 nucleobases, wherein the complementary nucleobase sequence comprises corresponds to the subsequence identified in step B.

45. The method according to claim 44, wherein step A comprises the comparison of the nucleic acid sequences which, in a human or mammalian cell, encode three non-identical mammalian p21 Ras protein; and wherein step B comprises identifying a subsequence of at least 8 consecutive nucleotides which is present in each of the nucleic acid sequences encoding the three non-identical mammalian p21 Ras proteins, wherein said at least 8 consecutive nucleotides may comprise no more than 1 mismatch when compared to each of the corresponding nucleic acids which encode said non-identical mammalian p21 Ras proteins.

46. The method according to claim 44 or 45, wherein step B or C further comprise an additional step, wherein said additional step comprises the identification of either a 3' or a 5' flanking sequence, or both a 5' and a 3' flanking sequence, which are contiguous with said subsequence, wherein said flanking sequence, or sequences, consist of a total of between 2 and 22 nucleobase units, and wherein when said flanking sequence or sequences are

combined with the sub-sequence identified in step B, the combined nucleobase sequence is at least at least 80% homologous to the corresponding sequences of each of said nucleic acids which encode said mammalian p21 ras family members.

47. The method according to any one of claims claim 44 - 46 wherein; the comparison performed in step a) is in the form of a sequence alignment.

48. The method according to any one of claims 44-47, wherein said non-identical mammalian p21 Ras proteins are as defined in any one of claims 1 - 43.

49. The method according to any one of claims 44-48, wherein said nucleic acids (sequences) which encode said mammalian p21 Ras proteins are as defined in any one of claims 1 - 43.

50. The method according to any one of claims 44-49, wherein said subsequence is as defined in any one of claims 1 - 43.

51. The method according to any one of claims 44-50, wherein said complementary nucleobase sequence is as defined in any one of claims 1 - 43.

52. The method according to any one of claims 44-51, wherein said flanking sequence or sequences are as defined in any one of claims 1 - 43.

53. A method for the preparation of a compound capable of down regulation of at least three non-identical Ras proteins in a human or mammalian cell, comprising the sequential steps of A to C according to claims 44-52, and a further step D, which comprises preparing the compound; and a further optional step E, which comprises testing said compound prepared in step D to determine efficacy of concurrent or simultaneous down regulation of at least three non-identical Ras proteins in a human or animal cell.

54. A compound identified or prepared according to the methods of any one of claims 44-53.

55. A conjugate comprising the compound according to any one of the claims 1-43 or claim 54 and at least one non-nucleotide or non-polynucleotide moiety covalently attached to said compound

56. A pharmaceutical composition comprising a compound as defined in any of claims 1-43 or claim 54 or a conjugate as defined in claim 55, and a pharmaceutically acceptable diluent, carrier, salt or adjuvant

57. The pharmaceutical composition according to claim 56 further comprising at least one chemotherapeutic agent.

58. A pharmaceutical composition according to claims 56 or 57, wherein the compound is constituted as a pro-drug.

59. A pharmaceutical composition according to any one of claims 56-58, which further comprises an anti-inflammatory compounds and/or antiviral compounds.

60. Use of a compound as defined in any one of the claims 1-43 or 54, or a conjugate as defined in claim 55, for the manufacture of a medicament for the treatment of cancer.

61. A method for treating cancer, said method comprising administering a compound as defined in any one of the claims 1-43 or 54, or a conjugate as defined in claim 55, or a pharmaceutical composition as defined in any one of the claims 56-59, to a patient in need thereof.

62. A method of inhibiting the expression of p21 Ras in a cell or a tissue, the method comprising the step of contacting said cell or tissue with a compound as defined in any any one of the claims 1-43 or 54, or a conjugate as defined in claim 55, or a pharmaceutical composition as defined in any one of the claims 56-59, so that expression of p21 Ras is inhibited.

63. A method of modulating expression of a gene involved in a cancer disease comprising contacting the gene or RNA from the gene with the compound according to any one of the claims 1-43 or 54, or a conjugate as defined in claim 55, or a pharmaceutical composition as defined in any one of the claims 56-59, so that gene expression is modulated.

64. A method of treating a mammal suffering from, or being susceptible to, a cancer disease, comprising the step of administering to the mammal an therapeutically effective amount of an compound targeted to at least three members of the mammalian p21 ras family, such as three members of the mammalian p21 ras family, that comprises one or more LNA units, such as any one of the compounds according to any one of the preceding claims

65. A method of modulating the red blood cell proliferation, cellular proliferation, ion metabolism, glucose and energy metabolism, pH regulation or matrix metabolism, the method comprising the step of contacting a cell with an antisense compound as defined in any one of the claims 1-43 or 54, or a conjugate as defined in claim 55, or a pharmaceutical composition as defined in any one of the claims 56-59, so that the cell is modulated.

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FIGURE 1A

15PC3

T24

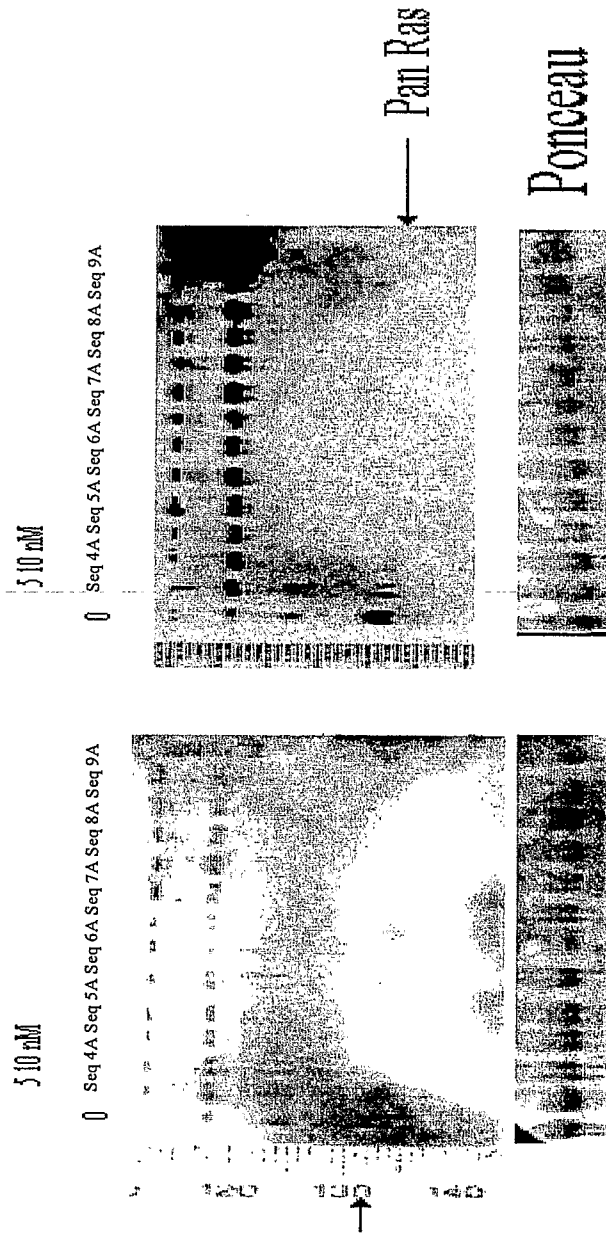
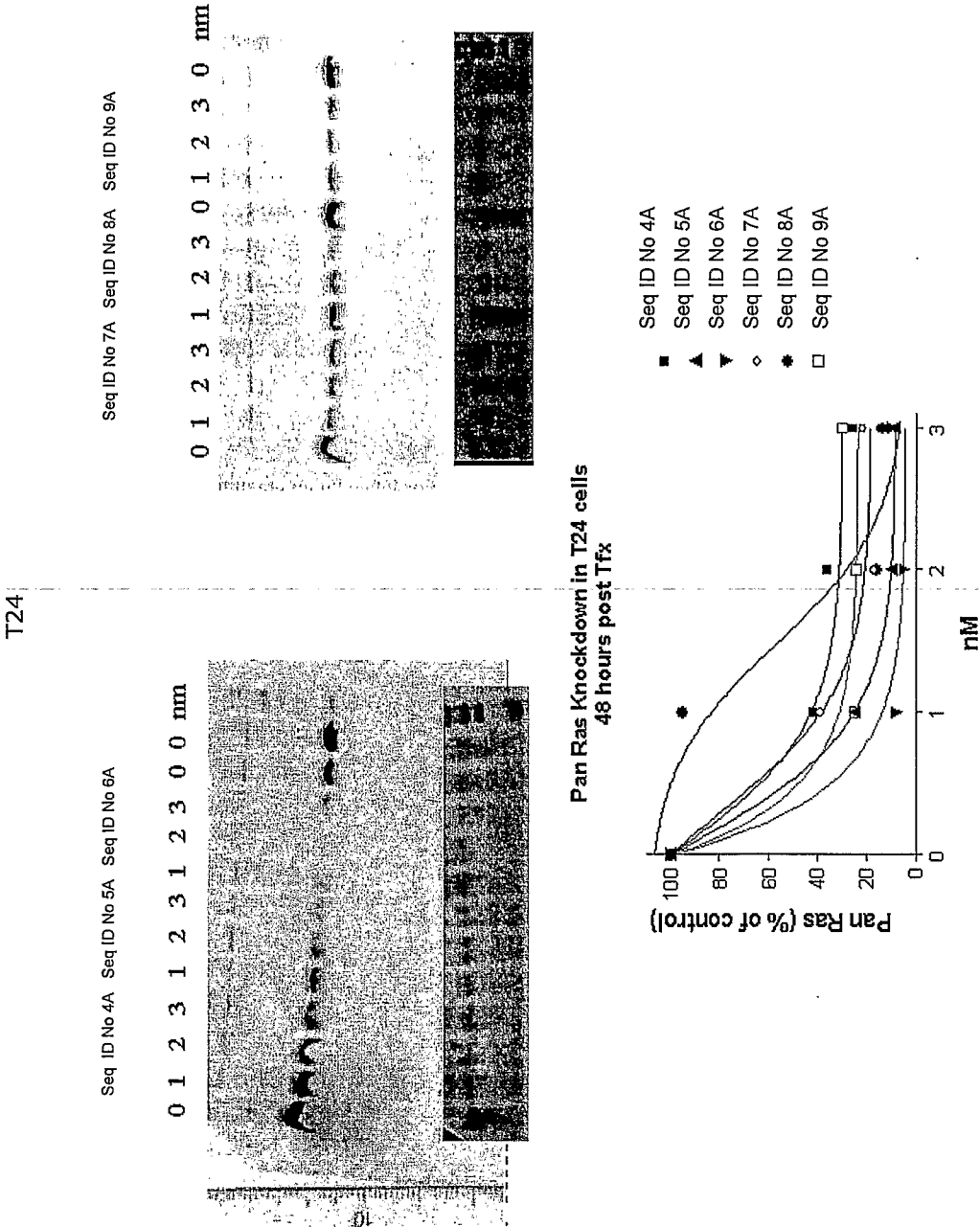


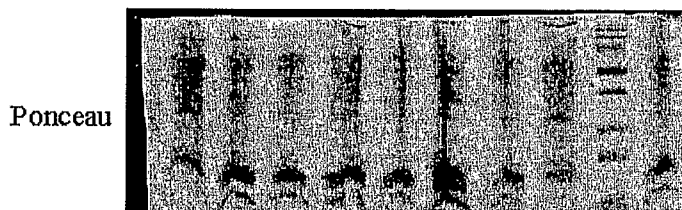
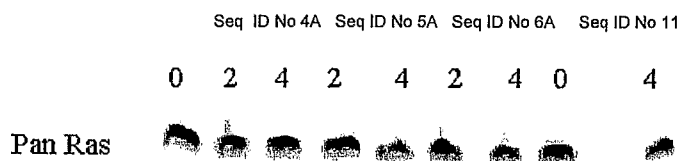
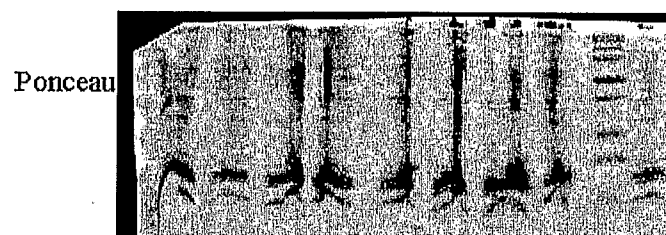
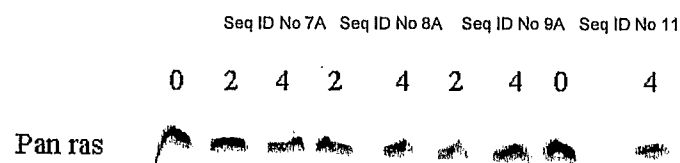
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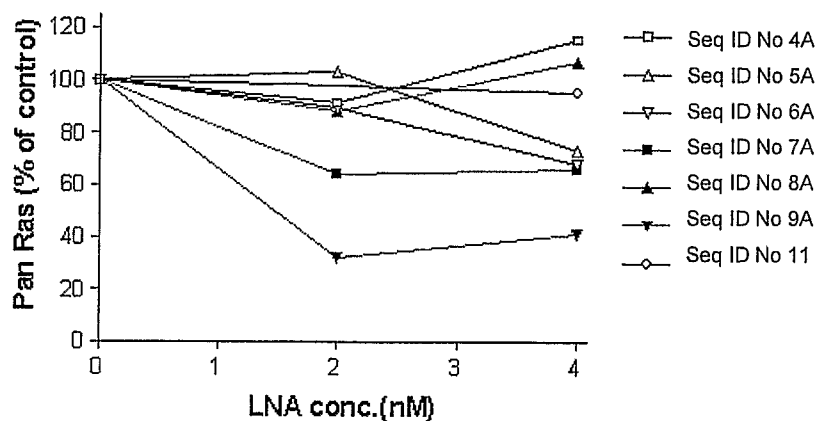
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FIGURE 1C

Mouse Fibroblasts



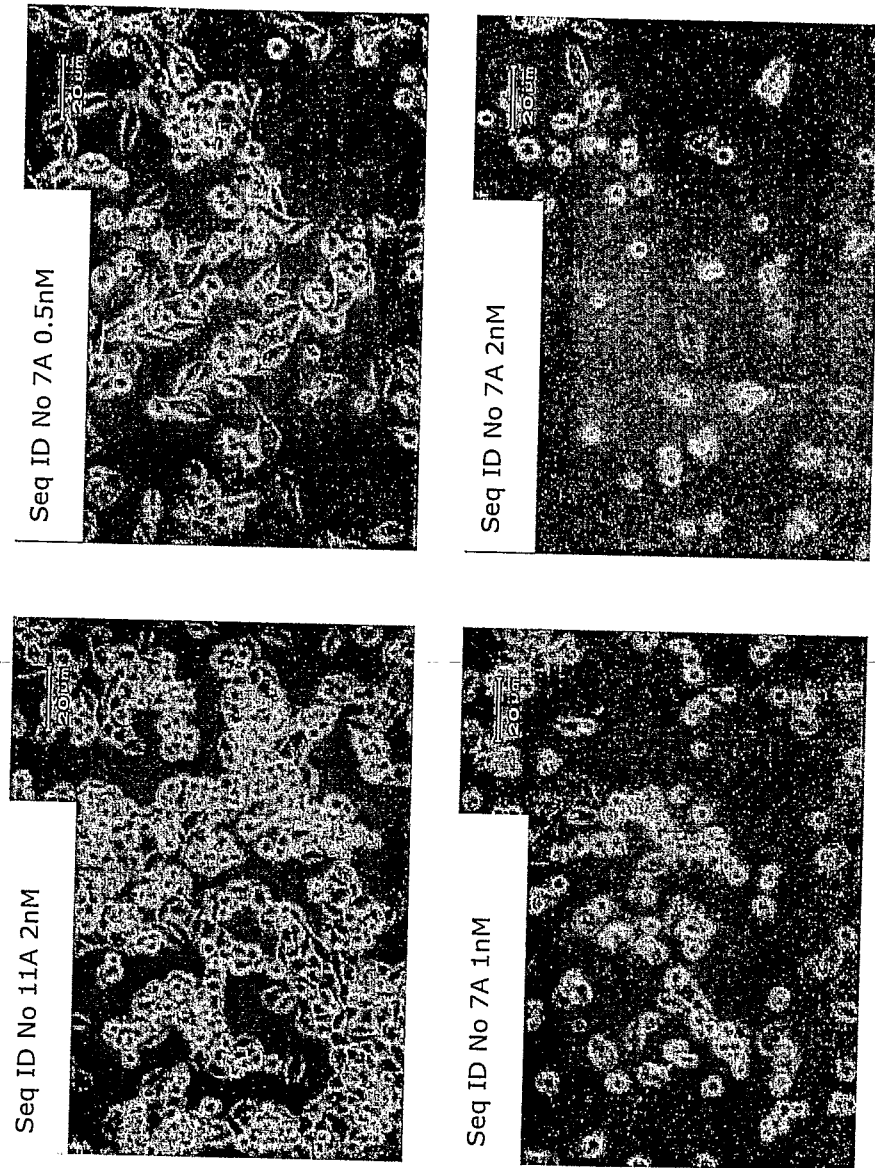
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FIGURE 2A

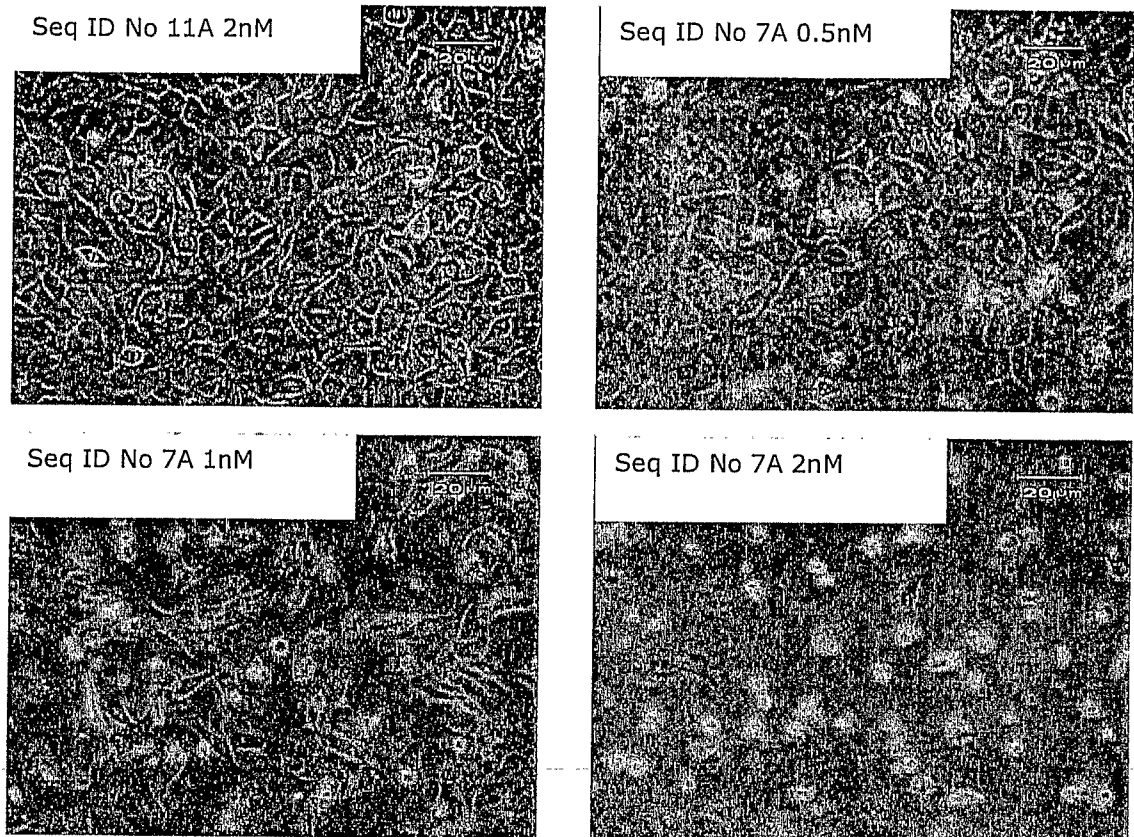
MP



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FIGURE 2B

T24



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FIGURE 2C

Mouse Fibroblasts



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FIGURE 3A

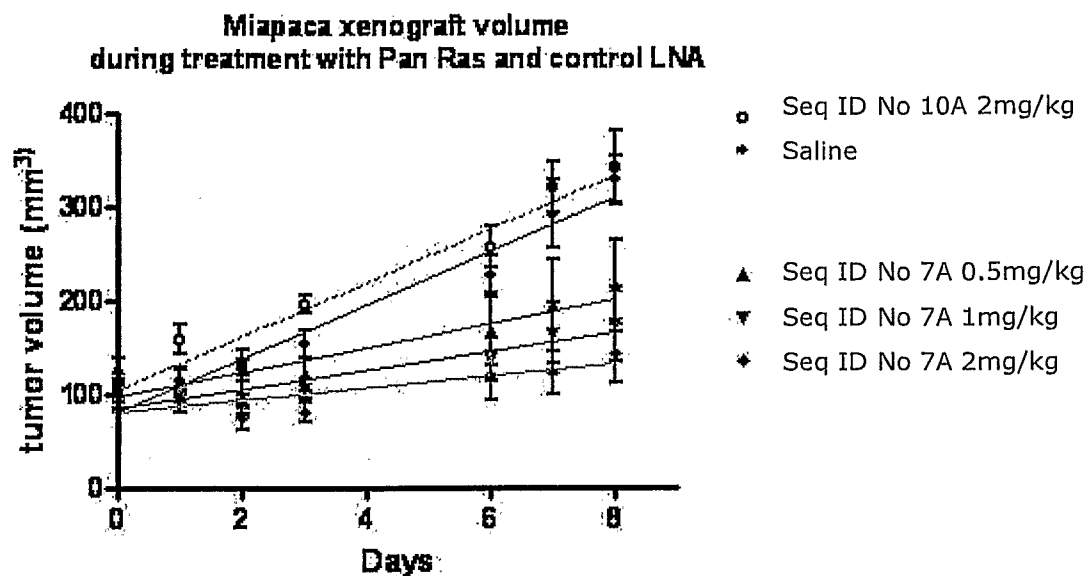
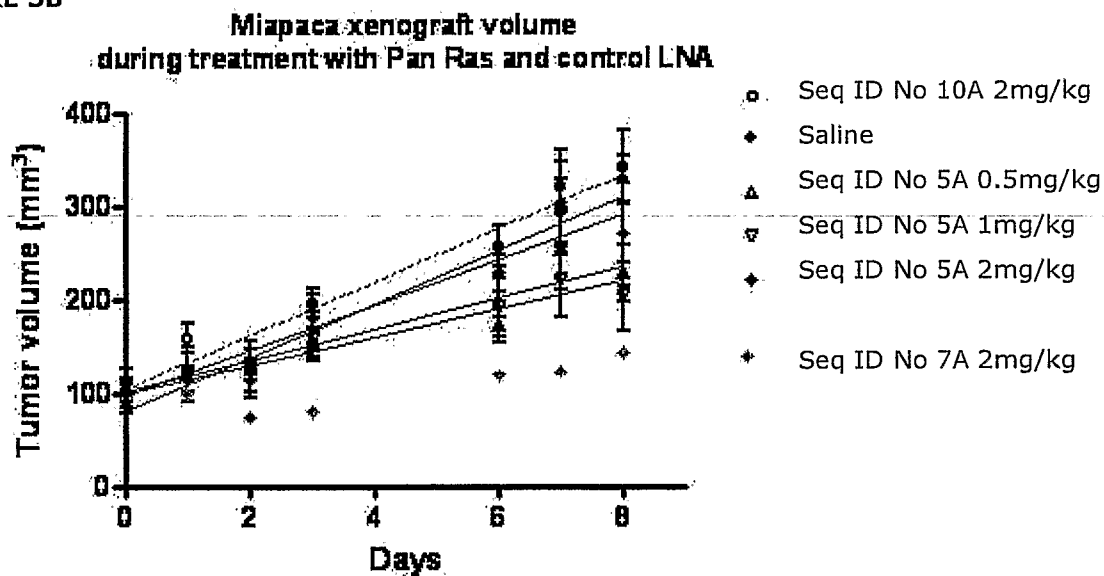


FIGURE 3B



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FIGURE 3C

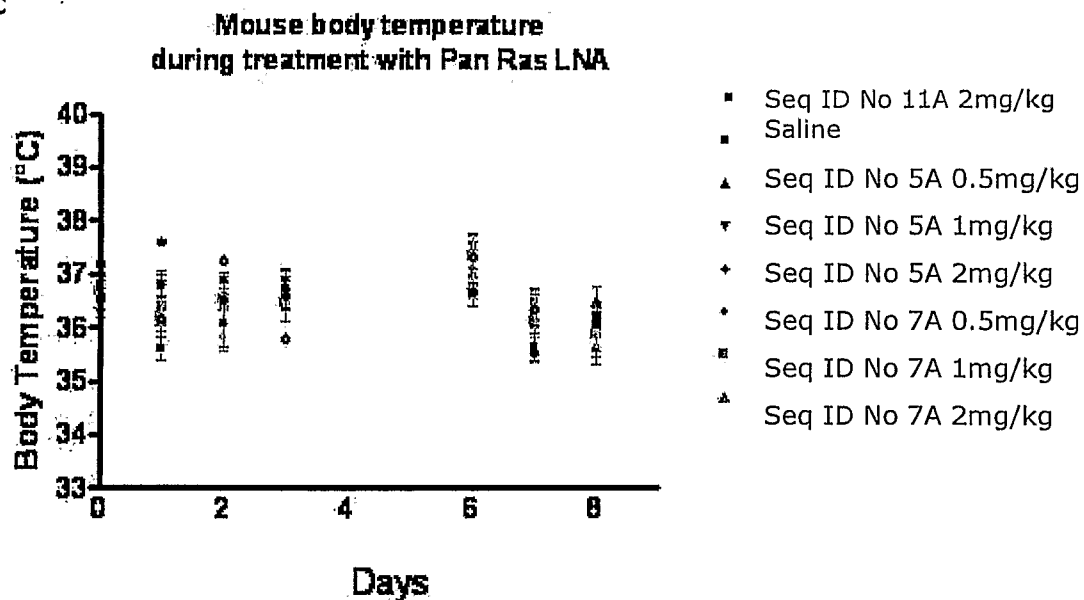
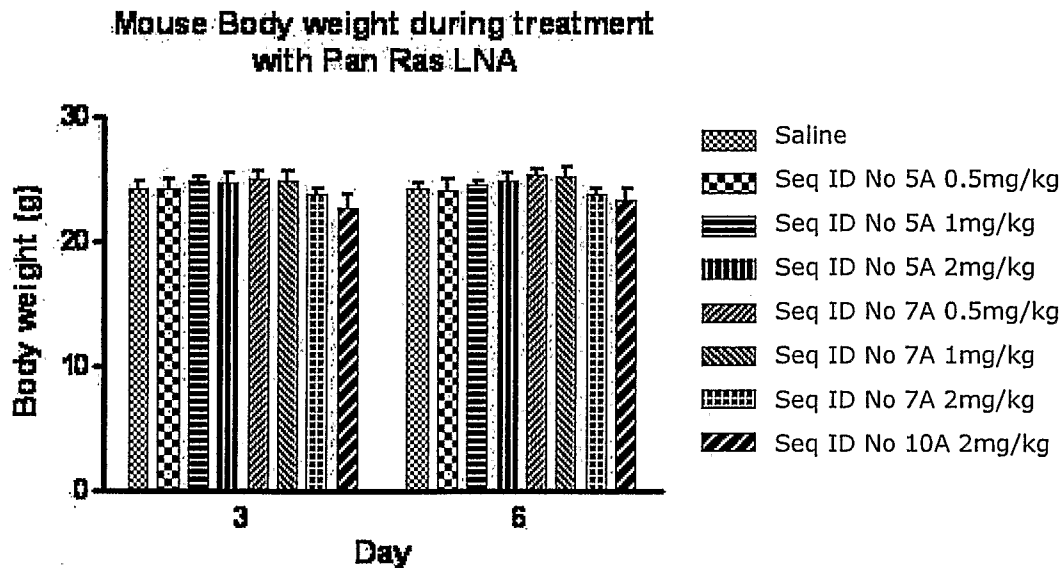


FIGURE 3D



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[illegible]

FIGURE 4

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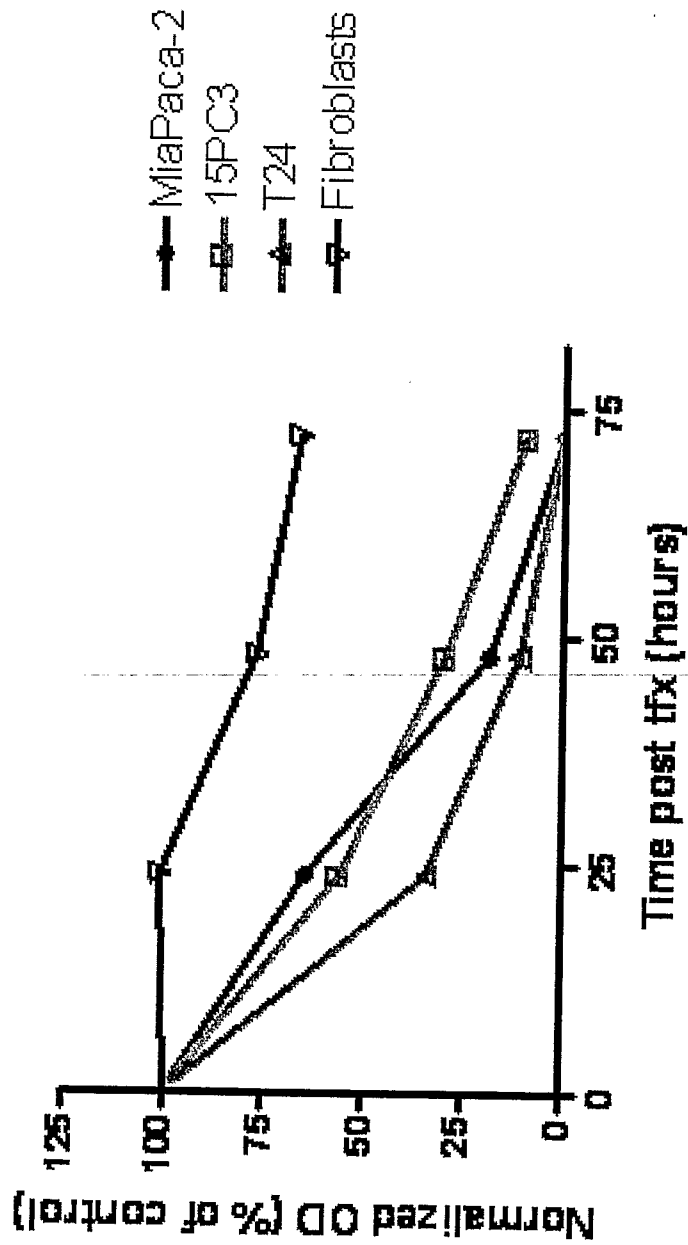
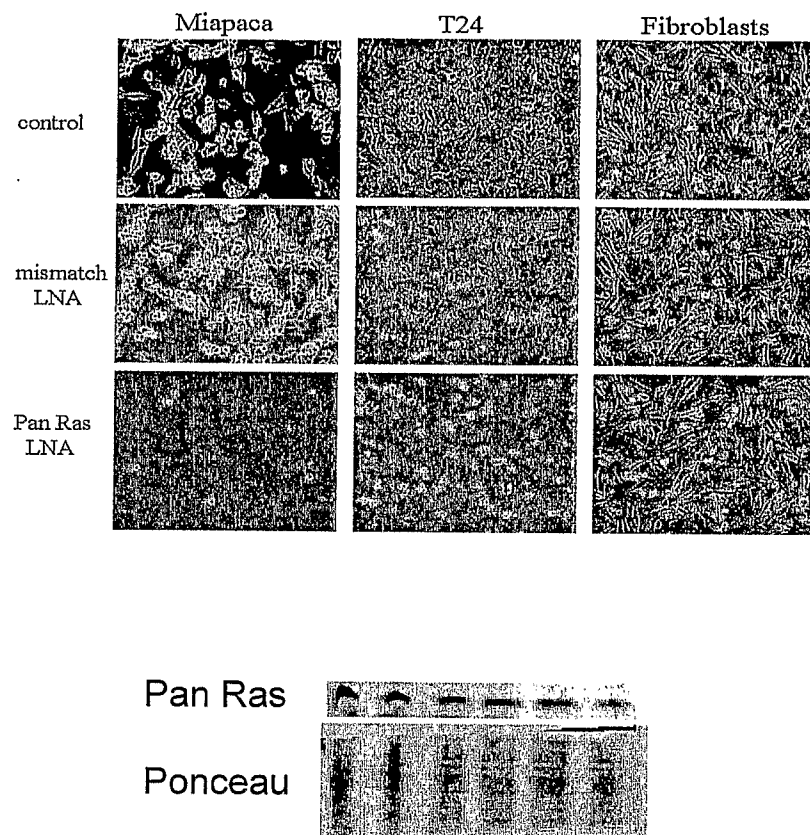


FIGURE 5

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**FIGURE 6**

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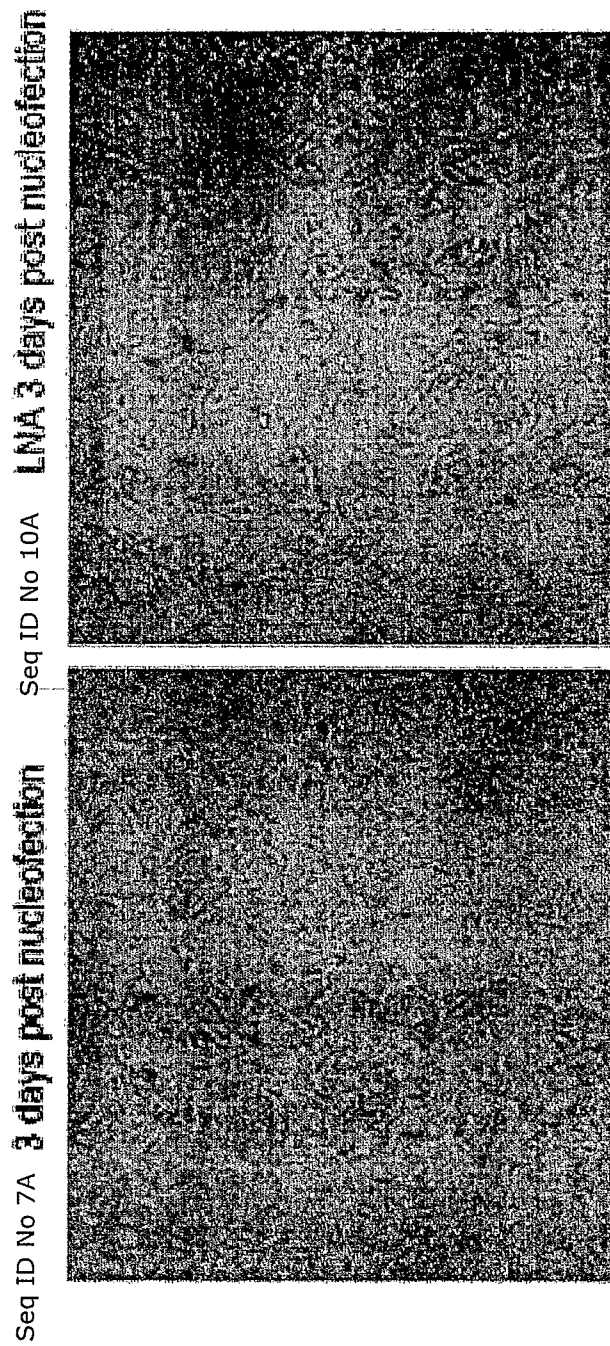


FIGURE 7

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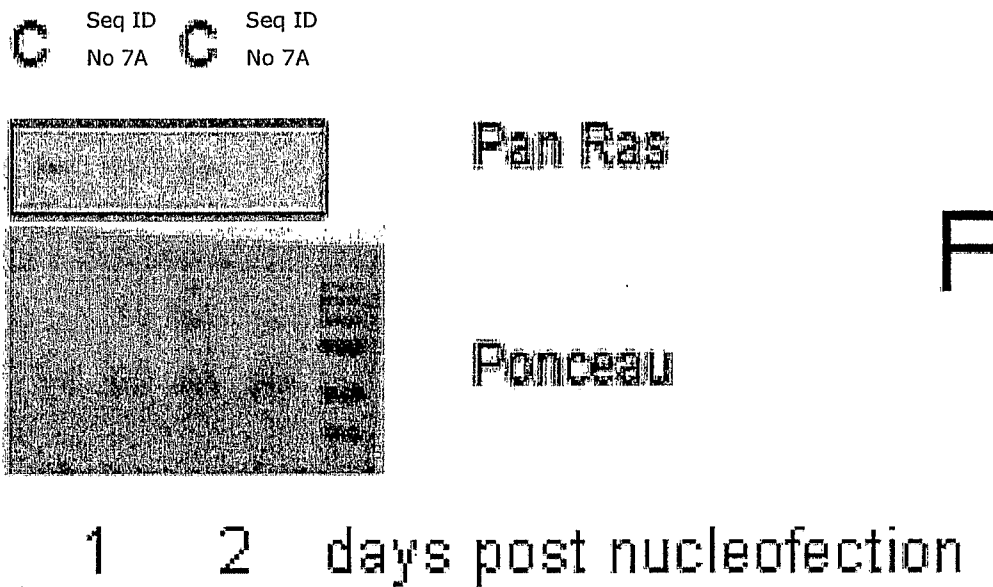


FIGURE 8

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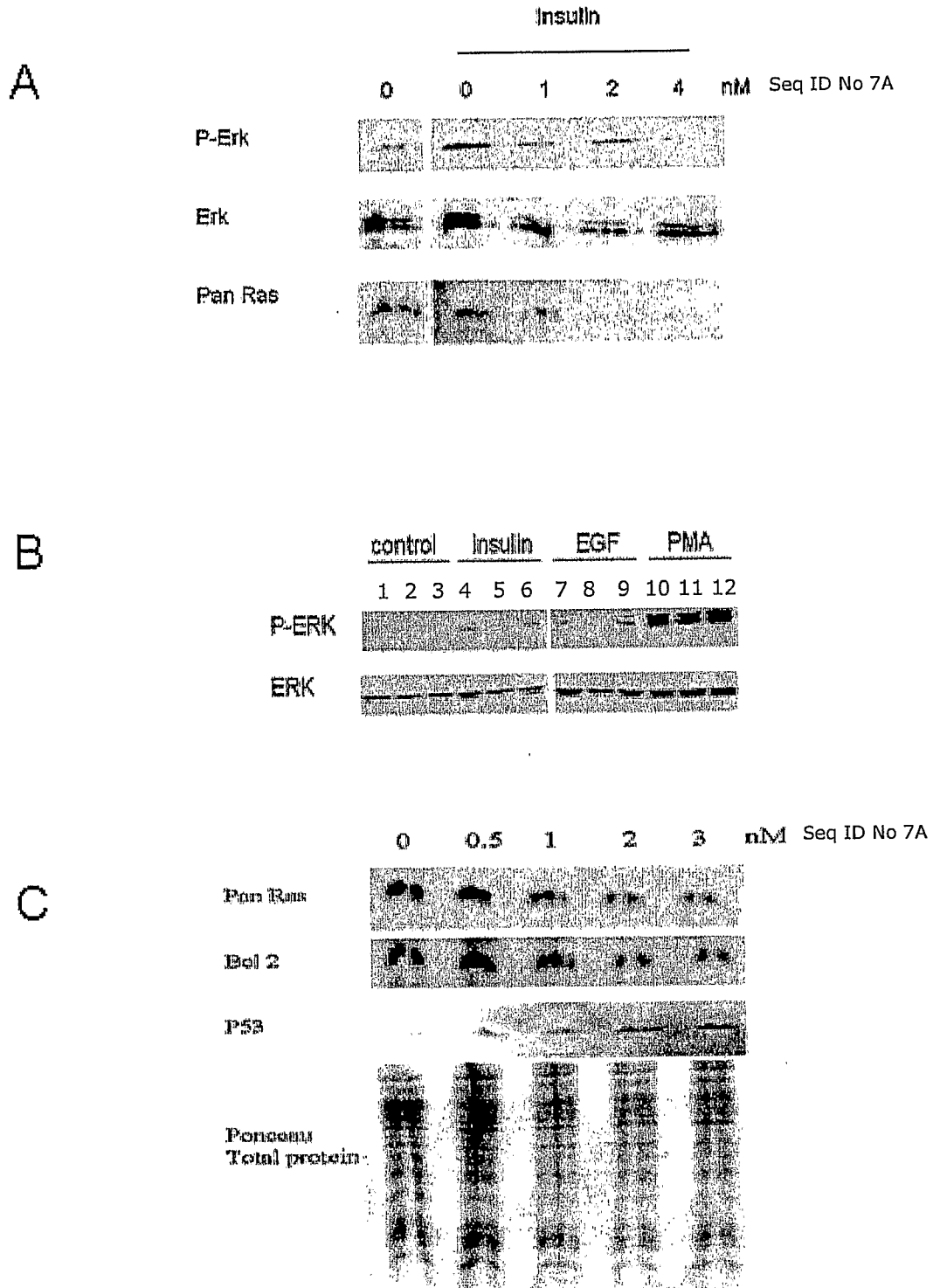


FIGURE 9

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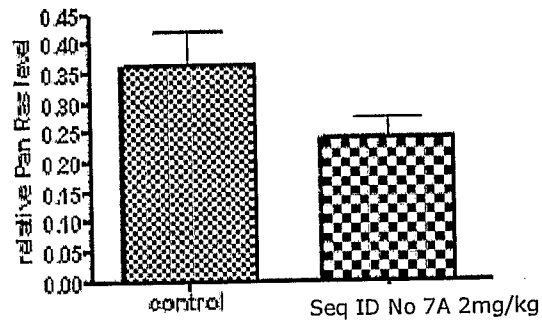


FIGURE 10

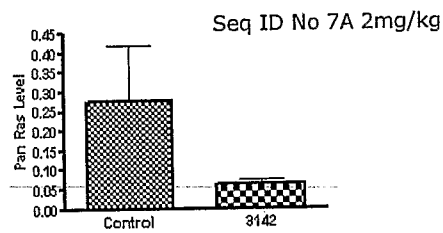


FIGURE 11

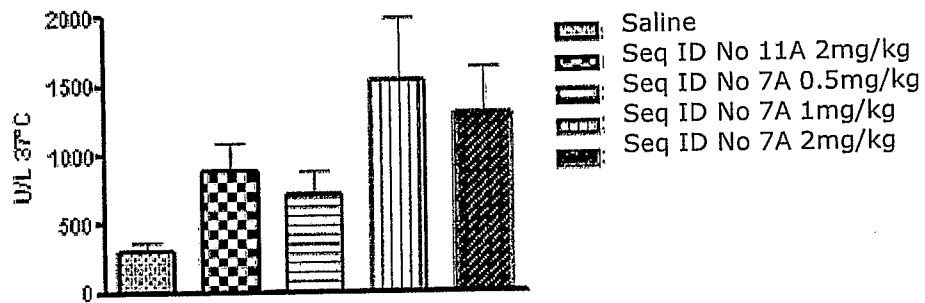


FIGURE 12

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Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly
 35 40 45

Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly His Glu Glu Tyr
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 35 40 45

Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr
 50 55 60

Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
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 35 40 45
 Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr
 50 55 60
 Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
 65 70 75 80
 Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His Leu Tyr
 85 90 95
 Arg Glu Gln Ile Lys Arg Val Lys Asp Ser Asp Asp Val Pro Met Val
 100 105 110
 Leu Val Gly Asn Lys Cys Asp Leu Pro Ala Arg Thr Val Asp Thr Lys
 115 120 125
 Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Glu Thr
 130 135 140
 Ser Ala Lys Thr Arg Gln Gly Val Glu Asp Ala Phe Tyr Thr Leu Val
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