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(54) Title: TREATING CANCER BY MODULATING A MNK

(57) Abstract: The present invention relates to a method for treating cancer in a subject using a mTOR inhibitor in combination with a therapeutically effective amount of a modulator of a MNK.



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Treating cancer by modulating a MNK**Field of the Invention**

The present invention relates to a method of treating cancer.

Background of the Invention

Cancers and malignant tumors are characterized by continuous cell proliferation and cell death and are related causally to both genetics and the environment. Genes whose expression are associated with cancer, and the products of said genes, are of potentially great importance as cancer markers in the early diagnosis and prognosis of various cancers, as well as potential targets in cancer treatment. Cancer is the a leading cause of human death next to coronary disease. In the United States, cancer causes the death of over a half-million people each year and about two million new cases of cancer are diagnosed each year. Glioblastoma multiform (GBM) is the most aggressive and lethal form of brain cancer with a mean patient survival time of less than 12 months. An estimated 10,000 patients are diagnosed with GBM each year in EU member countries.

The identification of new genes essential for the growth of tumors has been an objective of cancer research over the past several decades. Many factors involved in regulation of protein synthesis have been found to be mutated, differentially expressed or post-translationally modified in cancer cells supporting cancer cell growth and survival (Watkins and Norbury, 2002, Br J Cancer. 86:1023-7).

The overall rate of protein synthesis is an important factor modulating cell and tissue metabolism. In addition, the translational machinery plays key roles in controlling gene-specific expression in eukaryotic cells. The regulatory mechanisms involve changes in the activities of components of translational complexes that lead to translation of specific subsets of messenger RNAs. Modulations of translational activity are primarily mediated by changes in the phosphorylation states of translation factors or RNA-binding proteins promoting specific modes of translation (Proud, 2007, Biochem J. 403:217-34). Kinases closely associated with translation initiation complexes have a huge potential to regulate translation. MAP kinase interacting kinases (MNKs), which function downstream of p38 and ERK MAP kinases, bind to translation initiation factor eIF4G, and phosphorylate the cap-binding protein, translation initiation factor eIF4E (Buxade et al., 2008, Front Biosci. 1:5359-73.). Recent key finding demonstrates that eIF4E phosphorylation at Ser209 by MNK kinases is absolutely required for eIF4E action in opposing apoptosis and promoting tumorigenesis *in vivo* (Wendel et al., 2007, Genes Dev. 21:3232-7).

Summary of the invention

The present inventors have now surprisingly found that a MNK is significantly elevated in human brain tumors and that the inhibition of said MNK in a cancer cell lines leads to a drastic increase of the sensitivity of these cells to the mTOR inhibitor rapamycin.

The present invention hence provides a method for treating cancer in a subject, said method using a mTOR inhibitor for treating cancer in the subject, together with a therapeutically effective amount of a modulator of a MNK.

In an embodiment of the invention, the modulator of said MNK is an inhibitor, for instance a small molecule, an antibody or a siRNA.

In an embodiment of the invention, the subject is a mammal, for instance a human subject.

In yet another embodiment of the invention, the cancer is a cancer of the brain, for example an astrocytoma, a glioblastoma or an oligodendroglioma.

The present invention also encompasses a small molecule, a siRNA and/or an antibody, decreasing or silencing said MNK, for use as a medicament to treat cancer in combination with a mTOR inhibitor, as well as compositions comprising both a MNK inhibitor, for instance a MNK1 inhibitor, and mTOR inhibitors together.

The present invention also encompasses a method for the identification of a substance that modulates the expression of expression and/or biological activity of a MNK, which method comprises: (i) contacting a MNK polypeptide or a fragment thereof having the biological activity of said MNK, a polynucleotide encoding such a polypeptide or polypeptide fragment, an expression vector comprising such a polynucleotide or a cell comprising such an expression vector, and a test substance in the presence of a mTOR inhibitor, under conditions that in the absence of the test substance would permit expression and/or biological activity of said MNK; and (ii) determining the amount of expression and/or biological activity of said MNK, to determine whether the test substance modulates biological activity and/or expression of said MNK in the presence of the mTOR inhibitor, wherein a test substance which modulates biological activity and/or expression of said MNK in the presence of the mTOR inhibitor is a potential therapeutical agent to treat cancer.

In an embodiment of the invention the biological activity of the MNK is assessed through its kinase activity.

In some embodiments of the invention, the MNK is MNK1.

These and other aspects of the present invention should be apparent to those skilled in the art, from the teachings herein.

Brief Description of the Drawings

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1: Effect of pharmacological co-inhibition of MNK1 and mTOR in human glioblastoma BS125 and LN319 cells.

Total number of BS125 or LN319 cells was measured after incubation with 10 μ M CGP57380 (MNK1 inhibitor) and/or with 1nM rapamycin at indicated time points. Results are expressed as means $\pm$ SD of three independent experiments.

Figure 2: Treatment of BS125 and LN319 cells with 10 μ M CGP57380 and with increasing amounts of rapamycin significantly increases the inhibitory effect on cell proliferation in a dose-dependent manner.

Cell proliferation was assayed using MTT-based method 5 days after treatment. Growth in untreated cells was used as a reference. Data shown are the mean $\pm$ SD values of at least three separate experiments.

Figure 3: Effect on phosphorylation of S6K and eIF4E in BS125 treated cells monitored using Western blot analysis.

BS125 cells were treated with increasing amounts of MNK1 inhibitor, CGP57380 or with mTOR inhibitor, rapamycin and phosphorylation of eIF4E or S6K were analyzed using phospho-specific antibodies.

Figure 4: Over-expression of full-length MNK1-Flag fusion protein reduces growth inhibition of rapamycin treated BS125 cells.

BS125 cells were transfected either with construct for overexpression of MNK1-Flag protein or with control empty vector. Twenty-four hours after transfection cells were treated with rapamycin at different concentration (as indicated) for another forty-eight hours and used for viability determination by using an MTT-based assay. Growth in control-transfected and untreated cells was used as a reference. Results are expressed as means $\pm$ SD of three independent experiments.

Figure 5: MNK1-specific knockdown sensitizes BS125 cells to rapamycin.

BS125 cells were transfected either with duplex siRNA oligonucleotides against the *MNK1* gene (si MNK1) or with control duplex (Control). Twenty-four hours after transfection cells were treated with rapamycin at different concentration (as indicated) for another forty-eight hours and used for viability determination by using an MTT-based assay. Growth in control-transfected and untreated cells was used as a reference. Results are expressed as means $\pm$ SD of three independent experiments.

Figure 6: MNK1 protein level in BS125 cells transfected with full-length MNK1-Flag fusion protein determined using Western blot analysis.

BS125 cells were transfected either with construct for overexpression of MNK1-Flag protein or with control vector. Twenty-four hours after transfection cells were treated with rapamycin at different concentration (as indicated) for another forty-eight hours and protein lysates were subjected to immunoblotting using MNK1-specific antibody.

Figure 7: MNK1 protein level in BS125 cells transfected MNK1-specific siRNA determined using Western blot analysis.

BS125 cells were transfected either with duplex siRNA oligonucleotides against the *MNK1* gene (si MNK1) or with control duplex (Control). Twenty-four hours after transfection cells were treated with rapamycin at different concentration (as indicated) for another forty-eight hours and protein lysates were subjected to immunoblotting using MNK1-specific antibody.

Figure 8: MNK1 is overexpressed in human gliomas.

MNK1 transcript levels in primary brain tumors were obtained by microarray analysis. HNA: human normal astrocytes; Oligo: oligodendrioglioma; Astro: astrocytoma; GBM: primary glioblastoma and GBM; NB: normal brain.

Detailed Description of the Invention

The present inventors have now surprisingly found that the inhibition of a MNK in a cancer cell lines leads to a drastic increase of the sensitivity of these cells to the mTOR inhibitor rapamycin.

The present inventors have now surprisingly found that a MNK is significantly elevated in human brain tumors and that the inhibition of said MNK in a cancer cell lines leads to a drastic increase of the sensitivity of these cells to the mTOR inhibitor rapamycin.

The present invention hence provides a method for treating cancer in a subject, said method using a mTOR inhibitor for treating cancer in the subject, together with a therapeutically effective amount of a modulator of a MNK.

In an embodiment of the invention, the modulator of said MNK is an inhibitor, for instance a small molecule, an antibody or a siRNA.

In an embodiment of the invention, the subject is a mammal, for instance a human subject.

In yet another embodiment of the invention, the cancer is a cancer of the brain, for example an astrocytoma, a glioblastoma or an oligodendroglioma.

The present invention also encompasses a small molecule, a siRNA and/or an antibody, decreasing or silencing the MNK, for use as a medicament to treat cancer in combination with a mTOR inhibitor, as well as compositions comprising both MNK inhibitor, for instance a MNK1 inhibitor, and mTOR inhibitor together.

The present invention also encompasses a method for the identification of a substance that modulates the expression of expression and/or biological activity of a MNK, which method comprises: (i) contacting a MNK polypeptide or a fragment thereof having the biological activity of said MNK, a polynucleotide encoding such a polypeptide or polypeptide fragment, an expression vector comprising such a polynucleotide or a cell comprising such an expression vector, and a test substance in the presence of a mTOR inhibitor, under conditions that in the absence of the test substance would permit expression and/or biological activity of said MNK; and (ii) determining the amount of expression and/or biological activity of said MNK, to determine whether the test substance modulates biological activity and/or expression of said MNK in the presence of the mTOR inhibitor, wherein a test substance which modulates the biological activity and/or expression said MNK in the presence of the mTOR inhibitor is a potential therapeutical agent to treat cancer.

In an embodiment of the invention the biological activity of said MNK is assessed through its kinase activity.

In some embodiments of the invention, the MNK is MNK1.

In addition, the present invention also encompasses the modulators of the expression of expression and/or of its biological activity of a MNK identified using a method of screening of the invention.

Another embodiment of the invention encompasses the use of a MNK as a biomarker for cancer, brain cancer in particular. In this embodiment, the expression level or protein concentration of said MNK is measured in a sample from a subject and compared to the expression level or protein concentration in a normal subject, whereas said normal subject can be a pool of subjects.

These and other aspects of the present invention should be apparent to those skilled in the art, from the teachings herein.

The following definitions are provided to facilitate understanding of certain terms used throughout this specification.

In the present invention, "isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state. For example, an isolated polynucleotide could be part of a vector or a composition of matter, or could be contained within a cell, and still be "isolated" because that vector, composition of matter, or particular cell is not the original environment of the polynucleotide. The term "isolated" does not refer to genomic or cDNA libraries, whole cell total or mRNA preparations, genomic DNA preparations (including those separated by electrophoresis and transferred onto blots), sheared whole cell genomic DNA preparations or other compositions where the art demonstrates no distinguishing features of the polynucleotide/sequences of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or

substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. However, a nucleic acid contained in a clone that is a member of a library (e.g., a genomic or cDNA library) that has not been isolated from other members of the library (e.g., in the form of a homogeneous solution containing the clone and other members of the library) or a chromosome removed from a cell or a cell lysate (e.g., a "chromosome spread", as in a karyotype), or a preparation of randomly sheared genomic DNA or a preparation of genomic DNA cut with one or more restriction enzymes is not "isolated" for the purposes of this invention. As discussed further herein, isolated nucleic acid molecules according to the present invention may be produced naturally, recombinantly, or synthetically.

In the present invention, a "secreted" protein refers to a protein capable of being directed to the ER, secretory vesicles, or the extracellular space as a result of a signal sequence, as well as a protein released into the extracellular space without necessarily containing a signal sequence. If the secreted protein is released into the extracellular space, the secreted protein can undergo extracellular processing to produce a "mature" protein. Release into the extracellular space can occur by many mechanisms, including exocytosis and proteolytic cleavage.

"Polynucleotides" can be composed of single-and double-stranded DNA, DNA that is a mixture of single-and double-stranded regions, single-and double-stranded RNA, and RNA that is mixture of single-and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single-and double-stranded regions. In addition, polynucleotides can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. Polynucleotides may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

The expression "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

"Stringent hybridization conditions" refers to an overnight incubation at 42 degree C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 50 degree C. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, moderately high stringency conditions include an overnight incubation at 37 degree C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH₂PO₄; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50 degree C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

The terms "fragment," "derivative" and "analog" when referring to polypeptides means polypeptides which either retain substantially the same biological function or activity as such polypeptides. An analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons).

Polypeptides can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include, but are not limited to, acetylation, acylation, biotinylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, derivatization by known protecting/blocking groups, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, linkage to an antibody molecule or other cellular ligand, methylation, myristoylation, oxidation, pegylation, proteolytic processing (e.g., cleavage), phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS-STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. I-12 (1983); Seifter et al., Meth Enzymol 182:626-646 (1990); Rattan et al., Ann NY Acad Sci 663:48-62 (1992).)

A polypeptide fragment "having biological activity" refers to polypeptides exhibiting activity similar, but not necessarily identical to, an activity of the original polypeptide, including mature forms, as measured in a particular biological assay, with or without dose dependency. In the case where dose dependency does exist, it need not be identical to that of the polypeptide, but rather substantially similar to the dose-dependence in a given activity as compared to the original polypeptide (i.e., the candidate polypeptide will exhibit greater activity or not more than about 25-fold less and, in some embodiments, not more than about tenfold less activity, or not more than about three-fold less activity relative to the original polypeptide.)

Species homologs may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source for the desired homologue.

"Variant" refers to a polynucleotide or polypeptide differing from the original polynucleotide or polypeptide, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the original polynucleotide or polypeptide.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or 100% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=-1, Joining Penalty=-30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=-5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter. If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 impaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99%, or 100% identical to, for instance, the amino acid sequences shown in a sequence or to the amino acid sequence encoded by deposited DNA clone can be determined conventionally using known computer programs. A preferred method for determining, the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=-1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=-5, Gap Size Penalty=-0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. If the subject sequence is shorter than the query sequence due to N-or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N-and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N-and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N-and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N-and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N-and C-terminal residues of the subject sequence. Only residue positions outside the N-and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

Naturally occurring protein variants are called "allelic variants," and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. (Genes 11, Lewin, B., ed., John Wiley & Sons, New York (1985).) These allelic variants can vary at either the polynucleotide and/or polypeptide level. Alternatively, non-naturally occurring variants may be produced by mutagenesis techniques or by direct synthesis. Using known methods of protein engineering and recombinant DNA technology, variants may be generated to improve or alter the characteristics of polypeptides. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of a secreted protein without substantial loss of biological function. The authors of Ron et al., J. Biol. Chem. 268: 2984-2988 (1993), reported variant KGF proteins having heparin binding activity even after deleting 3, 8, or 27 amino-terminal amino acid residues. Similarly, Interferon gamma exhibited up to ten times higher activity after deleting 8-10 amino acid residues from the carboxy terminus of this protein (Dobeli et al., J. Biotechnology 7:199-216 (1988)). Moreover, ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. For example, Gayle and co-workers (J. Biol. Chem 268:22105-22111 (1993)) conducted extensive mutational analysis of human cytokine IL-1a. They used random mutagenesis to generate over 3,500 individual IL-1a mutants that averaged 2.5 amino acid changes per variant over the entire length of the molecule. Multiple mutations were examined at every possible amino acid position. The investigators found that "[most of the molecule could be altered with little effect on either [binding or

biological activity]." (See, Abstract.) In fact, only 23 unique amino acid sequences, out of more than 3,500 nucleotide sequences examined, produced a protein that significantly differed in activity from wild-type. Furthermore, even if deleting one or more amino acids from the N-terminus or C-terminus of a polypeptide results in modification or loss of one or more biological functions, other biological activities may still be retained. For example, the ability of a deletion variant to induce and/or to bind antibodies which recognize the secreted form will likely be retained when less than the majority of the residues of the secreted form are removed from the N-terminus or C-terminus. Whether a particular polypeptide lacking N-or C-terminal residues of a protein retains such immunogenic activities can readily be determined by routine methods described herein and otherwise known in the art.

In one embodiment where one is assaying for the ability to bind or compete with full-length MNK polypeptide for binding to MNK antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination, assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody.

In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Assays described herein and otherwise known in the art may routinely be applied to measure the ability of MNK polypeptides and fragments, variants derivatives and analogs thereof to elicit MNK-related biological activity (either *in vitro* or *in vivo*).

The term "epitopes," as used herein, refers to portions of a polypeptide having antigenic or immunogenic activity in an animal, in some embodiments, a mammal, for instance in a human. In an embodiment, the present invention encompasses a polypeptide comprising an epitope, as well as the polynucleotide encoding this polypeptide. An "immunogenic epitope," as used herein, is defined as a portion of a protein that elicits an antibody response in an animal, as determined by any method known in the art, for example, by the methods for generating antibodies described *infra*. (See, for example, Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1983)). The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art, for example, by the immunoassays described herein. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic.

Fragments which function as epitopes may be produced by any conventional means. (See, e.g., Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985), further described in U.S. Patent No. 4,631,211).

As one of skill in the art will appreciate, and as discussed above, polypeptides comprising an immunogenic or antigenic epitope can be fused to other polypeptide sequences. For example, polypeptides may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof), or albumin (including but not limited to recombinant albumin (see, e.g., U.S. Patent No. 5,876, 969, issued March 2, 1999, EP Patent 0 413 622, and U.S. Patent No. 5,766,883, issued June 16, 1998)), resulting in chimeric polypeptides. Such fusion proteins may facilitate purification and may increase half-

life *in vivo*. This has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See, e.g., EP 394,827; Traunecker et al., *Nature*, 331:84-86 (1988).

Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (e.g., insulin) conjugated to an FcRn binding partner such as IgG or Fc fragments (see, e.g., PCT Publications WO 96/22024 and WO 99/04813). IgG Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion disulfide bonds have also been found to be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. See, e.g., Fountoulakis et al., *J. Biochem.*, 270:3958-3964 (1995). Nucleic acids encoding the above epitopes can also be recombined with a gene of interest as an epitope tag (e.g., the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni²⁺ nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers. Additional fusion proteins may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to modulate the activities of polypeptides of the invention, such methods can be used to generate polypeptides with altered activity, as well as agonists and antagonists of the polypeptides. See, generally, U.S. Patent Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., *Curr. Opin. Biotechnol.* 8:724-33 (1997); Harayama, *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, et al., *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo and Blasco, *Biotechniques* 24(2):308-13 (1998).

Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

In addition, in the context of the present invention, the term "antibody" shall also encompass alternative molecules having the same function, e.g. aptamers and/or CDRs grafted onto alternative peptidic or non-peptidic frames.

In some embodiments the antibodies are human antigen-binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')₂, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. In some embodiments, the antibodies are human, murine (e.g., mouse and rat), donkey, sheep rabbit, goat, guinea pig, camel, shark, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a

human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described *infra* and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati et al. The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multi specificity. Multispecific antibodies may be specific for different epitopes of a polypeptide or may be specific for both a polypeptide as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Patent Nos. 4, 474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide which they recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be specified as described herein, e.g., by N-terminal and C-terminal positions, by size in contiguous amino acid residues.

Antibodies may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of a polypeptide of the present invention are included. Antibodies that bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide are also included in the present invention. In specific embodiments, antibodies of the present invention cross-react with murine, rat and/or rabbit homologs of human proteins and the corresponding epitopes thereof. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide are also included in the present invention.

Antibodies may also be described or specified in terms of their binding affinity to a polypeptide

Antibodies may act as agonists or antagonists of the recognized polypeptides. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or of one of its down-stream substrates by immunoprecipitation followed by western blot analysis (for example, as described *supra*). In specific embodiments, antibodies are provided that inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex. Likewise, encompassed by the invention are antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides disclosed herein. The above antibody agonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Patent No. 5,811, 097; Deng et al., Blood 92(6):1981-1988 (1998); Chen et al., Cancer Res. 58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4):1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. III(Pt2) :237-247 (1998); Pitard et al., J. Immunol. Methods 205(2):177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997); Carlson et al., J. Biol. Chem.

272(17):11295-11301 (1997); Taryman et al., *Neuron* 14(4):755-762 (1995); Muller et al., *Structure* 6(9):1153-1167 (1998); Bartunek et al., *Cytokine* 8(1):14-20 (1996).

As discussed in more detail below, the antibodies may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionuclides, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396, 387.

The antibodies as defined for the present invention include derivatives that are modified, i. e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen-of-interest can be produced by various procedures well known in the art. For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen.

Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')₂ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). F(ab')₂ fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

For example, the antibodies can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry

the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., *J. Immunol. Methods* 182:41-50 (1995); Ames et al., *J. Immunol. Methods* 184:177-186 (1995); Kettleborough et al., *Eur. J. Immunol.* 24:952-958 (1994); Persic et al., *Gene* 187 9-18 (1997); Burton et al., *Advances in Immunology* 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5, 698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821, 047; 5,571, 698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108. As described in these references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')₂ fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax. et al., *BioTechniques* 12(6):864-869 (1992); and Sawai et al., *AJRI* 34:26-34 (1995); and Better et al., *Science* 240:1041-1043 (1988).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al., *Methods in Enzymology* 203:46-88 (1991); Shu et al., *PNAS* 90:7995-7999 (1993); and Skerra et al., *Science* 240:1038-1040 (1988). For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, *Science* 229:1202 (1985); Oi et al., *BioTechniques* 4:214 (1986); Gillies et al., (1989) *J. Immunol. Methods* 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and a framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, and/or improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modelling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; Riechmann et al., *Nature* 332:323 (1988).) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592, 106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-498 (1991); Studnicka et al., *Protein Engineering* 7(6):805-814 (1994); Roguska. et al., *PNAS* 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716, 111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741.

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol.* 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e. g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569, 825; 5, 661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., *Bio/technology* 12:899-903 (1988)).

Furthermore, antibodies can be utilized to generate anti-idiotypic antibodies that "mimic" polypeptides using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, *FASEB J.* 7(5):437-444; (1989) and Nissinoff, *J. Immunol.* 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization. and/or binding of a polypeptide to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization. and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide and/or to bind its ligands/receptors, and thereby block its biological activity.

Polynucleotides encoding antibodies, comprising a nucleotide sequence encoding an antibody are also encompassed. These polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a

polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., *BioTechniques* 17:242 (1994)), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

The amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well known in the art, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, e.g., into human framework regions to humanize a non-human antibody, as described supra. The framework regions may be naturally occurring or consensus framework regions, and in some embodiments, human framework regions (see, e.g., Chothia et al., *J. Mol. Biol.* 278: 457-479 (1998) for a listing of human framework regions). In some embodiments, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide. In some embodiments, as discussed supra, one or more amino acid substitutions may be made within the framework regions, and, in some embodiments, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present description and within the skill of the art.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci.* 81:851-855 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described supra, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778; Bird, *Science* 242:423-42 (1988); Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883 (1988); and Ward et al., *Nature* 334:544-54 (1989)) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra et al., *Science* 242:1038-1041 (1988)).

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, in some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, in some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide).

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety, for instance to increase their therapeutical activity. The conjugates can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor

necrosis factor, α -interferon, B-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF- α , TNF- β , AIM I (See, International Publication No. WO 97/33899), AIM 11 (See, International Publication No. WO 97/34911), Fas Ligand (Takahashi et al., *Int. Immunol.*, 6:1567-1574 (1994)), VEGF (See, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors. Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676, 980.

The present invention is also directed to antibody-based therapies which involve administering antibodies of the invention to an animal, in some embodiments, a mammal, for example a human, patient to treat cancer. Therapeutic compounds include, but are not limited to, antibodies (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof and anti-idiotypic antibodies as described herein). Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

The invention also provides methods for treating cancer in a subject by inhibiting a MNK, e.g. MNK1, by administration to the subject of an effective amount of an inhibitory compound or pharmaceutical composition comprising such inhibitory compound. In some embodiments, said inhibitory compound is an antibody or an siRNA. In an embodiment, the compound is substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is in some embodiments, an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is in some embodiments, a mammal, for example human.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a compound, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e. g., Wu and Wu, *J. Biol. Chem.* 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it

may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*) In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Rev. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., *Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

The present invention also provides pharmaceutical compositions for use in the treatment of cancer by inhibiting a MNK, e.g. MNK1. Such compositions comprise a therapeutically effective amount of an inhibitory compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U. S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as

pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, in some embodiments, in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In an embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anaesthetic such as lidocaine to ease pain at the site of the injection.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent.

Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms.

Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. The amount of the compound which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. In some embodiments, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, for example 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

Also encompassed is a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The antibodies as encompassed herein may also be chemically modified derivatives which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased

immunogenicity (see U. S. Patent No. 4,179,337). The chemical moieties for derivatisation may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The antibodies may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100000 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog). For example, the polyethylene glycol may have an average molecular weight of about 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,600, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, or 100,000 kDa. As noted above, the polyethylene glycol may have a branched structure. Branched polyethylene glycols are described, for example, in U. S. Patent No. 5,643, 575; Morpurgo et al., *Appl. Biochem. Biotechnol.* 56:59-72 (1996); Vorobjev et al., *Nucleosides Nucleotides* 18:2745-2750 (1999); and Caliceti et al., *Bioconjug. Chem.* 10:638-646 (1999). The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g., EP 0 401 384 (coupling PEG to G-CSF), see also Malik et al., *Exp. Hematol.* 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group. As suggested above, polyethylene glycol may be attached to proteins via linkage to any of a number of amino acid residues. For example, polyethylene glycol can be linked to proteins via covalent bonds to lysine, histidine, aspartic acid, glutamic acid, or cysteine residues. One or more reaction chemistries may be employed to attach polyethylene glycol to specific amino acid residues (e.g., lysine, histidine, aspartic acid, glutamic acid, or cysteine) of the protein or to more than one type of amino acid residue (e.g., lysine, histidine, aspartic acid, glutamic acid, cysteine and combinations thereof) of the protein. As indicated above, pegylation of the proteins of the invention may be accomplished by any number of means. For example, polyethylene glycol may be attached to the protein either directly or by an intervening linker. Linkerless systems for attaching polyethylene glycol to proteins are described in Delgado et al., *Crit. Rev. Thera. Drug Carrier Sys.* 9:249-304 (1992); Francis et al., *Intern. J. of Hematol.* 68:1-18 (1998); U.S. Patent No. 4,002,53 1; U.S. Patent No. 5,349,052; WO 95/06058; and WO 98/32466.

By "biological sample" is intended any biological sample obtained from an individual, body fluid, cell line, tissue culture, or other source which contains the polypeptide of the present invention or mRNA. As indicated, biological samples include body fluids (such as semen, lymph, sera, plasma, urine, synovial fluid and spinal fluid) which

contain the polypeptide of the present invention, and other tissue sources found to express the polypeptide of the present invention. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

"RNAi" is the process of sequence specific post-transcriptional gene silencing in animals and plants. It uses small interfering RNA molecules (siRNA) that are double-stranded and homologous in sequence to the silenced (target) gene. Hence, sequence specific binding of the siRNA molecule with mRNAs produced by transcription of the target gene allows very specific targeted knockdown of gene expression.

"siRNA" or "small-interfering ribonucleic acid" according to the invention has the meanings known in the art, including the following aspects. The siRNA consists of two strands of ribonucleotides which hybridize along a complementary region under physiological conditions. The strands are normally separate. Because of the two strands have separate roles in a cell, one strand is called the "anti-sense" strand, also known as the "guide" sequence, and is used in the functioning RISC complex to guide it to the correct mRNA for cleavage. This use of "anti-sense", because it relates to an RNA compound, is different from the antisense target DNA compounds referred to elsewhere in this specification. The other strand is known as the "anti-guide" sequence and because it contains the same sequence of nucleotides as the target sequence, it is also known as the sense strand. The strands may be joined by a molecular linker in certain embodiments. The individual ribonucleotides may be unmodified naturally occurring ribonucleotides, unmodified naturally occurring deoxyribonucleotides or they may be chemically modified or synthetic as described elsewhere herein.

In some embodiments, the siRNA molecule is substantially identical with at least a region of the coding sequence of the target gene to enable down-regulation of the gene. In some embodiments, the degree of identity between the sequence of the siRNA molecule and the targeted region of the gene is at least 60% sequence identity, in some embodiments at least 75% sequence identity, for instance at least 85% identity, 90% identity, at least 95% identity, at least 97%, or at least 99% identity.

Calculation of percentage identities between different amino acid/polypeptide/nucleic acid sequences may be carried out as follows. A multiple alignment is first generated by the ClustalX program (pairwise parameters: gap opening 10.0, gap extension 0.1, protein matrix Gonnet 250, DNA matrix IUB; multiple parameters: gap opening 10.0, gap extension 0.2, delay divergent sequences 30%, DNA transition weight 0.5, negative matrix off, protein matrix gonnet series, DNA weight IUB; Protein gap parameters, residue-specific penalties on, hydrophilic penalties on, hydrophilic residues GPSNDQERK, gap separation distance 4, end gap separation off). The percentage identity is then calculated from the multiple alignment as $(N/T) \times 100$, where N is the number of positions at which the two sequences share an identical residue, and T is the total number of positions compared. Alternatively, percentage identity can be calculated as $(N/S) \times 100$ where S is the length of the shorter sequence being compared. The amino acid/polypeptide/nucleic acid sequences may be synthesised de novo, or may be native amino acid/polypeptide/nucleic acid sequence, or a derivative thereof. A substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to any of the nucleic acid sequences referred to herein or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to filter-bound DNA or RNA in 6x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2x SSC/0.1% SDS at approximately 5-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the peptide sequences according to the present invention. Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence could be varied or changed without substantially affecting the sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of

different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequences which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine; large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine; the polar neutral amino acids include serine, threonine, cysteine, asparagine and glutamine; the positively charged (basic) amino acids include lysine, arginine and histidine; and the negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The accurate alignment of protein or DNA sequences is a complex process, which has been investigated in detail by a number of researchers. Of particular importance is the trade-off between optimal matching of sequences and the introduction of gaps to obtain such a match. In the case of proteins, the means by which matches are scored is also of significance. The family of PAM matrices (e.g., Dayhoff, M. et al., 1978, Atlas of protein sequence and structure, Natl. Biomed. Res. Found.) and BLOSUM matrices quantify the nature and likelihood of conservative substitutions and are used in multiple alignment algorithms, although other, equally applicable matrices will be known to those skilled in the art. The popular multiple alignment program ClustalW, and its windows version ClustalX (Thompson et al., 1994, Nucleic Acids Research, 22, 4673-4680; Thompson et al., 1997, Nucleic Acids Research, 24, 4876-4882) are efficient ways to generate multiple alignments of proteins and DNA.

Frequently, automatically generated alignments require manual alignment, exploiting the trained user's knowledge of the protein family being studied, e.g., biological knowledge of key conserved sites. One such alignment editor programs is Align (<http://www.gwdg.de/dhepper/download/>; Hepperle, D., 2001: Multicolor Sequence Alignment Editor. Institute of Freshwater Ecology and Inland Fisheries, 16775 Stechlin, Germany), although others, such as JalView or Cinema are also suitable.

Calculation of percentage identities between proteins occurs during the generation of multiple alignments by Clustal. However, these values need to be recalculated if the alignment has been manually improved, or for the deliberate comparison of two sequences. Programs that calculate this value for pairs of protein sequences within an alignment include PROTDIST within the PHYLIP phylogeny package (Felsenstein; <http://evolution.gs.washington.edu/phylip.html>) using the "Similarity Table" option as the model for amino acid substitution (P). For DNA/RNA, an identical option exists within the DNADIST program of PHYLIP.

The dsRNA molecules in accordance with the present invention comprise a double-stranded region which is substantially identical to a region of the mRNA of the target gene. A region with 100% identity to the corresponding sequence of the target gene is suitable. This state is referred to as "fully complementary". However, the region may also contain one, two or three mismatches as compared to the corresponding region of the target gene, depending on the length of the region of the mRNA that is targeted, and as such may be not fully complementary. In an embodiment, the RNA molecules of the present invention specifically target one given gene. In order to only target the desired mRNA, the siRNA reagent may have 100% homology to the target mRNA and at least 2 mismatched nucleotides to all other genes present in the cell or organism. Methods to analyze and identify siRNAs with sufficient sequence identity in order to effectively inhibit expression of a specific target sequence are known in the art. Sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for

example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group).

The length of the region of the siRNA complementary to the target, in accordance with the present invention, may be from 10 to 100 nucleotides, 12 to 25 nucleotides, 14 to 22 nucleotides or 15, 16, 17 or 18 nucleotides. Where there are mismatches to the corresponding target region, the length of the complementary region is generally required to be somewhat longer. In an embodiment, the inhibitor is a siRNA molecule and comprises between approximately 5bp and 50 bp, in some embodiments, between 10 bp and 35 bp, or between 15 bp and 30 bp, for instance between 18 bp and 25bp. In some embodiments, the siRNA molecule comprises more than 20 and less than 23 bp.

Because the siRNA may carry overhanging ends (which may or may not be complementary to the target), or additional nucleotides complementary to itself but not the target gene, the total length of each separate strand of siRNA may be 10 to 100 nucleotides, 15 to 49 nucleotides, 17 to 30 nucleotides or 19 to 25 nucleotides.

The phrase "each strand is 49 nucleotides or less" means the total number of consecutive nucleotides in the strand, including all modified or unmodified nucleotides, but not including any chemical moieties which may be added to the 3' or 5' end of the strand. Short chemical moieties inserted into the strand are not counted, but a chemical linker designed to join two separate strands is not considered to create consecutive nucleotides.

The phrase "a 1 to 6 nucleotide overhang on at least one of the 5' end or 3' end" refers to the architecture of the complementary siRNA that forms from two separate strands under physiological conditions. If the terminal nucleotides are part of the double-stranded region of the siRNA, the siRNA is considered blunt ended. If one or more nucleotides are unpaired on an end, an overhang is created. The overhang length is measured by the number of overhanging nucleotides. The overhanging nucleotides can be either on the 5' end or 3' end of either strand.

The siRNA according to the present invention display a high in vivo stability and may be particularly suitable for oral delivery by including at least one modified nucleotide in at least one of the strands. Thus the siRNA according to the present invention contains at least one modified or non-natural ribonucleotide. A lengthy description of many known chemical modifications are set out in published PCT patent application WO 200370918. Suitable modifications for delivery include chemical modifications can be selected from among:

- a) a 3' cap;
- b) a 5' cap,
- c) a modified internucleoside linkage; or
- d) a modified sugar or base moiety.

Suitable modifications include, but are not limited to modifications to the sugar moiety (i.e. the 2' position of the sugar moiety, such as for instance 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group) or the base moiety (i.e. a non-natural or modified base which maintains ability to pair with another specific base in an alternate nucleotide chain). Other modifications include so-called 'backbone' modifications including, but not limited to, replacing the phosphoester group (connecting adjacent ribonucleotides) with for instance phosphorothioates, chiral phosphorothioates or phosphorodithioates.

End modifications sometimes referred to herein as 3' caps or 5' caps may be of significance. Caps may consist of simply adding additional nucleotides, such as "T-T" which has been found to confer stability on a siRNA. Caps may consist of more complex chemistries which are known to those skilled in the art.

Design of a suitable siRNA molecule is a complicated process, and involves very carefully analysing the sequence of the target mRNA molecule. On exemplary method for the design of siRNA is illustrated in WO2005/059132.

Then, using considerable inventive endeavour, the inventors have to choose a defined sequence of siRNA which has a certain composition of nucleotide bases, which would have the required affinity and also stability to cause the RNA interference.

The siRNA molecule may be either synthesised *de novo*, or produced by a micro-organism. For example, the siRNA molecule may be produced by bacteria, for example, *E. coli*. Methods for the synthesis of siRNA, including siRNA containing at least one modified or non-natural ribonucleotides are well known and readily available to those of skill in the art. For example, a variety of synthetic chemistries are set out in published PCT patent applications WO2005021749 and WO200370918. The reaction may be carried out in solution or, in some embodiments, on solid phase or by using polymer supported reagents, followed by combining the synthesized RNA strands under conditions, wherein a siRNA molecule is formed, which is capable of mediating RNAi.

It should be appreciated that siNAs (small interfering nucleic acids) may comprise uracil (siRNA) or thymidine (siDNA). Accordingly the nucleotides U and T, as referred to above, may be interchanged. However it is preferred that siRNA is used.

Gene-silencing molecules, i.e. inhibitors, used according to the invention are in some embodiments, nucleic acids (e.g. siRNA or antisense or ribozymes). Such molecules may (but not necessarily) be ones, which become incorporated in the DNA of cells of the subject being treated. Undifferentiated cells may be stably transformed with the gene-silencing molecule leading to the production of genetically modified daughter cells (in which case regulation of expression in the subject may be required, e.g. with specific transcription factors, or gene activators). The gene-silencing molecule may be either synthesised *de novo*, and introduced in sufficient amounts to induce gene-silencing (e.g. by RNA interference) in the target cell. Alternatively, the molecule may be produced by a micro-organism, for example, *E. coli*, and then introduced in sufficient amounts to induce gene silencing in the target cell.

The molecule may be produced by a vector harbouring a nucleic acid that encodes the gene-silencing sequence. The vector may comprise elements capable of controlling and/or enhancing expression of the nucleic acid. The vector may be a recombinant vector. The vector may for example comprise plasmid, cosmid, phage, or virus DNA. In addition to, or instead of using the vector to synthesise the gene-silencing molecule, the vector may be used as a delivery system for transforming a target cell with the gene silencing sequence.

The recombinant vector may also include other functional elements. For instance, recombinant vectors can be designed such that the vector will autonomously replicate in the target cell. In this case, elements that induce nucleic acid replication may be required in the recombinant vector. Alternatively, the recombinant vector may be designed such that the vector and recombinant nucleic acid molecule integrates into the genome of a target cell. In this case nucleic acid sequences, which favour targeted integration (e.g. by homologous recombination) are desirable. Recombinant vectors may also have DNA coding for genes that may be used as selectable markers in the cloning process.

The recombinant vector may also comprise a promoter or regulator or enhancer to control expression of the nucleic acid as required. Tissue specific promoter/enhancer elements may be used to regulate expression of the nucleic acid in specific cell types, for example, endothelial cells. The promoter may be constitutive or inducible.

Alternatively, the gene silencing molecule may be administered to a target cell or tissue in a subject with or without it being incorporated in a vector. For instance, the molecule may be incorporated within a liposome or virus particle (e.g. a retrovirus, herpes virus, pox virus, vaccinia virus, adenovirus, lentivirus and the like).

Alternatively a "naked" siRNA or antisense molecule may be inserted into a subject's cells by a suitable means e.g. direct endocytotic uptake.

The gene silencing molecule may also be transferred to the cells of a subject to be treated by either transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by: ballistic transfection with coated gold particles; liposomes containing a siNA molecule; viral vectors comprising a gene silencing sequence or means of providing direct nucleic acid uptake (e.g. endocytosis) by application of the gene silencing molecule directly.

In an embodiment of the present invention siNA molecules may be delivered to a target cell (whether in a vector or "naked") and may then rely upon the host cell to be replicated and thereby reach therapeutically effective levels. When this is the case the siNA is in some embodiments, incorporated in an expression cassette that will enable the siNA to be transcribed in the cell and then interfere with translation (by inducing destruction of the endogenous mRNA coding the targeted gene product).

Inhibitors according to any embodiment of the present invention may be used in a monotherapy (e.g. use of siRNAs alone). However it will be appreciated that the inhibitors may be used as an adjunct, or in combination with other therapies.

The inhibitors of MNK or of mTOR may be contained within compositions having a number of different forms depending, in particular on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micelle, transdermal patch, liposome or any other suitable form that may be administered to a person or animal. It will be appreciated that the vehicle of the composition of the invention should be one which is well tolerated by the subject to whom it is given, and in some embodiments, enables delivery of the inhibitor to the target site.

The inhibitors of MNK or of mTOR may be used in a number of ways.

For instance, systemic administration may be required in which case the compound may be contained within a composition that may, for example, be administered by injection into the blood stream. Injections may be intravenous (bolus or infusion), subcutaneous, intramuscular or a direct injection into the target tissue (e.g. an intraventricular injection-when used in the brain). The inhibitors may also be administered by inhalation (e.g. intranasally) or even orally (if appropriate).

The inhibitors of the invention may also be incorporated within a slow or delayed release device. Such devices may, for example, be inserted at the site of a tumour, and the molecule may be released over weeks or months. Such devices may be particularly advantageous when long term treatment with an inhibitor of MNK is required and which would normally require frequent administration (e.g. at least daily injection).

It will be appreciated that the amount of an inhibitor that is required is determined by its biological activity and bioavailability which in turn depends on the mode of administration, the physicochemical properties of the molecule employed and whether it is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the above-mentioned factors and particularly the half-life of the inhibitor within the subject being treated.

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular inhibitor in use, the strength of the preparation, and the mode of administration.

Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

When the inhibitor is a nucleic acid conventional molecular biology techniques (vector transfer, liposome transfer, ballistic bombardment etc) may be used to deliver the inhibitor to the target tissue.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. in vivo experimentation, clinical trials, etc.), may be used to establish specific formulations for use according to the

invention and precise therapeutic regimes (such as daily doses of the gene silencing molecule and the frequency of administration).

Generally, a daily dose of between 0.01 µg/kg of body weight and 0.5 g/kg of body weight of an inhibitor of MNK or of mTOR may be used for the treatment of cancer in the subject, depending upon which specific inhibitor is used. When the inhibitor is an siRNA molecule, the daily dose may be between 1 pg/kg of body weight and 100 mg/kg of body weight, in some embodiments, between approximately 10 pg/kg and 10 mg/kg, or between about 50 pg/kg and 1mg/kg.

When the inhibitor (e.g. siRNA) is delivered to a cell, daily doses may be given as a single administration (e.g. a single daily injection).

Various assays are known in the art to test dsRNA for its ability to mediate RNAi (see for instance Elbashir et al., Methods 26 (2002), 199-213). The effect of the dsRNA according to the present invention on gene expression will typically result in expression of the target gene being inhibited by at least 10%, 33%, 50%, 90%, 95% or 99% when compared to a cell not treated with the RNA molecules according to the present invention.

Similarly, various assays are well-known in the art to test antibodies for their ability to inhibit the biological activity of their specific targets. The effect of the use of an antibody according to the present invention will typically result in biological activity of their specific target being inhibited by at least 10%, 33%, 50%, 90%, 95% or 99% when compared to a control not treated with the antibody.

The term "cancer" refers to a group of diseases in which cells are aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues), and sometimes metastatic (spread to other locations in the body). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited in their growth and don't invade or metastasize (although some benign tumor types are capable of becoming malignant). A particular type of cancer is a cancer forming solid tumours. Such cancer forming solid tumours can be breast cancer, prostate carcinoma or oral squamous carcinoma. Other cancer forming solid tumours for which the methods and inhibitors of the invention would be well suited can be selected from the group consisting of adrenal cortical carcinomas, angiomatoid fibrous histiocytoomas (AFH), squamous cell bladder carcinomas, urothelial carcinomas, bone tumours, e.g. adamantinomas, aneurysmal bone cysts, chondroblastomas, chondromas, chondromyxoid fibromas, chondrosarcomas, fibrous dysplasias of the bone, giant cell tumours, osteochondromas or osteosarcomas, breast tumours, e.g. secretory ductal carcinomas, chordomas, clear cell hidradenomas of the skin (CCH), colorectal adenocarcinomas, carcinomas of the gallbladder and extrahepatic bile ducts, combined hepatocellular and cholangiocarcinomas, fibrogenesis imperfecta ossium, pleomorphic salivary gland adenomas head and neck squamous cell carcinomas, chromophobe renal cell carcinomas, clear cell renal cell carcinomas, nephroblastomas (Wilms tumor), papillary renal cell carcinomas, primary renal ASPSCR1-TFE3 t(X;17)(p11;q25) tumors, renal cell carcinomas, laryngeal squamous cell carcinomas, liver adenomas, hepatoblastomas, hepatocellular carcinomas, non-small cell lung carcinomas, small cell lung cancers, malignant melanoma of soft parts, medulloblastomas, meningiomas, neuroblastomas, astrocytic tumours, ependymomas, peripheral nerve sheath tumours, neuroendocrine tumours, e.g. pheochromocytomas, neurofibromas, oral squamous cell carcinomas, ovarian tumours, e.g. epithelial ovarian tumours, germ cell tumours or sex cord-stromal tumours, pericytomas, pituitary adenomas, posterior uveal melanomas, rhabdoid tumours, skin melanomas, cutaneous benign fibrous histiocytoomas, intravenous leiomyomatosis, aggressive angiomyxomas, liposarcomas, myxoid liposarcomas, low grade fibromyxoid sarcomas, soft tissue leiomyosarcomas, biphasic synovial sarcomas, soft tissue chondromas, alveolar soft part sarcomas, clear cell sarcomas, desmoplastic small round cell tumours, elastofibromas, Ewing's tumours,

extraskelatal myxoid chondrosarcomas, inflammatory myofibroblastic tumours, lipoblastomas, lipoma, benign lipomatous tumours, liposarcomas, malignant lipomatous tumours, malignant myoepitheliomas, rhabdomyosarcomas, synovial sarcomas, squamous cell cancers, subungual exostosis, germ cell tumours in the testis, spermatocytic seminomas, anaplastic (undifferentiated) carcinomas, oncocytic tumours, papillary carcinomas, carcinomas of the cervix, endometrial carcinomas, leiomyoma as well as vulva and/or vagina tumours. In an embodiment of the invention, the cancer is a cancer of the pancreas, large intestine, small intestine, lungs or ovary. In a particular embodiment, the cancer is a cancer of the brain, for instance an astrocytoma, a glioblastoma or an oligodendroglioma.

In one embodiment, the cancer is a MNK-dependent cancer. MNK-dependent cancers are cancers where a MNK has become an essential gene. MNK-dependent cancers can be easily identified by depleting the cells of MNK, e.g. MNK1, expression, and identifying the cancers that are not able to grow in the absence of it.

The MKNK (MAP kinase-interacting serine/threonine kinase) family, or MNK family, is found in *C. elegans*, *Drosophila*, and mammals. The MKNK family contains Gprk7 (a member of a subfamily of G protein-coupled receptor kinases), which may help regulate chemoattractant receptors in neutrophils and contains a DLG motif preceded by a DFD motif and has low similarity with the Gprk family in the AGC group. The functions of *C. elegans* MKNK family is yet unknown. MKNKs are activated by RAS signaling in *Drosophila* and mammals and are activated by p38 signaling in mammals (Huang and Rubin 2000; Fukunag and Hunter 1997). The function as a MAP kinase activated or MAP kinase-interacting kinase is conserved from SC:RCK2 (Bilsland-Marchesan et al. 2000), which is a substrate of HOG1; MAP kinase of osmotic stress pathway. HOG1 is believed to have been evolved to p38 and JNK pathway. RAS1 pathway signaling in *Drosophila melanogaster*. (Genetics 156:1219-1230) See also *Frontiers in Bioscience* 5359-5374, May 1, 2008] for review.

The term "MNK", as used herein, refers to all the member of the MNK family, e.g. MNK1, MNK2, GPRK7, and or LK6.

"MNK1" (Entrez Gene 8569) or MAP kinase interacting serine/threonine kinase 1, also known as MKNK1, Mnk1, EC 2.7.11.1, is a kinase interacting with members of the MAP kinase family (Mitogen-activated protein (MAP) kinases (EC 2.7.11.24) are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis).

Protein kinases are important enzymes involved in the regulation of many cellular functions. The LK6-serine/threonine-kinase gene of *Drosophila melanogaster* was described as a short-lived kinase which can associate with microtubules (J. Cell Sci. 1997, 110(2): 209-219). Genetic analysis in the development of the compound eye of *Drosophila* suggested a role in the modulation of the RAS signal pathway (Genetics 2000 156(3): 1219-1230). The closest human homologues of *Drosophila* LK6-kinase are the MAP-kinase interacting kinase 2 (Mnk2, e.g. the variants Mnk2a and Mnk2b) and MAP-kinase interacting kinase 1 (Mnk1) and variants thereof. These kinases are mostly localized in the cytoplasm. Mnk1s are phosphorylated by the p42 MAP kinases Erk1 and Erk2 and the p38-MAP kinases. This phosphorylation is triggered in a response to growth factors, phorbol esters and oncogenes such as Ras and Mos, and by stress signaling molecules and cytokines. The phosphorylation of Mnk proteins stimulates their kinase activity towards eukaryotic initiation factor 4E (eIF4E) (EMBO J. 16: 1909-1920, 1997; Mol Cell Biol 19, 1871-1880, 1999; Mol Cell Biol 21, 743-754, 2001). Simultaneous disruption of both, the Mnk1 and Mnk2 gene in mice diminishes basal and stimulated eIF4E phosphorylation (Mol Cell Biol 24, 6539-6549, 2004). Phosphorylation of eIF4E results in a regulation of the protein translation (Mol Cell Biol 22: 5500-5511, 2001). There are different hypotheses describing the mode of

the stimulation of the protein translation by Mnk proteins. Most publications describe a positive stimulatory effect on the cap-dependent protein translation upon activation of MAP kinase-interacting kinases. Thus, the activation of Mnk proteins can lead to an indirect stimulation or regulation of the protein translation, e.g. by the effect on the cytosolic phospholipase 2 alpha (BBA 1488:124-138, 2000). WO 03/037362 discloses a link between human Mnk genes, particularly the variants of the human Mnk2 genes, and diseases which are associated with the regulation of body weight or thermogenesis. It is postulated that human Mnk genes, particularly the Mnk2 variants are involved in diseases such as e.g. metabolic diseases including obesity, eating disorders, cachexia, diabetes mellitus, hypertension, coronary heart disease, hypercholesterolemia, dyslipidemia, osteoarthritis, biliary stones, cancer of the genitals and sleep apnea, and in diseases connected with the ROS defense, such as e.g. diabetes mellitus and cancer. WO 03/03762 moreover discloses the use of nucleic acid sequences of the MAP kinase-interacting kinase (Mnk) gene family and amino acid sequences encoding these and the use of these sequences or of effectors of Mnk nucleic acids or polypeptides, particularly Mnk inhibitors and activators in the diagnosis, prophylaxis or therapy of diseases associated with the regulation of body weight or thermogenesis. WO 02/103361 describes the use of kinases 2a and 2b (Mnk2a and Mnk2b) interacting with the human MAP kinase in assays for the identification of pharmacologically active ingredients, particularly useful for the treatment of diabetes mellitus type 2. Moreover, WO 02/103361 discloses also the prophylaxis and/or therapy of diseases associated with insulin resistance, by modulation of the expression or the activity of Mnk2a or Mnk2b. Apart from peptides, peptidomimetics, amino acids, amino acid analogues, polynucleotides, polynucleotide analogues, nucleotides and nucleotide analogues, 4- hydroxybenzoic acid methyl ester are described as a substance which binds the human Mnk2 protein. Inhibitors of Mnk (referred to as CGP57380 and CGP052088) have been described (cf. Mol. Cell. Biol. 21 , 5500, 2001 ; Mol Cell Biol Res Comm 3, 205, 2000; Genomics 69, 63, 2000). CGP052088 is a staurosporine derivative having an IC₅₀ of 70 nM for inhibition of in vitro kinase activity of Mnk1. CGP57380 is a low molecular weight selective, non-cytotoxic inhibitor of Mnk2 (Mnk2a or Mnk2b) or of Mnk1 . Other inhibitors of MNK1 are also disclosed in WO2008/006547, WO2007/115822, WO2006/136402, and/or WO2006/066937.

The addition of CGP57380 to cell culture cells, transfected with Mnk2 (Mnk2a or Mnk2b) or Mnk1 showed a strong reduction of phosphorylated eIF4E. Evidence for a role of Mnks in inflammation was provided by studies demonstrating activation of Mnk1 by proinflammatory stimuli. The cytokines TNF α and IL-1 β trigger the activation of Mnk1 in vitro (Fukunaga and Hunter, EMBO J 16(8): 1921-1933, 1997) and induce the phosphorylation of the Mnk-specific substrate eIF4E in vivo (Ueda et al., Mol Cell Biol 24(15): 6539-6549, 2004). In addition, administration of lipopolysaccharide (LPS), a potent stimulant of the inflammatory response, induces activation of Mnk1 and Mnk2 in mice, concomitant with a phosphorylation of their substrate eIF4E (Ueda et al., Mol Cell Biol 24(15): 6539-6549, 2004). Furthermore, Mnk1 has been shown to be involved in regulating the production of proinflammatory cytokines. Mnk1 enhances expression of the chemokine RANTES (Nikolcheva et al., J Clin Invest 110, 119-126, 2002). RANTES is a potent chemottractant of monocytes, eosinophils, basophiles and, natural killer cells. It activates and induces proliferation of T lymphocytes, mediates degranulation of basophils and induces the respiratory burst in eosinophils (Conti and DiGiacchino, Allergy Asthma Proc 22(3): 133-7, 2001). WO 2005/00385 and Buxade et al., Immunity 23: 177-189, August 2005 both disclose a link between Mnks and the control of TNF α biosynthesis. The proposed mechanism is mediated by a regulatory AU-rich element (ARE) in the TNF α mRNA. Buxade et al. demonstrate proteins binding and controlling ARE function to be phosphorylated by Mnk1 and Mnk2. Specifically Mnk-mediated phosphorylation of the ARE-binding protein hnRNP A1 has been suggested to enhance translation of the TNF α mRNA. TNF α is not the only cytokine regulated by an ARE.

Functional AREs are also found in the transcripts of several interleukins, interferones and chemokines (Khabar, J Interf Cytokine Res 25: 1-10, 2005). The Mnk-mediated phosphorylation of ARE-binding proteins has thus the potential to control biosynthesis of cytokines in addition to that of TNF α . Current evidence demonstrates Mnks as down stream targets of inflammatory signalling as well as mediators of the inflammatory response. Their involvement in the production of TNF α , RANTES, and potentially additional cytokines suggests inhibition of Mnks as strategy for anti-inflammatory therapeutic intervention.

A mammalian target of rapamycin (mTOR) inhibitor is a compound that decreases the activity of the target of rapamycin (mTOR) pathway. A decrease in activity of the target of rapamycin pathway is defined by a reduction of a biological function of the target of rapamycin. A target of rapamycin biological function includes for example, inhibition of the response to interleukin-2 (IL-2) or blocking the activation of T- and B-cells. A mTOR inhibitor acts for example by binding to protein FK-binding protein 12 (FKBP 12). mTOR inhibitors are known in the art or are identified using methods described herein. The m-TOR inhibitor is for example a macrolide antibiotic such as rapamycin, temsirolimus (2,2-bis(hydroxymethyl)propionic acid;CCI-779) or everolimus (RAD001); AP23573 or mimetics or derivatives thereof. Mimetics and derivatives of rapamycin are known in the art such as those describes in US Patent Nos. RE37,421; 5,985,890; 5,912,253; 5,728,710; 5,712,129; 5,648,361; 7,332,601; 7,282,505; 6,680,330.

The growth of cells is inhibited, e.g. reduced or apoptosis is induced by contacting a cell with a composition containing a MNK inhibitor, for instance a MNK1 inhibitor, and an mTOR inhibitor. By inhibition of cell growth is meant the cell proliferates at a lower rate or has decreased viability compared to a cell not exposed to the composition. Cell growth is measured by methods know in the art such as, the MTT cell proliferation assay. By inducing apoptosis is meant an increase of oxidative stress induced cell death. The process of apoptosis is characterized by, but not limited to, several events. Cells lose their cell junctions and microvilli, the cytoplasm condenses and nuclear chromatin marginates into a number of discrete masses. As the nucleus fragments, the cytoplasm contracts and mitochondria and ribosomes become densely compacted. After dilation of the endoplasmic reticulum and its fusion with the plasma membrane, the cell breaks up into several membrane-bound vesicles, apoptotic bodies, which are usually phagocytosed by adjacent bodies. As fragmentation of chromatin into oligonucleotides fragments is characteristic of the final stages of apoptosis, DNA cleavage patterns can be used as and in vitro assay for its occurrence (Cory, Nature 367: 317-18, 1994). Many methods for measuring apoptosis, including those described herein, are known to the skilled artisan including, but not limited to, the classic methods of DNA ladder formation by gel electrophoresis and of morphologic examination by electron microscopy. The more recent and readily used method for measuring apoptosis is flow cytometry.

Cells are directly contacted with an inhibitor. Alternatively, the inhibitor is administered systemically. Inhibitors are administered in an amount sufficient to decrease (e.g., inhibit) cell proliferation or induce apoptosis. The MNK inhibitor, for instance a MNK1 inhibitor, and the mTOR inhibitor are administered concurrently. Alternatively, the MNK inhibitor, for instance MNK1 inhibitor, and the mTOR inhibitor are administered sequentially.

The combined administration of the MNK inhibitor, for instance an inhibitor of MNK1, and mTOR inhibitor inhibits cell death or induced apoptosis to a greater extent that when either the MNK or the mTOR inhibitor is administered alone. By greater is meant 1, 2, 3, 4, 5, 10, 20, 30, 40 or 50-fold greater.

The present invention also provides a method of screening compounds to identify those which might be useful for treating cancer in a subject by inhibiting a MNK as well as the so-identified compounds.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials

similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Examples

Patients

Frozen tissue samples of primary gliomas obtained from the operating room were processed as previously described (Maier et al., 1997), according to the guidelines of the Ethical Committee of the University Hospitals of Basel and Düsseldorf, approved by all patients. Tumors were diagnosed and graded according to the WHO Classification of Tumors of the Nervous System (Kleihues and Sobin, 2000). All patients received open tumor resection, grade III and IV tumors external beam radiotherapy.

RNA extraction and microarray analysis

Total RNA was extracted from the primary tumor samples using Trizol™ reagent, according to manufacturer's instructions, and resuspended in diethylpyrocarbonate (DEPC) treated water. Total RNA was further purified using the RNeasy™ kit and following the protocol for Total RNA cleanup. RNA was then amplified and labeled using the Affymetrix 2-cycle amplification protocol as per manufacturer's instructions (Affymetrix). Samples were hybridized to Affymetrix U133v2.0 GeneChips and scanned using an Affymetrix GeneChip scanner as per manufacturer's instructions. Expression values were estimated using the GC-RMA implementation found in Genedata's Refiner 4.1 (Genedata) package. Data-mining and visualization was performed using Genedata's Analyst 4.1 package. Only median fold ratio values with $P < 0.05$ using t test were used for subsequent analysis. All samples were quantile normalized and median scaled to correct for minor variations in their expression distributions.

Quantitative real-time PCR analysis

The expression levels of selected genes were analyzed by real-time PCR using 2X SYBR Green Master Mix (Applied Biosystems) on an ABI Prism 7000 sequence detection system (Applied Biosystems). Reverse transcription of 1 µg of DNase-treated RNA was performed with oligo(dT). Primer sets were designed across intron:exon boundaries to derive transcript-specific amplicons. Dissociation curves were performed on all reactions to verify product purity. Relative quantification of MNK1 was performed using the standard curve method and amount of each gene was normalized to amount of eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), a transcript that showed little variation based on microarray data. MNK1 forward primer sequence was 5'-AGAAACAAGCAGGGCACAGT-3' (SEQ ID NO:1) and the reverse primer sequence was 5'-TGCTTTTGCTTCTGGATGTG-3' (SEQ ID NO:2). EEF1A1 was amplified with forward (5'-AATGGTGACAACATGCTGGA-3' (SEQ ID NO:3)) and reverse (5'-AACGTTGACTGGAGCAAAGG-3' (SEQ ID NO:4)) primers. Amplicons of 227 and 141 bp corresponding to EEF1A1 and MNK1, respectively, were confirmed by sequencing. Experiments were performed in triplicate for each data point.

Cell culture, transfection and viability assay

Human brain tumor cell lines; BS125 and LN319 were derived from human patients. The “BS” series were generated at the University of Basel, Switzerland while the “LN” series was generated in Lausanne, Switzerland (Ishii *et al.*, 2007, Brain Pathol. 9:469-79). These cells were cultured in DMEM supplemented with 10% FCS and standard antibiotics in an incubator at 37°C and 5% CO₂ humidified atmosphere. Transfection was accomplished using Lipofectamine (Invitrogen) according to the supplier’s instructions. For overexpression experiments, previously reported MNK1-Flag construct (Knauf *et al.*, 2001, Mol Cell Biol. 21:5500-11) were used. For MNK1 knock-down gene specific commercially-available siRNA duplex against MNK1 (Qiagen) or control duplex against luciferase (Qiagen) were used at a final concentration of 100 nM in Optimem (Gibco). The MNK1 target sequence lies 25 nt downstream from the translation initiation codon. For viability assays, cells at 60-80% confluency were transfected on 6-well plates and on the following day, the cells were treated with an appropriate inhibitor. After indicated time points CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used according to manufacturer’s instruction.

Western blotting

Cells were homogenized in lysis buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 1% Nonidet P-40, 40 mM β-glycerophosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 25 mM NaF and 2 μM microcystin-LR). After centrifugation at 15,000 g for 15 min at 4°C, supernatants were collected and soluble protein concentrations were determined using the Coomassie blue dye-binding method and the BioRad prepared reagent. 40-60 μg of protein extracts were separated on 8 or 10% SDS-PAGE and transferred onto PVDF membrane (Millipore) by electroblotting. Membranes were blocked with 5% BSA in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and incubated overnight with the primary antibody. A rabbit polyclonal antibody that recognizes MNK1 was purchased from Abgent. Antibodies against phosphorylated form of p70 S6 kinase and eIF4E (Ser209) were obtained from Cell Signaling. Actin antibody (I-19) was from Santa Cruz Biotechnology and antibody against α-tubulin was generated in house using standard methods, and used as a hybridoma supernatant. Each primary antibodies were used at a 1:1000 v:v dilution in TBST. Subsequently, membranes were incubated with appropriate HRP-linked secondary antibody (1:2000 v:v dilution) in TBST for 1 h at room temperature. Blots were analyzed using enhanced chemiluminescence reagents and optical density of the obtained signals was analysed using ImageJ software (National Institutes of Health).

Cell growth assays

For cell proliferation MTT-based assays in 96-well plates, about ~5000 cells were seeded 1 day before drug treatment. The following day, cells were treated with the indicated amount of CGP57380 and/or rapamycin. The number of viable cells was measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) used according to manufacturer’s instruction. In addition, cell growth was also monitored by counting cell number with a Vi-Cell XR cell viability analyzer (Beckman Coulter). In these experiments, ~100000 cells were seeded in 6-wells plates. The following day, cells were treated with the indicated amount of DMSO, CGP 57380 and/or Rapamycin. Cells were counted every day over a 5-day period.

Claims

1. A method using a mTOR inhibitor for treating cancer in a subject, said method being characterized in that a therapeutically effective amount of a modulator of MNK is administered in combination with said mTOR inhibitor to said subject.
2. The method of claim 1 wherein MNK is modulated by an inhibitor.
3. The method of claim 2 wherein the inhibitor is a small molecule.
4. The method of claim 1 or 2 wherein the inhibitor is an antibody.
5. The method of claims 1 or 2 wherein the inhibitor decreases or silences the expression of MNK.
6. The method of claim 5 wherein the inhibitor is a siRNA.
7. The method of any of claims 1 to 6 wherein the subject is a mammal, for instance a human subject.
8. The method of any of claims 1 to 7 wherein the cancer is a cancer of the brain, for instance an astrocytoma, a glioblastoma or an oligodendroglioma.
9. The method of any of claims 1 to 8 wherein the cancer of the brain is a glioblastoma.
10. The method of any of claims 1 to 9 wherein the MNK is MNK1.
11. A siRNA decreasing or silencing a MNK, for use as a medicament to treat cancer in combination with a mTOR inhibitor.
12. An antibody specifically binding to a MNK, for use as a medicament to treat cancer in combination with a mTOR inhibitor.
13. The antibody of claim 9 or the siRNA of claim 11, wherein said antibody or siRNA inhibits or decreases the kinase activity of MNK1.
14. A method for the identification of a substance that modulates the expression of a MNK expression and/or biological activity, which method comprises:
 - (i) contacting a MNK polypeptide or a fragment thereof having the biological activity of said MNK, a polynucleotide encoding such a polypeptide or polypeptide fragment, an expression vector comprising such a polynucleotide or a cell comprising such an expression vector, and a test substance in the presence of a mTOR inhibitor, under conditions that in the absence of the test substance would permit MNK expression and/or biological activity; and

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(ii) determining the amount of expression and/or biological activity, for example the kinase activity, of the MNK, to determine whether the test substance modulates biological activity and/or expression of said MNK in the presence of the mTOR inhibitor,
wherein a test substance which modulates biological activity and/or expression of the MNK in the presence of the mTOR inhibitor is a potential therapeutical agent to treat cancer.

15. The method of claim 14 wherein the MNK is MNK1.

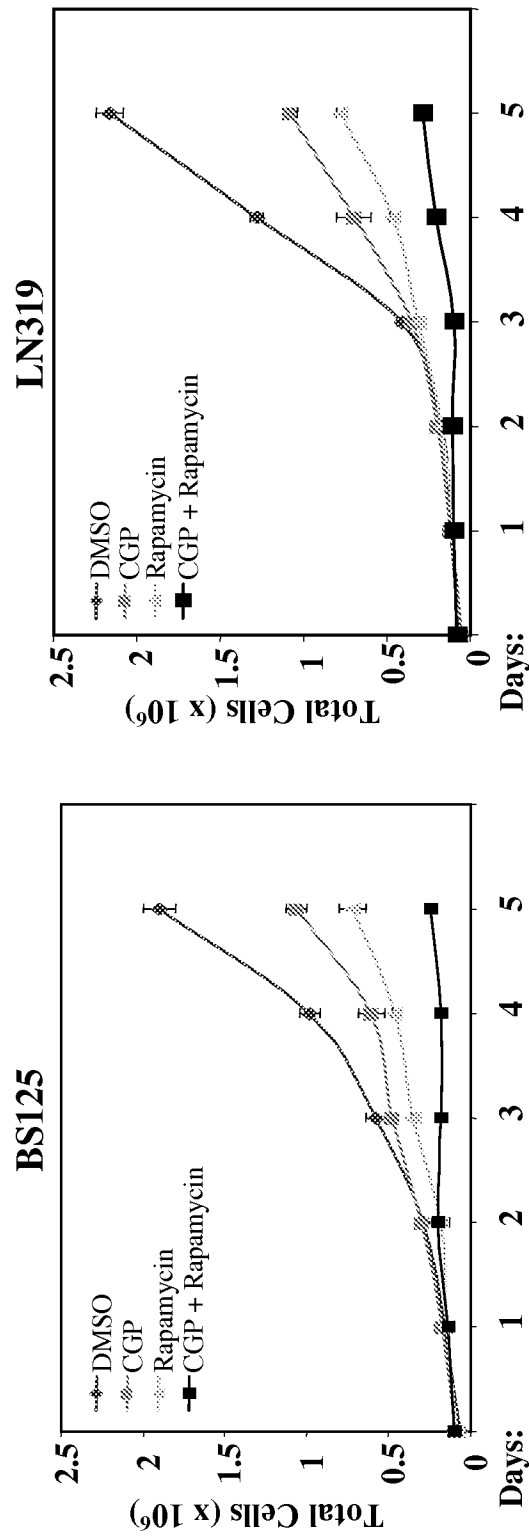


Figure 1

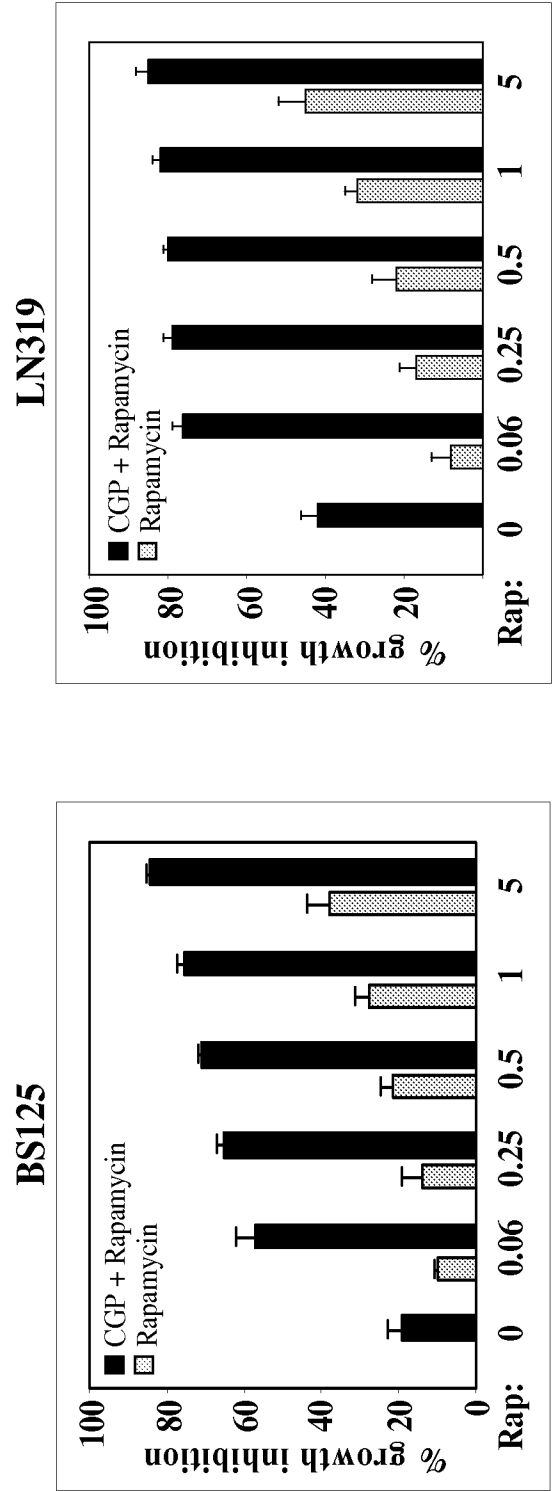


Figure 2

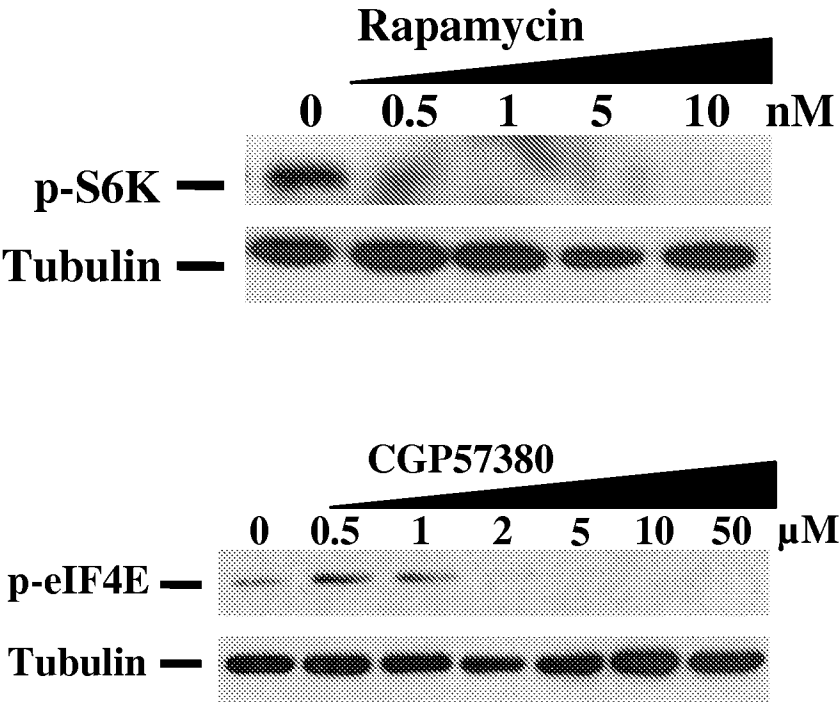


Figure 3

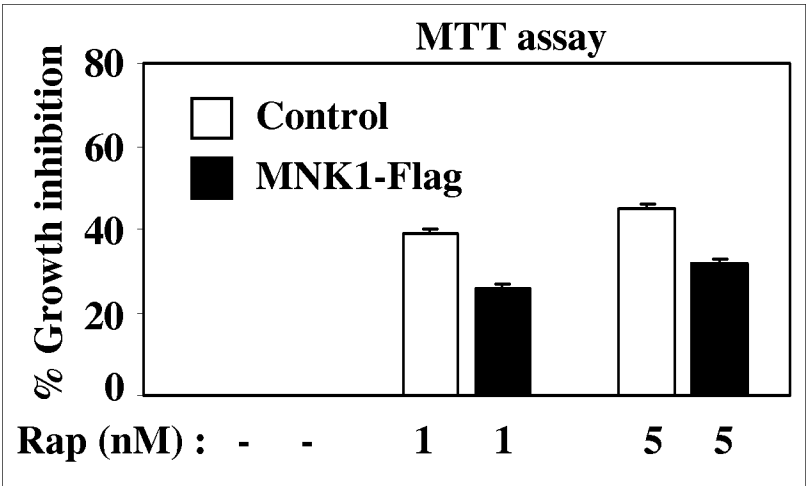


Figure 4

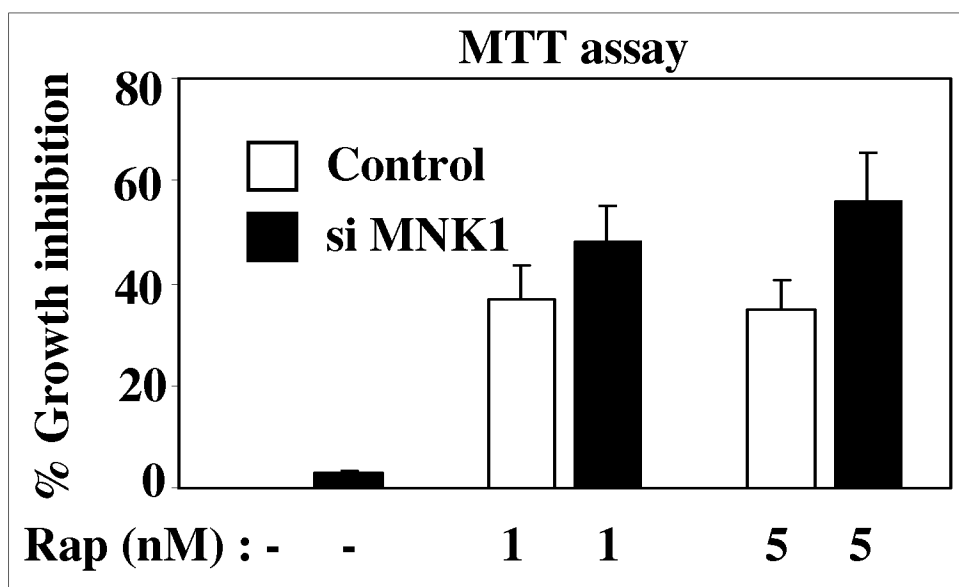


Figure 5

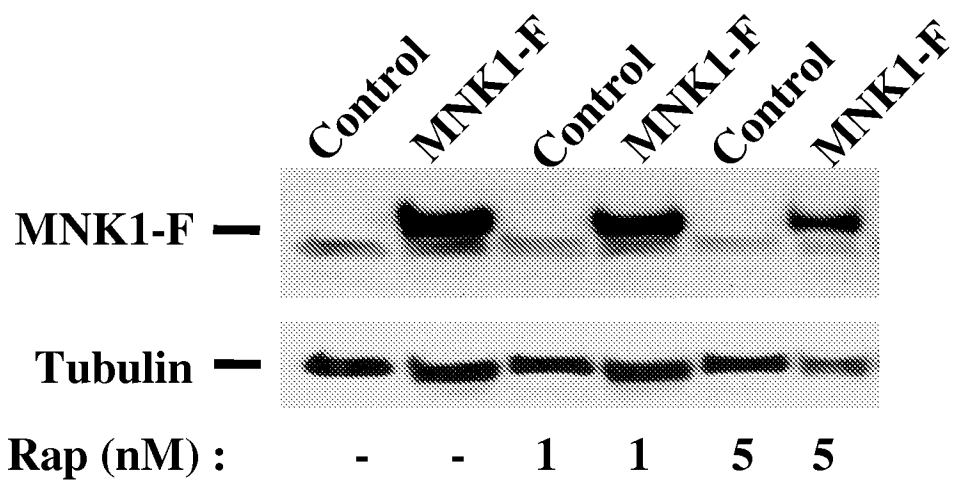


Figure 6

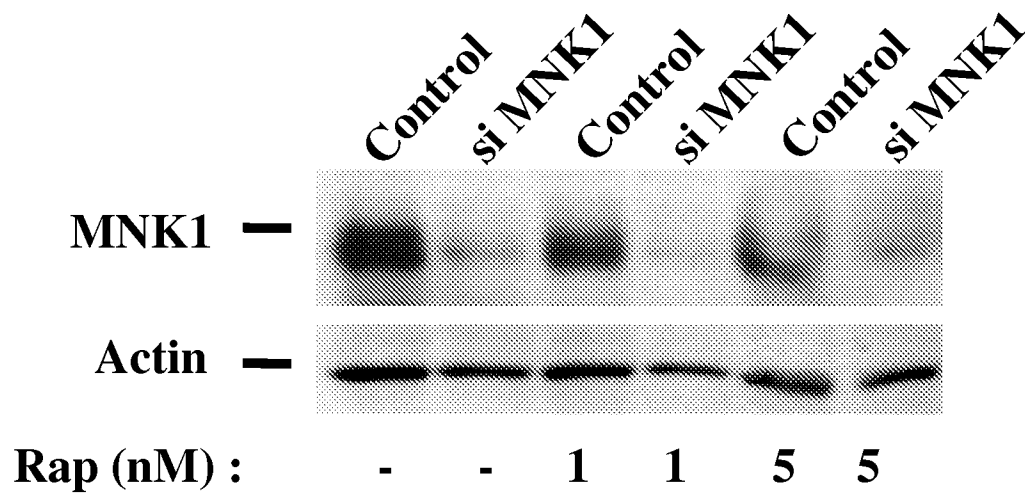


Figure 7

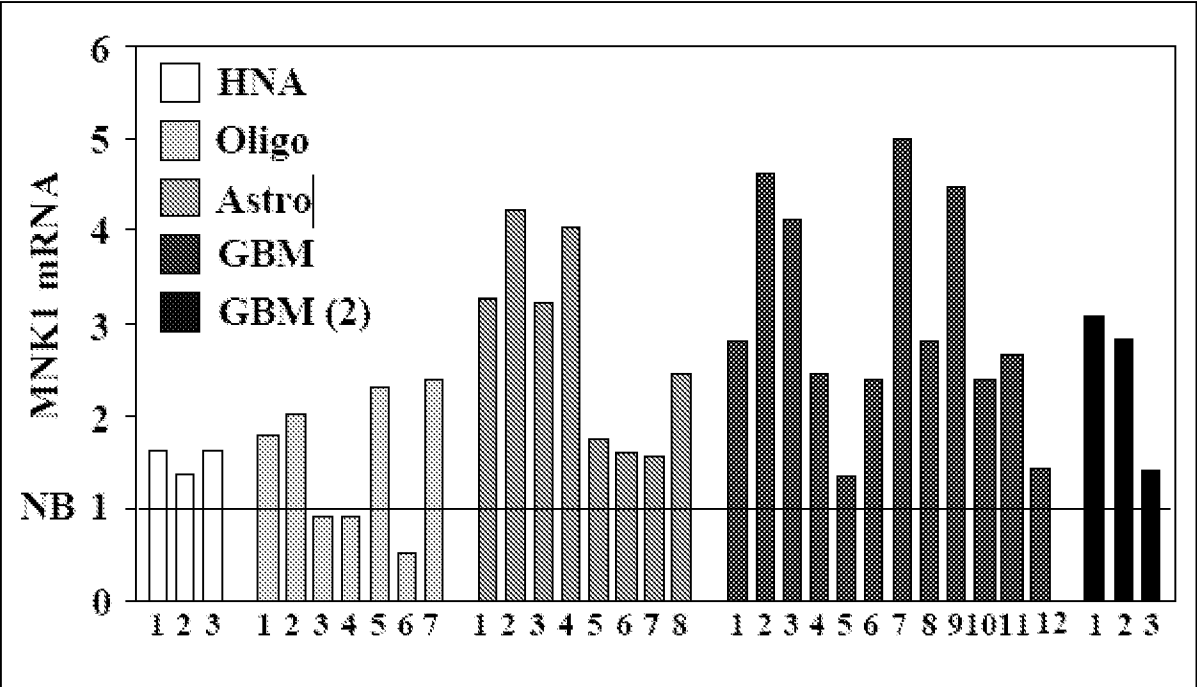


Figure 8