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(54) Title: METHOD FOR TREATING BONE MARROW DISORDERS

(57) Abstract: The present invention relates to methods for the treatment of bone marrow disorders including myelodysplastic syndromes and adult and pediatric leukemias. The methods comprise systemically administering an oligonucleotide which reduces or inhibits expression of a gene associated with a bone marrow disorder to a subject in need thereof. The present invention further provides the use of an oligonucleotide inhibitor for the preparation of a medicament for promoting recovery in a subject suffering from a bone marrow disorder.



WO 2008/126085 A2

METHOD FOR TREATING BONE MARROW DISORDERS

RELATED APPLICATION

5 This application claims priority of U.S. Provisional Patent Application No. 60/923,074, filed April 12, 2007, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

10 The present invention relates to a method for the treatment of bone marrow disorders, including myelodysplastic syndromes and adult and pediatric leukemias. The method comprises systemically administering an oligonucleotide, which reduces or inhibits expression of a gene associated with the bone marrow disorder to a subject in need thereof.

BACKGROUND OF THE INVENTION

Bone marrow and bone marrow disease

 Bone marrow is a specialized tissue that produces a plurality of different cell types, including stromal cells, hematopoietic lineage cells and cells involved in bone remodeling. Bone marrow is the primary source of red blood cells (erythrocytes) and
20 white blood cells in the body.

 Hematopoiesis is an ongoing process whereby highly specialized blood cells are generated from hematopoietic stem cells (HSC). The specialized cells fall within two functionally distinct groups termed myeloid and lymphoid cells. During normal human adult life, myeloid cells are produced exclusively within the bone marrow. Myeloid stem
25 cells produce the progenitor cells for the neutrophil, monocyte, macrophage, eosinophil, erythrocyte, megakaryocyte, mast cell, platelet and basophil cell types.

 Cells of the lymphoid lineage, which develop into B-cells (lymphocytes) or T-cells (Th or CTL), are produced to varying degrees in the bone marrow, spleen, thymus and lymph nodes. The principle function of B-cells lies in the production of antibodies.

30 Many human malignancies are myeloid or lymphoid in origin and arise from transforming events that occur in hematopoietic progenitors situated in the bone marrow. Understanding normal B cell development affords the opportunity to learn how the

transformation process interferes with normal B cell signaling mechanisms. In normal B cell development, rearrangement of the immunoglobulin heavy chain gene occurs during pro-B cell stages (reviewed in LeBien, 2000 1: *Blood*. 96(1):9-23). The late pro-B cell normally completes the rearrangement of heavy chain (V to D-J) joining. The cell then progresses through the pre-B cell stage, producing IgM heavy chain (μ) protein and undergoing proliferation. If the B cell precursor fails to produce a productive VDJ arrangement at both alleles, the cell undergoes apoptosis, which is the fate of the vast majority of (>90%) early pre-B cells.

Chromosomal translocations leading to the expression of chimeric proteins are often involved in malignant transformation of hematopoietic cells. Various leukemias including Acute Lymphocytic Leukemia (ALL), Acute Myelocytic Leukemia (AML), Chronic Lymphocytic Leukemia (CLL) and Chronic Myelocytic Leukemia (CML) result from oncogenic transformation of fusion proteins. Several of those fusion genes and their resulting proteins have been identified as valid targets in therapeutic strategies. The Philadelphia translocation, characteristic for CML and ALL, generates the *bcr/abl* fusion gene, which encodes a BCR/ABL constitutive active kinase, which is able to induce and maintain leukemic transformation.

Chromosomal translocations involving the mixed lineage leukemia (MLL) gene are frequently observed in AML and ALL patients. The N-terminus of MLL can fuse in-frame to a number of partner proteins including *inter alia* AF4, AF9, AF10, EEN, ENL, and ELL. Recent studies suggest that the MLL fusion protein is able to inhibit p53, a tumor suppressor involved in the cell cycle and apoptotic pathways (Wiederschain, et al, *J. Biol. Chem.* 280(26):24315-24321).

RNA interference

RNA interference (RNAi) is a phenomenon involving double-stranded RNA (dsRNA) dependent, gene-specific posttranscriptional silencing. Originally, attempts to study this phenomenon *in vivo* were thwarted by an active, non-specific antiviral defense mechanism (Gil et al. 2000, *Apoptosis*, 5:107-114). Later it was discovered that synthetic RNA duplexes of 21 nucleotides in length could mediate gene specific RNAi in mammalian cells, while obviating the generic antiviral defense mechanisms (Elbashir et al. 2001 *Nature*, 411:494-498; Caplen et al. 2001, *PNAS* 98:9742-9747). As a result, small interfering RNAs (siRNAs), which are short double-stranded RNAs, have become powerful tools in attempting to understand gene function and to treat disease.

Thus, RNA interference (RNAi) refers to the process of sequence-specific post-transcriptional gene silencing in mammals mediated by small interfering RNAs (siRNAs) (Fire, et al., 1998, *Nature* 391, 806) or microRNAs (miRNAs) (Ambros, 2004, *Nature* 431:350-355; Bartel, 2004, *Cell* 116(2): 281-97).

5 The selection and synthesis of siRNA corresponding to known genes has been widely reported. Examples of targets for successful silencing of cancer associated genes has been summarized by Gage (2005, *Future Oncol.* 1(1):101-111) and RNAi in hematopoietic cells has been reviewed by Venturini, et al., (2006, *J. Biomed. Biotech.*, 2006:87340). Venturini, et al., (2006, *ibid*) assert that although targeting the fusion
10 products of translocations by RNAi is feasible, the use of RNAi in primary hematopoietic cells is limited by the delivery of the molecules to the cells. The implementation of various techniques, *inter alia*, lentiviral transduction, retroviral delivery and electroporation, was disclosed.

For effective therapeutic application of siRNA, three requirements must be met:
15 delivery of the molecule to the target tissue responsible for the pathology; delivery of the molecule to the correct intracellular location and interaction of the molecule with the RNAi machinery within the target cell. Each of these levels of targeting poses a significant barrier.

Various methods for targeted delivery of siRNAs have been proposed (Oliveira, et
20 al., *J. Biomed. Biotech.*, 2006:1-9; Aigner, *J. Biomed. Biotech.*, 2006, 71659:1-15). Oliveira et al., (2006, *ibid*;) report that naked siRNA fails to produce gene silencing effects *in vivo*, even after prolonged incubation of cells with high siRNA concentrations *in vitro*. That disclosure further notes that rapid removal of naked siRNA from the circulation after intravenous administration, with more than 99% of the injected dose
25 renally excreted and taken up by the liver Kuppfer cells within minutes, makes a very small percentage of the administered dose available to the target tissue.

One promising direction includes local injection to the site of pathology and the complexation of siRNAs with the polyethylenimine (PEI), which efficiently stabilizes siRNAs and, upon systemic administration, leads to the delivery of the intact siRNAs
30 into different organs. Other delivery methods for siRNAs include coupling to a cholesterol moiety for targeting a siRNA molecule to the liver and jejunum (Soutschek et al., 2004, *Nature*, 432(7014):173-178); liposome delivery (Morrissey, et al., 2005, *Nature Biotech.*, 23(8):1002-1007) and antibody mediated delivery (Song et al., 2005, *Nature Biotech.* 23(6):709-717).

Wilda, et al., (2002, *Oncogene* 21(37):5716) disclose 21 base pair double stranded siRNA molecules that target the M-bcr/abl fusion gene in leukemic cells.

International Patent Application Publication No. WO 2006/073602 teaches RNAi inhibition of two specific *bcr/abl* transcript variants associated with CML and AML and provides siRNA molecules which target the *bcr/abl* fusion site.

US Patent Application Publication No. US 2005/0245475 teaches siRNA molecules effective at inhibiting Bcl-2 expression.

None of the above references teaches a method for the targeted delivery of naked siRNA to the bone marrow.

10 SUMMARY OF THE INVENTION

The present invention provides novel methods for treating disorders of the bone marrow. The present invention overcomes certain of the limitations of the prior art by providing an *in vivo* method of treating bone marrow disorders by directly targeting hematopoietic cells expressing genes associated with bone marrow disorders.

15 According to one aspect, the present invention provides a method of treating a subject afflicted with a bone marrow disorder in a subject in need thereof, which comprises systemically administering to the subject an oligonucleotide which reduces or inhibits expression of a gene associated with the disorder in the bone marrow of the subject in an amount effective to treat the disorder. In preferred embodiments the method
20 comprises contacting a bone marrow cell of the subject with an oligonucleotide which reduces or inhibits expression of a gene associated with the disorder.

In another embodiment the oligonucleotide comprises a sufficient number of consecutive nucleotides having a sequence of sufficient homology to a nucleic acid sequence present within the gene to hybridize to the gene and reduce or inhibit
25 expression of the gene in the subject.

The method of the present invention has utility in treating a bone marrow disorder wherein the bone marrow cell is selected from a stromal cell or a hematopoietic lineage cell. In some embodiments the bone marrow disorder is associated with a cell type selected from the group consisting of neutrophil, monocyte, macrophage, eosinophil,
30 erythrocyte, megakaryocyte, mast cell, platelet and basophil cell types.

In some embodiments, a disorder is selected from the group consisting of a leukemia, a lymphoma, a myeloproliferative disease, a myelodysplastic syndrome and a plasma cell disorder.

In some embodiments the leukemia is selected from the group consisting of Acute Lymphocytic Leukemia (ALL), Acute Myelocytic Leukemia (AML), Chronic Lymphocytic Leukemia (CLL), Chronic Myelocytic Leukemia (CML), Chronic Myelomonocytic Leukemia (CMML) and stem cell leukemia.

In some embodiments the lymphoma is selected from the group consisting of Anaplastic Large-Cell Lymphoma (ALCL), Hodgkin's lymphoma and non-Hodgkin's lymphoma.

In various embodiments the myelodysplastic syndrome is selected from the group consisting of Refractory anemia (RA), Refractory anemia with ringed sideroblasts (RARS), Refractory cytopenia with multilineage dysplasia (RCMD), Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS), Refractory anemia with excess blasts I and II; 5q- syndrome and myelodysplasia unclassifiable.

In other embodiments the myeloproliferative disorder is selected from Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Chronic Idiopathic Myelofibrosis (MF).

In yet other embodiments the plasma cell disease is a plasma cell neoplasm selected from the group consisting of multiple myeloma, plasmacytoma, macroglobulinemia, monoclonal gammopathy of undetermined significance (MGUS).

According to certain embodiments the bone marrow disorder is a hemoglobinopathy selected from the group consisting of sickle-cell disease and a thalassemia.

In some embodiments the gene associated with the bone marrow disorder is an aberrantly expressed gene. In various embodiments the aberrantly expressed gene comprises a dominant mutant oncogene, a chromosomal translocation or an amplified gene. In some embodiments the disorder is associated with a viral oncogene.

In some embodiments the oligonucleotide reduces or inhibits expression of a gene associated with a late stage bone marrow disorder. In other embodiments the oligonucleotide reduces or inhibits expression of a gene associated with an early stage bone marrow disorder.

In some embodiments the oligonucleotide reduces or inhibits expression of the gene by inhibiting transcription of an mRNA which encodes a polypeptide associated with progression of the bone marrow disorder. In other embodiments the oligonucleotide reduces or inhibits expression of the gene by inhibiting transcription of an mRNA which encodes a polypeptide associated with etiology of the bone marrow disease.

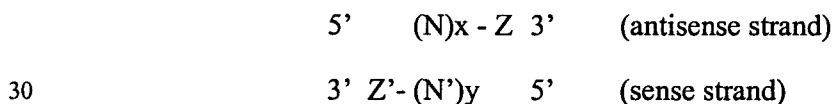
The method of the present invention is suitable for the treatment of non cancerous indications related to stem cells, for example, targeting genes which inhibit mobilization of stem cells into the blood stream and targeting genes which inhibit non-proliferating stem cells from proliferating.

The present application further provides a method for the delivery of siRNA to a bone marrow cell in a subject suffering from a bone marrow disorder, comprising systemically administering to the subject a naked siRNA which reduces or inhibits expression of a gene associated with the bone marrow disorder in an amount effective to treat the disorder.

In various embodiments the oligonucleotide is selected from the group consisting of a siRNA, an antisense oligonucleotide, an aptamer, and a ribozyme. In preferred embodiments the oligonucleotide molecule is an siRNA. In some embodiments the siRNA is selected from the group consisting of a naked siRNA, a vector comprising an siRNA, a vector which expresses an siRNA and an RNA which is endogenously processed into an siRNA. In preferred embodiments the siRNA is naked siRNA.

The siRNA according to the present invention comprises a sense strand and an antisense strand which form an RNA duplex, and wherein the sense strand comprises ribonucleotides having a nucleotide sequence complementary to target sequence of about 18 to about 40 consecutive nucleotides in the mRNA transcribed from the gene associated with a bone marrow disorder. In some embodiments the siRNA comprises at least one modified ribonucleotide so as to stabilize it against nuclease degradation.

According to some embodiments the present invention provides an siRNA molecule having the structure



wherein each N and N' is a ribonucleotide which may be modified or unmodified in its sugar residue and (N)_x and (N')_y is an oligomer in

which each consecutive N or N' is joined to the next N or N' by a covalent bond;

wherein each of x and y is an integer between 18 and 40;

5 wherein each of Z and Z' may be present or absent, but if present is dTdT and is covalently attached at the 3' terminus of the strand in which it is present;

and wherein the sequence of (N)_x comprises an antisense sequence relative to the mRNA transcribed from the gene associated with the bone marrow disorder.

10 In some embodiments $x = y$. In preferred embodiments $x = y = 19$. In some embodiments the siRNA consists of ribonucleotides unmodified in their sugar residues. In various other embodiments the siRNA comprises at least one modified ribonucleotide. According to some embodiments the modified nucleotide comprises a modified sugar residue. In some embodiments the modified nucleotide comprises a 2'-
15 O-methyl modification. In various embodiments the modified nucleotide is a spiegelmer. In various embodiments one strand is modified. In some embodiments both strands are modified.

In various embodiments one or more of the covalent bond joining the ribonucleotides in the oligonucleotide is modified. In one embodiment the
20 oligonucleotide comprises at least one phosphodiester bond. In some embodiments all the covalent bonds joining the ribonucleotides are modified.

While it may be possible for the oligonucleotide compounds to be administered *per se*, it is preferable to present them as a pharmaceutical composition. According to a further aspect, the present invention provides a pharmaceutical composition comprising
25 an oligonucleotide according to the present invention, together with one or more pharmaceutically acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The formulations include those suitable for oral, parenteral (including
30 subcutaneous, intradermal, intramuscular, intravenous and intraarticular), rectal and topical (including dermal, buccal, sublingual and intraocular) administration. The most suitable route may depend upon the condition and disorder of the recipient. The

formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical arts. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation. In certain preferred embodiments the oligonucleotide is administered parenterally. Parenteral administration comprises intravenous, intraarterial, intramuscular, intraperitoneal, or intranasal administration. A preferred method of administration comprises intravenous administration.

BRIEF DESCRIPTION OF THE FIGURES

10 Figs. 1A-1B: Fig. 1A-graphic representation of the normalized log ratio of p53 levels. Fig. 1B-normalized ratio of p53 levels. Fig. 1C-siRNA tissue distribution.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the treatment of bone marrow diseases and disorders, and in particular for the treatment of leukemias. The present invention is based in part on the unexpected discovery that naked siRNA molecules target the bone marrow when administered systemically. The discovery is surprising in view of the known obstacles to siRNA delivery.

For siRNA molecules to be effective in silencing mRNA of a target gene, the siRNA requires three levels of targeting: to the target tissue, to the target cell type and to the target subcellular compartment. The present invention now discloses systemic treatment of bone marrow diseases and disorders.

Definitions

For convenience and clarity certain terms employed in the specification, examples and claims are described herein.

25 By "small interfering RNA" (siRNA) is meant an RNA molecule which decreases or silences (prevents) the expression of a gene/mRNA of its endogenous cellular counterpart. Without wishing to be bound to theory, siRNA acts via a mechanism known as "RNA interference" (RNAi). RNA interference (RNAi) refers to the process of sequence-specific post-transcriptional gene silencing in mammals mediated by small interfering RNAs (siRNAs) (Fire et al, 1998, *Nature* 391, 806). The corresponding process in plants is commonly referred to as specific post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The RNA interference

response may feature an endonuclease complex containing an siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA may take place in the middle of the region
5 complementary to the antisense strand of the siRNA duplex (Elbashir et al 2001, *Genes Dev.*, 15, 188). For recent information on these terms and proposed mechanisms, see Bernstein et al. 2001. *Nature* 409, 363-6).

The term "naked siRNA" refers to an siRNA molecule that is free from any delivery vehicle that acts to assist, promote or facilitate entry into a target cell, including
10 transfection agents, viral sequences, viral particles, liposome formulations and the like. For example, siRNA in PBS is "naked siRNA". A transfection agent is a compound used in the prior art that mediates entry of nucleic acids into cells.

The term "polynucleotide" refers to any molecule composed of deoxynucleotides, ribonucleotides or a combination of both types, i.e. that comprises two or more of the
15 nucleotide bases, also known as nucleotides, guanine, cytosine, thymine, adenine, uracil or inosine, *inter alia*. A polynucleotide may include natural nucleotides, chemically modified nucleotides and synthetic nucleotides, or chemical analogs thereof. The term includes "oligonucleotides" and encompasses "nucleic acids".

The term "amino acid" refers to a molecule which consists of any one of the 20
20 naturally occurring amino acids, amino acids which have been chemically modified (see below), or synthetic amino acids. The term "polypeptide" refers to a molecule composed of two or more amino acid residues. The term includes peptides, polypeptides, proteins and peptidomimetics.

An "inhibitor" is a compound which is capable of inhibiting or reducing the
25 activity of a gene or the product of such gene to an extent sufficient to achieve a desired biological or physiological effect.

"Expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are known and/or commercially available. Selection of appropriate
30 expression vectors is within the knowledge of those having skill in the art.

The pharmaceutical composition of the present invention includes those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous and intraarticular), rectal and topical (including dermal, buccal, sublingual and intraocular)

administration. Parenteral administration is the currently preferred method of administration of the oligonucleotide.

Bone marrow

5 Bone marrow is the blood-forming tissue that fills the cavities of bones and contains *inter alia* hematopoietic cells, stromal cells and bone remodeling cells. Diseases or disorders that affect the bone marrow can affect the total counts of these cells.

Bone marrow stromal cells, are also known as mesenchymal stem cells, are a mixed population of cells derived from the non-blood forming fraction of bone marrow. Bone marrow stromal cells are capable of growth and differentiation into a number of
10 different cell types including bone, cartilage, fat and fibrous connective tissue.

Hematopoietic stem cells (HSC) are the precursors of mature blood cells that are defined by their ability to replace the bone marrow system following its obliteration (for example, by irradiation) and can continue to produce mature blood cells.

The present invention also relates to functional nucleic acids comprising a double-
15 stranded structure, their use for the manufacture of a medicament, a pharmaceutical composition comprising such functional nucleic acids and a method for the treatment of a subject. "Treating a disease" refers to administering a therapeutic substance effective to ameliorate symptoms associated with a disease, to lessen the severity or cure the disease, result in relief of symptoms or to prevent the disease from occurring. "Treatment" refers
20 to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (retard) a disease or disorder.

A "therapeutically effective dose" refers to an amount of a pharmaceutical compound or composition which is effective to achieve an improvement in a subject or
25 his physiological systems including, but not limited to, improved survival rate, more rapid recovery, or improvement or elimination of symptoms, and other indicators as are selected as appropriate determining measures by those skilled in the art.

The methods of treating the diseases disclosed herein and included in the present invention may include administering an oligonucleotide inhibitor in conjunction with or
30 in combination with an additional therapeutic agent, a substance which improves the pharmacological properties of the active ingredient as detailed below, or an additional compound known to be effective in the treatment of the bone marrow disease to be treated, such as *inter alia* AML, ALL, CML and CLL. By "in conjunction with" or "in

combination with” is meant “prior to”, “simultaneously” or “subsequent to”. Further detail on exemplary conjoined therapies is given below.

In another embodiment, the present invention provides for the use of a therapeutically effective dose of an oligonucleotide inhibitor for the preparation of a medicament for promoting recovery in a subject suffering from a bone marrow disease as detailed above, and the use of a therapeutically effective dose of an oligonucleotide inhibitor for the preparation of a medicament for treating said diseases and conditions. In certain preferred embodiments the oligonucleotide inhibitor is an siRNA compound.

In this embodiment, the nucleic acid inhibitor may target a gene (including a chromosomal translocation) disclosed in Table 1, and therefore, comprises an oligonucleotide which comprises consecutive nucleotides having a sequence which comprises an antisense sequence to the sequence of the mRNA of a gene/translocation set forth in Table 1. Additionally, the nucleic acid inhibitor may be an expression vector comprising a polynucleotide having a sequence which comprises an antisense sequence to the sequence of the mRNA of any of the genes/translocations set forth in Table 1. Table 1 provides a partial list of genes implicated in the pathogenesis or progression of bone marrow diseases.

Table 1: Partial list of target genes

Indication		Target gene
ALL		BCR/ABL (DIFFERENT FORMS)
		ALL/AF4
	t(1;19)	E2A/PBX1; wnt16
		TEL/AML1
	t(11;) MLL translocations	MLL-ENL, MLL-AF4, MLL-AF9, MLL-AF10, MLL-ELL, MLL-EEN, MLL-MEN
	Src; Lyn	
AML	t(8;21)	AML1-ETO
		AML1-MTG8
		FLT3-ITD
		CBFb/MYH11
		MLL-CALM
		CALM-AF10
	MLL-AF4	
CML	t(9;22)(q34;q11)	BCR-ABL
	t(11;?)	MLL-ENL, AF4, AF9, AF10, ELL
		Src; Lyn; Jak2

CMML		TEL/PDGFbR
CLL		Bcl-2 translocation; TCL1; Wnt16
ALCL		NPM1-ALK
APL	t(15;17)(q21;q11)	PML/RARa
	t(11;17)(q23;q11)	PLZF/RARa
	t(11;17)(q13;q11)	NuMA/RARa
	t(5;17)(q31;q11)	NPM/RARa
	t(17;17)	stat5b/RARa
Burkitt Lymphoma	t(8;14)	Ig/c-myc
DLBCL		c-rel; NF-KB, I-kB kinase, Bcl-10
MM		c-maf; FGFR3
Polycythemia vera		Jak2 V617F variant
β thalassemia		globin mutations
Sickle cell anemia		β -s-globin
Fanconi Anemia		FANCD2
Additional target genes		c-myb, c-raf, v-raf, bcl-2, c-myc, N-myc, Nrf2, c-kit, src, c-abl, cathepsin C and W; calpain small subunit; caspase-8; perforins; A 20; PAC-1; NGK2 receptors; RANTES; MIP-1 α ; MIP-1 β ; IL-1 β ; IL-8; IL-IRa; IFN- γ ; IL-18; IL-10; IL-12 p35; c-mer; ras, HMG-1

In some hematopoietic neoplasias, a chromosomal translocation is associated with the etiology of the disease. Late stage disease may or may not be associated with the primary translocation.

- 5 In one embodiment the present invention provides a method of treating a subject afflicted with a bone marrow disorder comprising administering to the subject a compound according to the present invention in an amount sufficient to ameliorate or alleviate the symptoms of the disorder, thereby treating the subject. A more detailed description of bone marrow disorders is provided *infra*.

10 B-cell Associated Malignancies

B cell neoplasms include precursor B-lymphoblastic leukemia/lymphoma (precursor B- cell acute lymphoblastic leukemia), B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, B-cell prolymphocytic leukemia, Lymphoplasmacytic lymphoma, Splenic marginal zone B-cell lymphoma, Hairy cell
15 leukemia, Plasma cell myeloma/plasmacytoma, Extranodal marginal zone B-cell

lymphoma of MALT type, Nodal marginal zone B-cell lymphoma, Follicular lymphoma, Mantle-cell lymphoma Diffuse large B-cell lymphoma, Monocytoid B-cell lymphoma and Multiple myeloma.

Multiple Myeloma

5 Multiple myeloma (MM) is a fatal hematopoietic malignancy of plasma cells, typically affecting people over 60 years old. Multiple myeloma is the second most prevalent blood cancer after non-Hodgkin's lymphoma, representing approximately 10% of all hematopoietic cancers. The etiology of multiple myeloma is unknown, yet current theories involve chronic antigenic stimulation of a plasma cell, which results in
10 transformation and the development of myeloma. Once a plasma cell is transformed, it produces clones, which spread hematogenously to other myelogenous areas. Once there, these neoplastic cells replace the normal bone marrow. The osteoblastic response in myeloma tends to be suppressed and the myeloma cells produce osteoclast-stimulating factor, a cytokine that results in bone destruction. The plasma cell activating factor
15 interleukin 6 is found within bone marrow, resulting in plasma cell proliferation.

Several genetic determinants have been shown to be responsible for the onset and progression of MM. Approximately 15%-20% of the MM cases are associated with a chromosomal translocation, t(4;14)(p16.3;q32), that deregulates the expression of MMSET from der(4) and FGFR3 from der(14). FGFR3 expresses at very high levels and
20 induces proliferative signals in myeloma cells. This translocation has been shown to be a primary event in MM and in some cases activating mutations of FGFR3 are acquired as the disease progresses. Recent studies demonstrate that patients with t(4;14) have a particularly poor prognosis

Leukemias

25 Leukemias are grouped into four main types: acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myelocytic leukemia (CML) according to how quickly they progress and the type of cell that becomes cancerous. Acute leukemias progress rapidly; chronic leukemias progress slowly. Lymphocytic leukemias develop from cancerous changes in lymphocytes or in
30 cells that normally produce lymphocytes; myelocytic (myeloid) leukemias develop from cancerous changes in cells that normally produce neutrophils, basophils, eosinophils, and monocytes.

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a malignant (clonal) disease of the bone marrow in which early lymphoid precursors proliferate and replace the normal hematopoietic cells of the marrow. ALL may be distinguished from other malignant lymphoid disorders by the immunophenotype of the cells, which is similar to B- or T-precursor cells. Immunochemistry, cytochemistry, and cytogenetic markers also may aid in categorizing the malignant lymphoid clone. Acute lymphocytic leukemia (ALL) occurs in people of all ages but is the most common cancer in children, accounting for 25% of all cancers in children younger than 15 years. ALL most often affects young children and adults older than 65.

Chromosomal rearrangements are associated with about 50% of adult ALL. Genomic signatures have been identified for the remaining cases yet the molecular mechanism of transformation is unknown (Chiaretti et al., 2005, Clin Cancer Res 11(20):7209-7218).

Acute Myelocytic Leukemia

Also known as acute myeloid, myeloblastic or myelogenous leukemia. Immunophenotyping can be used to help distinguish AML from ALL and further classify the subtype of AML and in some cases may even correlate with prognosis. Recently, several molecular abnormalities that are not detected with routine cytogenetics have been shown to have prognostic importance in patients with AML, including:

Fms-like tyrosine kinase 3 (FLT3) is the most commonly mutated gene in persons with AML and is constitutively activated in one third of AML cases. Internal tandem duplications (ITDs) in the juxtamembrane domain of FLT3 exist in 25% of AML cases. In other cases, mutations exist in the activation loop of FLT3. Most studies demonstrate that patients with AML and FLT3 mutations have a poor prognosis.

Mutations in CEBPA are detected in 15% of patients with normal cytogenetics findings and are associated with a longer remission duration and longer overall survival. Mutations in nucleophosmin (NPM) are associated with increased response to chemotherapy in patients with a normal karyotype.

Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL), also known as M3, is the most common subtype of AML associated with DIC. Acute promyelocytic leukemia (APL) is a malignancy of the bone marrow in which there is a deficiency of mature blood cells in

the myeloid line of cells and an excess of immature cells called promyelocytes. APL is associated with the t(15;17) translocation.

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia in Western countries, is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes. The cells of origin in the majority of patients with CLL are clonal B cells arrested in the B-cell differentiation pathway, between pre-B cells and mature B cells. Morphologically these cells resemble mature lymphocytes. B-CLL lymphocytes typically show B-cell surface antigens, as demonstrated by CD19, CD20, CD21, and CD24 monoclonal antibodies. In addition, they express CD5, which is more typically found on T cells. Because normal CD5+ B cells are present in the mantle zone (MZ) of lymphoid follicles, B-cell CLL is most likely a malignancy of an MZ-based subpopulation of anergic self-reactive cells devoted to the production of polyreactive natural autoantibodies.

B-CLL cells express extremely low levels of surface membrane immunoglobulin, most often immunoglobulin M (IgM) or IgM and immunoglobulin D (IgD). Additionally, they also express extremely low levels of a single immunoglobulin light chain (kappa or lambda). Recent studies have demonstrated that the proto-oncogene, *bcl-2*, is over-expressed in B-CLL, yet genetic translocations that are known to result in the over-expression of *bcl-2*, such as t(14;18), are not found in patients with CLL.

An abnormal karyotype is observed in the majority of patients with CLL. The most common abnormality is deletion of 13q, which occurs in more than 50% of patients. Patients showing 13q14 abnormalities have a relatively benign disease that usually manifests as stable or slowly progressive isolated lymphocytosis. The presence of trisomy 12, which is observed in 15% of patients, is associated with atypical morphology and progressive disease. Deletions of bands 11q22-q23, observed in 19% of patients, are associated with extensive lymph node involvement and aggressive disease. More sensitive techniques have demonstrated abnormalities of chromosome 12. Approximately 2-5% of patients with CLL exhibit a T-cell phenotype.

Chronic Myelocytic Leukemia

Also known as chronic myeloid or myelogenous leukemia. Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by increased proliferation of the granulocytic cell line without the loss of their capacity to differentiate.

Consequently, the peripheral blood cell profile shows an increased number of granulocytes and their immature precursors, including occasional blast cells.

CML is an acquired abnormality that involves the hematopoietic stem cell. It is characterized by a cytogenetic aberration consisting of a reciprocal translocation between the long arms of chromosomes 22 and 9; t(9;22). The translocation results in a shortened chromosome 22, an observation first described by Nowell and Hungerford and subsequently termed the Philadelphia (Ph) chromosome after the city of discovery. This translocation relocates an oncogene called *abl* from the long arm of chromosome 9 to the long arm of chromosome 22 in the BCR region. The resulting *bcr/abl* fusion gene encodes at least three different chimeric proteins, depending on exon usage of *bcr*. The expression of the BCR/ABL protein leads to the development of the CML phenotype through processes that are not yet fully understood. The presence of *bcr/abl* translocation is the hallmark of CML, and has been identified associated with other leukemias.

Myelodysplastic Syndrome (MDS)

Myelodysplastic Syndrome (MDS) is a clonal disorder of hematopoietic stem cells that results in excessive apoptosis. This leads to symptoms of anemia, infection, and excessive bleeding and bruising. Over time MDS tends to progress to acute myeloid leukemia. Several target genes have been identified as targets in the treatment of MDS including *inter alia* VEGF (vascular endothelial growth factor), VEGF receptor, TNF α (tumor necrosis factor), and farnesyl-transferase inhibitors yet no one therapy has been found to be effective in treating MDS (reviewed in Meletis et al., 2006. *Med Sci Monit.* 12(9):RA194-206).

The World Health Organization has classified the dysplastic syndromes under the a new WHO system as follows:

Refractory anemia (RA)

Refractory anemia with ringed sideroblasts (RARS)

Refractory cytopenia with multilineage dysplasia (RCMD)

Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS)

Refractory anemia with excess blasts I and II

5q- syndrome

Myelodysplasia unclassifiable (seen in those cases of megakaryocyte dysplasia with fibrosis and others)

Aplastic anemia

5 Aplastic anemia is associated with a loss of cell precursors (usually RBC), due to a defect in the stem cell producing them, or due to an injury to the bone marrow environment. Some aplastic anemias are caused by exposure to chemicals such as benzene, radiation, or certain drugs. A few are due to rare genetic abnormalities (such as Fanconi's anemia), or associated with an acute viral illness (such as human parvovirus), but for about half the cases the cause is unknown.

10 Myeloproliferative Disorders (MPD)

Myeloproliferative disorders (MPD) are a subset of bone marrow disorders characterized by the overproduction of a precursor of a marrow cell.

15 White blood cells (WBCs) help the body to fight infection, red blood cells (RBCs) carry oxygen, and platelets help the blood to clot. In MPD conditions, excessive production of a cell's precursor leads to an increased number of that type of mature cell, and a corresponding increase or decrease in the number of other blood cells (which may be inhibited and crowded out). This results in symptoms related to blood cell overproduction, shortages, and dysfunction throughout the body.

20 The three main myeloproliferative disorders are Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Chronic Idiopathic Myelofibrosis (MF).

Polycythemia Vera is a disease in which an excess of red blood cell precursors and erythrocytes are produced in the bone marrow. Symptoms include a swollen spleen and itching.

25 Agnogenic myeloid metaplasia (also known as Myeloid metaplasia with fibrosis, or myelofibrosis (MF)), is a disease characterized by overproduction of fiber creating cells, leading to excess fibrous tissue in the marrow. The dense network of fiber inhibits the normal formation and maturation of RBCs and myelocytes. The red blood cells that do enter the bloodstream look like teardrops. There may be too few normal mature red blood cells to carry oxygen, causing anemia. Fiber cells may also spread to the spleen,
30 causing it to swell.

Essential Thrombocythemia (ET) is characterized by an increased number of megakaryocytes, precursor to platelets. Essential thrombocythemia must be distinguished

from secondary thrombocytosis, increased numbers of platelets caused by non-marrow disorders (such as iron deficiency, rheumatoid arthritis, bleeding, or removal of the spleen).

Plasma Cell Disorders

5 Plasma cell disorders (plasma cell dyscrasias) are rare in children and young adults. They begin when a single group (clone) of plasma cells multiplies excessively and produces a large quantity of abnormal antibodies. Plasma cell disorders include monoclonal gammopathies of undetermined significance, multiple myeloma, macroglobulinemia, and heavy chain diseases.

10 Hematopoietic Stem Cell (HSC) associated malignancy

Hematopoietic stem cells (HSC) are a rare population of multipotent cells responsible for life-long regeneration of blood cells. All HSC have the dual properties of self-renewal and differentiation. Defects in either of these properties result in a lack of mature blood cell production. Little is known of the molecular mechanisms that
15 determine whether a HSC self-renews or commits to differentiation. Many infant leukemias are associated with a MLL translocation in HSC.

Osteoclast associated diseases

Bone is dynamic tissue that is remodeled constantly throughout life. Living bone tissue is replenished by the processes of resorption and deposition of bone matrix and
20 minerals, in a process termed bone remodeling. The remodeling process is initiated when osteoclasts are recruited from the bone marrow or the circulation to the bone surface. The matrix and minerals of the bone are subsequently replaced by osteoblasts recruited to the resorbed bone surface from the bone marrow. Resorption of bone is carried out mainly by osteoclasts, which are multinucleated cells that are formed by fusion of hematopoietic
25 stem cells related to the mononuclear phagocyte series. Osteoclasts have been linked to many diseases, including: marble disease, osteoporosis, fracture or trauma, bone metastasis, cancer, osteosarcoma, hypercalcemia and rheumatoid arthritis. Therefore, it is desirable to inhibit osteoclasts in various pathologies. In addition to TRAP (tartrate resistant acid phosphatase), osteoclasts have been shown to specifically express several
30 other genes, including those disclosed in US Patent 7,160,994.

Silencing RNA (siRNA)

An siRNA is a double-stranded RNA molecule which down-regulates or silences (prevents or inhibits) the expression of a gene/ mRNA of its endogenous (cellular) counterpart. RNA interference is based on the ability of dsRNA species to enter a specific protein complex, where it is then targeted to the complementary cellular mRNA and specifically degrades it. Thus, the RNA interference response features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having a sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA may take place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir et al 2001, *Genes Dev.*, 15:188). In more detail, longer dsRNAs are digested into short (17-29 bp) dsRNA fragments (also referred to as short inhibitory RNAs, "siRNAs") by type III RNAses (DICER, DROSHA, etc., Bernstein et al., 2001, *Nature* 409, 363-6; Lee et al., 2003, *Nature* 425, 415-9). The RISC protein complex recognizes these fragments and complementary mRNA. The whole process culminates in endonuclease cleavage of target mRNA (McManus & Sharp, 2002, *Nature Rev Genet* 3, 737-47; Paddison & Hannon, 2003, *Curr Opin Mol Ther.* 5(3):217-24). For additional information on these terms and proposed mechanisms, see Bernstein et al., (2001. *RNA* 7(11): 1509–1521).

Several groups have described the development of DNA-based vectors capable of generating siRNA within cells. The method generally involves transcription of short hairpin RNAs that are efficiently processed to form siRNAs within cells. (Paddison et al. 2002, *PNAS*, 99:1443-1448; Paddison et al. 2002, *Genes & Dev*, 16:948-958; Sui et al. 2002 *PNAS*, 8:5515-5520; and Brummelkamp et al. 2002 *Science*, 296:550-553.) Those reports describe methods to generate siRNAs capable of specifically targeting numerous endogenously and exogenously expressed genes. siRNA has recently been successfully used for inhibition in primates (Tolentino et al., 2004 *Retina* 24(1) I 132-138).

The present invention provides double-stranded oligoribonucleotides (siRNAs, aptamers, ribozymes), which down-regulate the expression of genes associated with bone marrow disease. An siRNA of the invention is a duplex oligoribonucleotide in which the sense strand is derived from the mRNA sequence of the gene associated with bone marrow disease, and the antisense strand is complementary to the sense strand. In general, some deviation from the target mRNA sequence is tolerated without compromising the siRNA activity (see e.g. Czauderna et al 2003 *NAR* 31(11), 2705-

2716). An siRNA of the invention inhibits gene expression on a post-transcriptional level with or without destroying the mRNA. Without being bound by theory, siRNA may target the mRNA for specific cleavage and degradation and/ or may inhibit translation from the targeted message.

5 Modified Oligonucleotides

Generally, the siRNAs used in the present invention comprise a ribonucleic acid comprising a double stranded structure, whereby the double-stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary
10 to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, whereby said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within the strand each group of modified nucleotides is flanked on one or both sides by a
15 flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides. Further, said first strand and/or said second strand may comprise said plurality of modified nucleotides and may comprises said plurality of groups of modified nucleotides.

20 The group of modified nucleotides and/or the group of flanking nucleotides may comprise a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides. In connection with any ranges specified herein it is to be understood that each range discloses any individual integer between the respective figures used to define the range including said two figures defining said range.
25 In the present case the group thus comprises one nucleotide, two nucleotides, three nucleotides, four nucleotides, five nucleotides, six nucleotides, seven nucleotides, eight nucleotides, nine nucleotides and ten nucleotides.

The pattern of modified nucleotides of said first strand may be shifted by one or more nucleotides relative to the pattern of modified nucleotides of the second strand.

30 The modifications discussed above may be selected from the group consisting of amino, fluoro, methoxy alkoxy, alkyl, amino, fluoro, chloro, bromo, CN, CF, imidazole, carboxylate, thioate, C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl or aralkyl, OCF₃, OCN, O-, S-, or N- alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂CH₃; ONO₂; NO₂, N₃; heterozycloalkyl; heterozycloalkaryl; aminoalkylamino; polyalkylamino or substituted

silyl, as, among others, described in European patents EP 0586520 B1 or EP 0618925 B1.

The oligonucleotide of the present invention may contain modified nucleosides such as DNA, LNA, PNA, arabinoside or one or more mirror nucleosides (spiegelmers).
5 The oligonucleotide may further comprise 2'O-methyl or 2'fluoro or 2'O-allyl or any other 2' modification, optionally on alternate positions. Other stabilizing patterns which do not significantly reduce the enzymatic activity are also possible (i.e. terminal modifications). The backbone of the active part of tandem oligonucleotides may comprise phosphate-D-ribose entities but may also contain thiophosphate-D-ribose
10 entities, triester, thioate, 5'-2' bridged backbone or any other type of modification. Terminal modifications on the 5' and/or 3' part of the tandem oligonucleotides are also possible. Such terminal modifications may be lipids, peptides, sugars or other molecules.

In an additional embodiment, the oligonucleotide is an oligoribonucleotide, comprising one or more backbone modifications selected from the group consisting of
15 thiophosphate, trimester or 5'-2' bridge.

Furthermore, the double stranded structure of the siRNA may be blunt ended, on one or both of its ends. More specifically, the double stranded structure may be blunt ended on the double stranded structure's side which is defined by the 5'- end of the first strand and the 3'-end of the second strand, or the double stranded structure may be blunt
20 ended on the double stranded structure's side which is defined by at the 3'-end of the first strand and the 5'-end of the second strand.

Additionally, at least one of the two strands may have an overhang of at least one nucleotide at the 5'-end; the overhang may consist of at least one deoxyribonucleotide. At least one of the strands may also optionally have an overhang of at least one nucleotide at
25 the 3'-end.

The length of each of the strands in the double-stranded structure of the siRNA is typically from about 17 to about 21 and preferably 18 or 19 bases. Furthermore, the length of said first strand and/or the length of said second strand may independently from each other be selected from the group comprising the ranges of from about 15 to about 23
30 bases, 17 to 21 bases and 18 or 19 bases.

Additionally, the complementarity between said first strand and the target nucleic acid may be perfect, or the duplex formed between the first strand and the target nucleic acid may comprise at least 15 nucleotides wherein there is one mismatch or two

mismatches between said first strand and the target nucleic acid forming said double-stranded structure.

In some cases both the first strand and the second strand each comprise at least one group of modified nucleotides and at least one flanking group of nucleotides, whereby
 5 each group of modified nucleotides comprises at least one nucleotide and whereby each flanking group of nucleotides comprising at least one nucleotide with each group of modified nucleotides of the first strand being aligned with a flanking group of nucleotides on the second strand, whereby the most terminal 5' nucleotide of the first strand is a nucleotide of the group of modified nucleotides, and the most terminal 3'
 10 nucleotide of the second strand is a nucleotide of the flanking group of nucleotides. Each group of modified nucleotides may consist of a single nucleotide and/or each flanking group of nucleotides may consist of a single nucleotide.

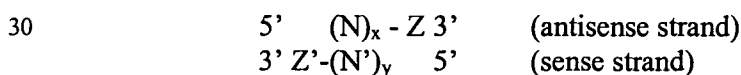
Additionally, it is possible that on the first strand the nucleotide forming the flanking group of nucleotides is an unmodified nucleotide which is arranged in a 3'
 15 direction relative to the nucleotide forming the group of modified nucleotides, and on the second strand the nucleotide forming the group of modified nucleotides is a modified nucleotide which is arranged in 5' direction relative to the nucleotide forming the flanking group of nucleotides.

Further, the first strand of the siRNA may comprise eight to twelve, preferably nine
 20 to eleven, groups of modified nucleotides, and the second strand may comprise seven to eleven, preferably eight to ten, groups of modified nucleotides.

The first strand and the second strand may be linked by a loop structure, which may be comprised of a non-nucleic acid polymer such as, *inter alia*, polyethylene glycol. Alternatively, the loop structure may be comprised of a nucleic acid.

25 Further, the 5'-terminus of the first strand of the siRNA may be linked to the 3'-terminus of the second strand, or the 3'-end of the first strand may be linked to the 5'-terminus of the second strand, said linkage being via a nucleic acid linker typically having a length between 10-2000 nucleobases.

In particular, the invention provides a compound having structure A:



wherein each of N and N' is a ribonucleotide which may be modified or unmodified in its sugar residue and (N)_x and (N')_y is oligomer in which each consecutive N or N' is joined to the next N or N' by a covalent bond ;

5 wherein each of x and y is an integer between 19 and 40;
 wherein each of Z and Z' may be present or absent, but if present is dTdT and is covalently attached at the 3' terminus of the strand in which it is present; and
 wherein the sequence of (N)_x comprises an antisense sequence relative to the
10 mRNA transcribed from the gene associated with the bone marrow disorder.

 It will be readily understood by those skilled in the art that the compounds of the present invention consist of a plurality of nucleotides, which are linked through covalent linkages. Each such covalent linkage may be a phosphodiester linkage, a phosphorothioate linkage, or a combination of both, along the length of the nucleotide
15 sequence of the individual strand. Other possible backbone modifications are described in, *inter alia*, U.S. Patent Nos. 5,587,361; 6,242,589; 6,277,967; 6,326,358; 5,399,676; 5,489,677; and 5,596,086.

 In particular embodiments, each of x and y is independently an integer between about 19 to about 27, most preferably from about 19 to about 23. In a particular
20 embodiment of the compound of the invention, x may be equal to y (*viz.*, $x = y$) and in preferred embodiments $x = y = 19$ or $x = y = 21$. In a particularly preferred embodiment $x = y = 19$.

 In one embodiment of the compound of the invention, Z and Z' are both absent; in another embodiment one of Z or Z' is present.

25 In one embodiment of the compound of the invention, all of the ribonucleotides of the compound are unmodified in their sugar residues.

 In preferred embodiments of the compound of the invention, at least one ribonucleotide is modified in its sugar residue, preferably a modification at the 2' position. The modification at the 2' position results in the presence of a moiety which is
30 preferably selected from the group comprising amino, fluoro, methoxy, alkoxy and alkyl groups. In a presently most preferred embodiment the moiety at the 2' position is methoxy (2'-O-methyl).

 In preferred embodiments of the invention, alternating ribonucleotides are modified in both the antisense and the sense strands of the compound. In particular, in some

embodiments the present invention provides an siRNA having 2' O-Me groups on the first, third, fifth, seventh, ninth, eleventh, thirteenth, fifteenth, seventeenth and nineteenth nucleotide of the antisense strand, whereby the same modification, i. e. a 2'-O-Me group was present at the second, fourth, sixth, eighth, tenth, twelfth, fourteenth, sixteenth and 5
eighteenth nucleotide of the sense strand. Additionally, it is to be noted that the in case of these particular nucleic acids according to the present invention the first stretch is identical to the first strand and the second stretch is identical to the second strand and these nucleic acids are also blunt ended.

According to one preferred embodiment of the invention, the antisense and the 10
sense strands of the siRNA molecule are both phosphorylated only at the 3'-terminus and not at the 5'-terminus. According to another preferred embodiment of the invention, the antisense and the sense strands are both non-phosphorylated both at the 3'-terminus and also at the 5'-terminus. According to yet another preferred embodiment of the invention, the 1st nucleotide in the 5' position in the sense strand is specifically modified to abolish 15
any possibility of *in vivo* 5'-phosphorylation.

In another embodiment of the compound of the invention, the ribonucleotides at the 5' and 3' termini of the antisense strand are modified in their sugar residues, and the ribonucleotides at the 5' and 3' termini of the sense strand are unmodified in their sugar residues.

20 The invention also provides a composition comprising one or more of the compounds of the invention in a carrier, preferably a pharmaceutically acceptable carrier. This composition may comprise a mixture of two or more different siRNAs.

The invention also provides a composition which comprises the above compound of the invention covalently or non-covalently bound to one or more compounds of the 25
invention in an amount effective to inhibit a gene associated with a bone marrow disease; and a physiologically acceptable carrier. This composition may be processed intracellularly by endogenous cellular complexes to produce one or more oligoribonucleotides of the invention.

The invention also provides a composition comprising a carrier and one or more of 30
the molecules of the invention in an amount effective to down-regulate expression a cell of a gene associated with bone marrow disease, wherein the molecule comprises a sequence substantially complementary to the sequence of (N)_x.

Additionally, the invention provides a method of down-regulating the expression of a gene associated with bone marrow disease by at least 50% as compared to a control, comprising contacting an mRNA transcript of a gene associated with bone marrow disease with one or more of the compounds of the invention.

5 In one embodiment the oligoribonucleotide is down-regulating a gene associated with bone marrow disease, whereby the down-regulation is selected from the group comprising down-regulation of gene function, down-regulation of polypeptide and down-regulation of mRNA expression.

Down-regulation refers to one or more of: down-regulation of function (which may
10 be examined by an enzymatic assay or a binding assay with a known interactor of the native gene / polypeptide, *inter alia*), down-regulation of protein (which may be examined by Western blotting, ELISA or immuno-precipitation, *inter alia*) and down-regulation of mRNA expression (which may be examined by, *inter alia*, Northern blotting, quantitative RT-PCR, in-situ hybridization or microarray hybridization).

15 The present invention also provides a method of treating a patient suffering from a disease accompanied by an elevated level of a polypeptide associated with bone marrow disease, the method comprising administering to the patient a composition of the invention in a therapeutically effective dose thereby treating the patient.

Aptamers

20 As disclosed herein, aptamers may also be used *per se* in the present invention or in combination with an siRNA disclosed herein. For example, an aptamer can be used with any one of the siRNA molecules disclosed herein in combination therapy for the treatment of any one of the conditions disclosed herein. The novel pharmaceutical composition employed for such a combination therapy, which is also part of the present
25 invention, may comprise an siRNA of the present invention covalently or non-covalently attached to an aptamer. Aptamers are RNA or DNA single-strand or double-strand oligonucleic acids, which bind to a target protein and do not generally exhibit non-specific effects. Aptamers can be modified for stability or other desired qualities in accordance with any nucleic acid modifications disclosed herein and/or known to one of
30 skill in the art. Modifications to aptamers can be introduced anywhere in the molecule, such as the 5' or 3' termini, or at any internally defined modification site. For example, RNA aptamers can be stabilized with 2'-fluoro or 2'-amino modified pyrimidines. Aptamers can also be linked to reporter molecules or linkers and can be attached to beads or other solid support if necessary (e.g., 5' or 3' amino, thiol ester or biotin groups).

Thioaptamers are aptamers which contain sulfur modifications at specific internucleoside phosphoryl sites, and may possess enhanced stability, nuclease resistance, target affinity and/or selectivity. Examples of thioaptamers include phosphoromonothioate (S-ODN) and phosphorodithioate (S2-ODN) oligodeoxy thioaptamers. For further information on aptamers and thioaptamers see US Patent Nos. 5,218,088 and 6,423,493.

Another compound of the invention comprises the above compound of the invention (structure A) covalently or non-covalently bound to one or more compounds of the invention (structure A). This compound may be delivered in a carrier, preferably a pharmaceutically acceptable carrier, and may be processed intracellularly by endogenous cellular complexes to produce one or more siRNAs of the invention.

The scope of the present invention also comprises a tandem double-stranded structure which comprises two or more siRNA sequences, which is processed intracellularly to form two or more different siRNAs, each one inhibiting a different gene associated with a bone marrow disease. In a related aspect, this invention also relates to a tandem double-stranded structure which comprises two or more siRNA sequences, which is degraded intracellularly to form two or more different siRNAs, both inhibiting the same gene associated with a bone marrow disease.

In particular, it is envisaged that a long oligonucleotide (typically about 80-500 nucleotides in length) comprising one or more stem and loop structures, where stem regions comprise the sequences of the oligonucleotides of the invention, may be delivered in a carrier, preferably a pharmaceutically acceptable carrier, and may be processed intracellularly by endogenous cellular complexes (e.g. by DROSHA and DICER as described above) to produce one or more smaller double stranded oligonucleotides (siRNAs) which are oligonucleotides of the invention. This oligonucleotide can be termed a tandem shRNA construct. It is envisaged that this long oligonucleotide is a single stranded oligonucleotide comprising one or more stem and loop structures, wherein each stem region comprises a sense and corresponding antisense siRNA sequence.

Antisense molecules

By the term "antisense" (AS) or "antisense fragment" is meant a polynucleotide fragment (comprising either deoxyribonucleotides, ribonucleotides or a mixture of both) having inhibitory antisense activity, said activity causing a decrease in the expression of the endogenous genomic copy of the corresponding gene. An AS polynucleotide is a polynucleotide which comprises consecutive nucleotides having a sequence of sufficient

length and homology to a sequence present within the sequence of the target gene to permit hybridization of the AS to the gene. The sequence of the AS is designed to complement a target mRNA of interest and form an RNA:AS duplex. This duplex formation can prevent processing, splicing, transport or translation of the relevant mRNA. Moreover, certain AS nucleotide sequences can elicit cellular RNase H activity when hybridized with their target mRNA, resulting in mRNA degradation (Calabretta et al, 1996 *Semin Oncol.* 23(1):78-87). In that case, RNase H will cleave the RNA component of the duplex and can potentially release the AS to further hybridize with additional molecules of the target RNA. An additional mode of action results from the interaction of AS with genomic DNA to form a triple helix which can be transcriptionally inactive.

Antisense intervention in the expression of specific genes can be achieved by the use of synthetic AS oligonucleotide sequences (see for example Lefebvre-d'Hellencourt et al, 1995. *Eur. Cytokine Netw.* 6:7; Agrawal, 1996. *TIBTECH*, 14:376.; Lev-Lehman et al., 1997. *In Antisense Therapeutics*, A. Cohen and S. Smicek, eds (Plenum Press, New York)). AS oligonucleotide sequences are designed to complement a target mRNA of interest and form an RNA:AS duplex. This duplex formation can prevent processing, splicing, transport or translation of the relevant mRNA. Moreover, certain AS nucleotide sequences can elicit cellular RNase H activity when hybridized with their target mRNA, resulting in mRNA degradation (Calabretta, et al, 1996. *Semin. Oncol.* 23:78). In that case, RNase H will cleave the RNA component of the duplex and can potentially release the AS to further hybridize with additional molecules of the target RNA. An additional mode of action results from the interaction of AS with genomic DNA to form a triple helix which may be transcriptionally inactive.

The sequence target segment for the antisense oligonucleotide is selected such that the sequence exhibits suitable energy related characteristics important for oligonucleotide duplex formation with their complementary templates, and shows a low potential for self-dimerization or self-complementation (Anazodo et al., 1996). For example, the computer program OLIGO (Primer Analysis Software, Version 3.4), can be used to determine antisense sequence melting temperature, free energy properties, and to estimate potential self-dimer formation and self-complimentary properties. The program allows the determination of a qualitative estimation of these two parameters (potential self-dimer formation and self-complimentary) and provides an indication of "no potential" or "some potential" or "essentially complete potential". Using this program target segments are generally selected that have estimates of no potential in these parameters. However,

segments can be used that have "some potential" in one of the categories. A balance of the parameters is used in the selection as is known in the art. Further, the oligonucleotides are also selected as needed so that analogue substitution does not substantially affect function.

5 Phosphorothioate antisense oligonucleotides do not normally show significant toxicity at concentrations that are effective and exhibit sufficient pharmacodynamic half-lives in animals (Agrawal, 1996. *TIBTECH*, 14:376) and are nuclease resistant. Antisense induced loss-of-function phenotypes related with cellular development have been shown for the glial fibrillary acidic protein (GFAP), for the establishment of tectal plate
10 formation in chick (Galileo et al., 1991. *J. Cell. Biol.*, 112:1285) and for the N-myc protein, responsible for the maintenance of cellular heterogeneity in neuroectodermal cultures (epithelial vs. neuroblastic cells, which differ in their colony forming abilities, tumorigenicity and adherence) (Rosolen et al., 1990. *Cancer Res.* 50:6316; Whitesell et al., 1991 *Mol. Cell. Biol.* 11:1360). Antisense oligonucleotide inhibition of basic
15 fibroblast growth factor (bFGF), having mitogenic and angiogenic properties, suppressed 80% of growth in glioma cells (Morrison, 1991. *J. Biol. Chem.* 266:728) in a saturable and specific manner. Being hydrophobic, antisense oligonucleotides interact well with phospholipid membranes (Akhter et al, 1991. *Nuc. Acid Res.* 19:5551-5559). Following their interaction with the cellular plasma membrane, they are actively (or passively)
20 transported into living cells (Loke et al, 1989. *PNAS USA* 86:3474.), in a saturable mechanism predicted to involve specific receptors (Yakubov et al, 1989. *PNAS USA* 86:6454).

Ribozyme

A "ribozyme" is an RNA molecule that possesses RNA catalytic ability (see Cech
25 for review) and cleaves a specific site in a target RNA. In accordance with the present invention, ribozymes which cleave mRNA may be utilized as inhibitors. This may be necessary in cases where antisense therapy is limited by stoichiometric considerations (Sarver et al., 1990, *Gene Regulation and Aids*, pp. 305-325). Ribozymes can then be used that will target a gene associated with a bone marrow disease. The number of RNA
30 molecules that are cleaved by a ribozyme is greater than the number predicted by stoichiometry. (Hampel and Tritz, 1989 *Biochemistry*, 28:4929; Uhlenbeck, 1987 *Nature*, 328:596).

Ribozymes catalyze the phosphodiester bond cleavage of RNA. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis

delta virus ribozyme, hammerhead ribozymes and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA (sTRSV) (Sullivan, 1994; U.S. Pat. No. 5,225,347). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication (Symons, 1989 *Trends Biochem Sci.* 1989 14 (11):445-50 and Symons 1992 *Annu Rev Biochem.* 61:641-71). Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). In general the ribozyme has a length of from about 30-100 nucleotides. Delivery of ribozymes is similar to that of AS fragments and/or siRNA molecules.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. The disclosures of these publications and patents and patent applications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

EXAMPLES

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the claimed invention in any way.

Abbreviations

RNAi: RNA interference; siRNA: small interfering RNA; dsRNA: double stranded RNA; ALL: Acute Lymphocytic Leukemia; AML: Acute Myelocytic (Myeloid, Myelogenous, Myeloblastic, Myelomonocytic) Leukemia; CLL: Chronic Lymphocytic Leukemia; CML: Chronic Myelocytic (Myeloid, Myelogenous, Granulocytic) Leukemia; CMML: Chronic Myelomonocytic Leukemia; ALCL: Anaplastic Large-Cell Lymphoma,

APL: Acute Promyelocytic Leukemia; DLBCL: Diffuse Large-B-Cell Lymphoma; MM: multiple myeloma; ITD: internal tandem duplication

Standard molecular biology protocols known in the art not specifically described herein are generally followed essentially as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New-York (1989, 1992), and in 5 Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1988).

Standard organic synthesis protocols known in the art not specifically described herein are generally followed essentially as in *Organic Syntheses: Vol. 1- 79*, editors vary, 10 J. Wiley, New York, (1941 - 2003); Gewert et al., *Organic Synthesis Workbook*, Wiley-VCH, Weinheim (2000); Smith & March, *Advanced Organic Chemistry*, Wiley-Interscience; 5th edition (2001).

Standard medicinal chemistry methods known in the art not specifically described herein are generally followed essentially as in the series "Comprehensive Medicinal 15 Chemistry", by various authors and editors, published by Pergamon Press.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

Example 1: Down regulation of p53 in bone marrow cells

20 The efficacy and duration of siRNA effect on p53 transcript were assayed in various rat tissues using real-time quantitative PCR (qPCR). A decrease in p53 level was observed in the bone marrow of treated animals. Surprisingly the level of p53 decreased over 24 and 48 hrs and was restored to basal level at only after 72 hrs, post administration.

25 p53 mRNA levels in tissues were determined by SYBR® green I-based quantitative real-time PCR (QPCR), performed on total RNA isolated from tissue/organs. Data processing was based on the standard curve method as disclosed

The p53 siRNA molecule contains a 19 base pair RNA duplex, where the antisense strand is complementary to the p53 gene. The p53 siRNA also contains alternating 2'-O-methyl modifications on the ribose sugar that confer resistance to nuclease degradation. 30 The p53 siRNA utilized in the present experiment, denoted QM5, has been disclosed in US Patent Application Publication No. 2006/0069056, assigned to the assignee of the present invention, the contents of which are hereby incorporated by reference.

Primers used for p53 amplification were:

GGAGCTGTTGCACATGTACT (REVERSE) and
ACAGCGTGGTGGTACCGTAT (FORWARD).

Primers used for s14 ribosomal protein amplification were:

5 ACCCTTCCTTCGAGTGCTGTCA (REVERSE) and
AGAGCTATGAGCTGCCTGAC (FORWARD).

The experiment was performed on male Sprague-Dawley (SD) rats. Following qPCR analysis of gene transcript levels, animals were further divided, according to basal transcript levels, into 2 sub-batches differing in tissue harvesting time (Ia and Ib). Dose
10 was 12 mg/kg, in a volume of 140 microliter for intravenous (i.v.) injection.

The measured p53 mRNA levels were corrected for errors arising from differences in sample preparation and reverse transcription efficiencies, using the expression levels of a housekeeping reference gene as an internal standard. Ribosomal protein S14 (RPS14) served as standard in BM samples.

15 Results:

The qPCR results passed QC standards, i.e. the value of the standard curve slope was in the interval [-4, -3], $R^2 > 0.99$, and no primer dimers.

qPCR analysis revealed differences in basal transcript levels of test gene (p53) and of reference genes, as measured in control PBS injected animals. A control PBS injected
20 group was included in each animal batch, which enabled the normalization of transcript level values measured in treatment groups to the levels measured in the corresponding PBS group.

Normalized p53 transcript levels, in experimental samples, are presented by 2 Box-plot figures. One portrays the level of p53 distribution in natural logarithm (Ln) of the
25 ratio between p53 and the reference gene transcript levels [$\text{Ln}(\text{p53}/ \text{reference gene})$] and the other portrays the distribution ratio. The results obtained from the two 24 hour groups were combined to one group following the normalization to their corresponding PBS controls.

30

Table 2 shows the mean p53 inhibition.

Tissue	Tested Gene	Reference Gene	Group	N	Ln(Ratio)		Ratio	
					Mean	Std	Mean	Std
BM	TP53	S14	24 hr	5	-0.605	0.509	0.60	0.33
			48 hr	7	-0.667	0.145	0.52	0.08
			72 hr	7	-0.006	0.377	1.05	0.36

Transcript levels are expressed as Ln quantity

Figure 1A is a graphic representation of the normalized log ratio of p53 levels.

Figure 1B shows the normalized ratio of p53 levels.

5 These results show that the siRNA was delivered specifically to the bone marrow and that the level of expression of the target gene was reduced.

Distribution of proprietary siRNA was measured in rat tissues and organs following four consequent bolus intravenous injections of 10 mg/kg siRNA (mouse p53) given over a period of 6 hrs with 1.5-2 hrs intervals. The analysis was done in normal rats and those
 10 with abnormal kidney function since oligonucleotides are mainly excreted through kidney. The siRNA concentration in tissues was measured at 3 hrs and 30 hrs after the last siRNA administration using a quantitative method developed for this particular siRNA molecule. As shown in Figure 1C, siRNA was detected in bone marrow (BM) cells at least as early as 3h after the last siRNA injection and was one of the highest
 15 among the organs tested, ~40 ng/g tissue. The siRNA concentration in BM appeared relatively stable and declined only by 30% over the next 27 hours (Fig. 1C lower). siRNA retention in BM was similar in both normal and 5/6 nephrectomized rats.

Example 2: Pharmacology and drug delivery

20 The compounds or pharmaceutical compositions of the present invention are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual subject, the disease to be treated, the site and method of administration, scheduling of administration, subject age, sex, body weight and other factors known to medical practitioners.

25 The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the subject species being treated. It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein.

5 The compounds of the present invention can be administered by any of the conventional routes of administration. A currently preferred mode of administration is parenteral, and, in particular, intravenous administration.

10 It should be noted that the compound can be administered as the compound or as pharmaceutically acceptable salt and can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, solvents, diluents, excipients, adjuvants and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants of the compounds are also useful. Liquid forms may be prepared for injection, the term
15 including subcutaneous, transdermal, intravenous, intramuscular, intrathecal, and other parental routes of administration. The liquid compositions include aqueous solutions, with and without organic cosolvents, aqueous or oil suspensions, emulsions with edible oils, as well as similar pharmaceutical vehicles. In addition, under certain circumstances the compositions for use in the novel treatments of the present invention may be formed
20 as aerosols, for intranasal and like administration. The subject being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, solvents, diluents, excipients, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

25 When administering the compound of the present invention parenterally, it is generally formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example,
30 water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil,

soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it is desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the subject in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the subject in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include U. S. Patent Nos. 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

A pharmacological formulation of the compound utilized in the present invention can be administered orally to the subject. Conventional methods such as administering the compound in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver the composition orally or intravenously and retain the biological activity are preferred.

In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The subject's levels are then maintained by an oral dosage form, although other forms of

administration, dependent upon the subject's condition and as indicated above, can be used.

In general, the active dose of compound for humans is in the range of from 1ng/kg to about 20-100 mg/kg body weight per day, preferably about 0.01 mg to about 2-10 mg/kg body weight per day, in a regimen of one dose per day or twice or three or more times per day for a period of 1-2 weeks or longer, preferably for 24-to 48 hrs or by continuous infusion during a period of 1-2 weeks or longer.

The therapeutic compositions of the present invention can be administered into the lung by inhalation of an aerosol containing these compositions / compounds, or by intranasal or intratracheal instillation of said compositions. Formulating the compositions in liposomes may benefit absorption. Additionally, the compositions may include a PFC liquid such as perflubron, and the compositions may be formulated as a complex of the compounds of the invention with polyethylemeimine (PEI).

For further information on pulmonary delivery of pharmaceutical compositions see Weiss et al., *Human gene therapy* 10:2287-2293 (1999); Densmore et al., *Molecular therapy* 1:180-188 (1999); Gautam et al., *Molecular therapy* 3:551-556 (2001); and Shahiwala & Misra, *AAPS PharmSciTech* 5 (2004).

The term "naked siRNA" refers to siRNA molecules that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. For example, siRNA in PBS is "naked siRNA".

Further, administration of any naked siRNA, oligonucleotide, combination of naked siRNAs or combination of naked siRNA and additional molecule in order to treat any of the diseases and conditions disclosed herein is within the scope of the present invention.

However, as disclosed herein, the siRNA molecules of the invention can also be delivered in liposome formulations and lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

Example 3: Animal Models

The following animal models are presented as examples for use in testing exemplary oligonucleotide molecules of the present invention. Other animal models may also be considered.

5 A. Transplantation in immunodeficient mice

The NOD/SCID mouse is defective in both lymphoid and myeloid function and will readily accept the long-term survival of human hematopoietic cells. Transplantation of human bone marrow into NOD/SCID mice to human/mouse chimeras, is well documented.

10 The NOD/SCID mice were used by Bertolini et al (2000. Blood 96-282) as a model high-grade non-Hodgkin lymphoma. The Namalwa cell line was used, which is derived from an Epstein-Barr virus-positive Burkitt non-Hodgkin lymphoma. The cells (10×10^6) were injected intraperitoneally into 6-8 weeks old mice. Intraperitoneal tumors were formed in the injection site which could be measured by calipers. The formula: "width² x length x 0.52" was applied to approximate the volume of a spheroid. (see Bohem et al
15 (1997) for further reference).

In the model of human AML described by Hall et al (1999) severe combined immunodeficiency (SCID) mice were injected intravenously with 1×10^7 HL-60 cells (AML-M2 cell line). The SCID mice developed abdominal masses, infiltration of the
20 liver and bone marrow, and peripheral blasts with a median survival of 42.5 days. This AML model was used to assess the therapeutic effect of diphtheria toxin fused to GM-CSF. This treatment caused an improvement in the survival of the mice.

The model used in the following studies is based on transgenic SCID mice expressing human GM-CSF. The expression of this cytokine enabled the successful
25 grafting of relevant cells lines in the SCID mice. Miyakawa et al (1996) details the production of the hGM-CSF SCID transgenic mice. Fukuchi et al (1998) shows that a retinoic-acid resistant leukemia can be established in these transgenic mice. The model consisted of UF-1 cells, an RA-resistant APL cell line established in that laboratory, which are transplanted into these transgenic SCID mice and cause the appearance of
30 subcutaneous tumors. Kinjo et al (2000) uses this model to test a specific treatment, arsenic trioxide, to be used in cases of RA resistant acute promyelocytic leukemia (APL).

The immunodeficient SCID mice are useful recipients of umbilical cord progenitors, but adult bone marrow can be engrafted only in the presence of human

stroma, supplying human cytokines. For a model of acute myeloblastic leukemia (AML), cytokines are also required when SCID mice are used. However, acute lymphoblastic leukemia cells graft efficiently without needing cytokines.

Lewis et al (1998) used the more profoundly immunodeficient mouse strain
5 NOD/SCID in which both T-cell and B-cells are functionally defective, and there is marked impairment of macrophage, natural killer cell, and hemolytic complement activity. These mice can be engrafted with cells taken from CML patients leading to a relatively high success rate and thus form a good model for the disease.

Graft-versus-host disease (GVHD) is a complication of DLI. One possibility of
10 reducing GVHD reactivity in favor of GVL reactivity may be the application of in vitro-selected cytotoxic T cells (CTLs) with relative specificity for leukemic cells.

Nijmeijer et al (2002) used NOD/SCID mice transplanted first with ALL cells and then with CTLs selected in-vitro to recognize the specific ALL cells used. The treatment with CTLs resulted in complete remission in 3/8 of the mice and in 5/8 of the mice partial
15 remission was observed. Thus, this system provides a model to test various modes of CTL treatments of leukemias and other cancers.

Acute promyelocytic leukemia (APL) is characterized by the specific chromosome translocation (15;17) and fusion protein PML-RARa as well as its unique response to the differentiation inducer, all-trans retinoic acid (ATRA). Clinical treatment with ATRA
20 results in a high rate of complete remission, but most patients treated with ATRA alone will develop early clinical relapse or require intensive chemotherapy to prevent relapse. The model described by Zhang et al (1996) involves the used of the NB-4 APL cell-line. Intraperitoneal injection of one million cells into SCID mice results in the appearance of ascites after 1 month. The ascites are taken and injected into SCID mice and in 100% of
25 the cases tumors appear. Treatment of the mice with ATRA caused a significant prolonged survival. Thus, this is a good model to follow additional therapeutic routes for PML treatment.

Naked 19-mer siRNA compounds having the alternating 2'O methylation motif as described above in Example 1, which specifically inhibit the genes described in the
30 models above, are tested in these transplantation animal models which show that these siRNAs treat and/or prevent symptoms of the associated disorder and thus may be used to treat this disorder.

B. Transgenic mouse models

Over expression of the Tcl-1 gene was found in a variety of human tumor-derived B-cell lines. The gene, found at 14q32.1, is also involved in various translocations and inversions in mature T-cell leukemias. To establish an animal model for cancers involving the gene in the relevant cells Bichi et al (2002) produced transgenic mice with the Tcl-1 gene under the control of the mouse VH promoter and IgH-m enhancer. The use of this promoter and enhancer supports expression in immature and mature B-cells. Expansion of specific populations of B-cells was noted in the transgenic mice and elder mice developed chronic lymphocytic leukemia (CLL)-like disorder resembling human B-CLL. Thus, these transgenic mice provide an animal model for CLL, the most common human leukemia. Naked 19-mer siRNA compounds having the alternating 2'O methylation motif as described above in Example 1, which specifically inhibit the Tcl-1 gene, are tested in these animal models which show that these siRNAs treat and/or prevent symptoms of CLL and thus may be used to treat this condition.

There is evidence for the involvement of the translocation t(9;12)(p24;p13) in ALL and myeloid malignancies. The translocation produces a fusion between the genes JAK2 and TEL. The former is involved in signaling pathways regulating hematopoiesis and the later is a gene of the ETS family (less known). A transgene of the TEL-JAK2 fusion, driven by the E μ SRa enhancer/promoter was used by Carron et al (2000) to generate transgenic mice. These transgenic mice develop a fatal CD81 T-cell leukemia at 4 to 22 weeks of age. Thus, these mice form a model for leukemias in which TEL-JAK2 is involved.

Honda et al (1998) describe a more stable model consisting of transgenic mice expressing a p210 bcr/abl transgene. The bcr/abl gene was driven by the tec promoter which directs expression to the hematopoietic lineage. It was shown that the mice developed both CML and ALL and constitute a model for these diseases.

Transgenic mice expressing p210 are also described by Voncken et al (1995). The authors compare the effects of p210 to those of p190 bcr/abl protein. The p190 protein is derived from translocations involving only exon 1 of bcr whereas the p210 protein is derived from translocation of exons 1 to 13 or 14 of the bcr gene. From studies in humans it appeared that p190 is associated only with ALL while p210 is associated with both ALL and CML. Transgenic mice harboring constructs expressing the p190 or the p210 proteins developed tumors. The p190 mice developed leukemia of B-cell origin after a relatively short period of latency. In comparison, the p210 mice developed

leukemia of B, T-lymphoid, or myeloid origin after a relatively long latency. Thus, both types of transgenic mice are good models of the relevant human diseases.

In most cases of acute promyelocytic leukemia (APML) a specific chromosomal translocation, t(15;17) (q22;q21) is detected. This translocation leads to fusion of the PML gene on chromosome 15 with RARa on chromosome 17. Grisolano et al (1997) describe the effects of a PML-RARa transgene on myeloid development in transgenic mice. The transgene was driven by regulatory sequences of the human cathepsin G (hCG) which directs expression to early myeloid cells. In the transgenic mice expressing the transgene altered myeloid development was characterized and 30% of them eventually developed acute myeloid leukemia after a long latent period. Thus, such mice (possibly similar mice expressing higher levels of the PML-RARa transgene) can be used as a model for APML.

The tax gene, encoded by the HTLV-I virus, is implicated in T-cell replication and activation. A tax transgene driven by the granzyme B promoter, targeting expression to mature T-lymphocytes, was used by Grossman et al (1995) to generate transgenic mice. Three expressing lines were examined and all developed leukemia. In one of the lines all progeny developed leukemia. Tumors developed on the ears, legs, and tails and involved the cervical, axillary, popliteal, and mesenteric lymph nodes. Accompanying the large tumors was massive splenomegaly (6- to 16-fold enlargement). Thus, these mice are a good model for tax or HTLV-I related leukemia. Additional papers presenting tax transgenic mice models with different features stemming from different expression patterns, include Nerenberg et al (1987) and Green et al (1989)

The t(14;18) translocation is a characteristic of follicular lymphoma. In this translocation the bcl-2 gene is fused to an immunoglobulin gene. This causes the deregulation of bcl-2, a well known survival factor that works by blocking programmed cell death. McDonnell et al (1991) generated transgenic mice expressing the bcl-2/immunoglobulin gene, mimicking the fusion gene generated by the translocation. Many of the transgenic mice developed lymphoma and thus are a good model for tumors that result from this specific translocation.

Naked 19-mer siRNA compounds having the alternating 2'O methylation motif as described above in Example 1, which specifically inhibit the genes described in the models above, are tested in the transgenic animal models above, which show that these siRNAs treat and/or prevent symptoms of the associated disorder and thus may be used to treat this disorder.

C. Infection with viral vectors

The Philadelphia chromosome is a hallmark of chronic myelogenous leukemia (CML). In the translocation of chromosomes 9 and 22, a fusion gene encoding p210 bcr/abl fusion protein is responsible for the emergence of CML. Daley et al (1990) used retroviruses harboring the bcr/abl fusion gene to infect murine bone marrow cells which were then implanted into mice. The mice developed a myeloproliferative syndrome closely resembling the chronic phase of human CML. In a follow-up work, Daley et al (1991) used the leukemic cells described in the first paper and transferred them into syngeneic mice. Some of the mice developed acute leukemia, reflecting evolution to a phase resembling blast crisis in the human disease.

A review on bcr/abl fusion protein, its involvement in CML and the various animal models that exist can be found in Ghaffari (1999).

Wolf and Ilaria (2001) describe an improved model of CML based on bone marrow (BM) retroviral transduction and transplantation. The basic model is not a good mimic of the human disease since the mice die in 3-4 weeks from overwhelming granulocytosis. In the model the mice are lethally irradiated and then reconstituted with syngeneic BM transduced with a replication-defective bcr/abl retrovirus. To prevent the rapid death described above the mice were treated with the tyrosine kinase inhibitor STI571. The treatment caused a significant prolonged survival of the mice and in some of the cases an almost complete normalization of the peripheral white blood count, reminiscent of human CML, was observed. This prolonged survival, as well as the physical properties seen in some of the mice, makes the mice a better model of CML.

Naked 19-mer siRNA compounds having the alternating 2'O methylation motif as described above in Example 1, which specifically inhibit the genes described in the models above, are tested in the viral animal models, which show that these siRNAs treat and/or prevent symptoms of the associated disorder and thus may be used to treat this disorder.

D. Other animal models

Fuchimoto et al (2000) state: "There are numerous clinical situations for which bone marrow transplantation (BMT) might be the treatment of choice. However, standard preparative regimens for BMT generally include whole body irradiation (WBI) to create space (1) or sufficient immunosuppression (2) for bone marrow engraftment. Such regimens are so toxic on their own that few patients are offered this option until they are

late in the course of their disease. These situations include patients with hemoglobinopathies, such as sickle cell disease and thalassemia (3–5), as well as patients with end-stage organ failure, who could be treated by allogeneic organ transplantation without the complications of long-term immunosuppression, if they could be made
5 tolerant by BMT.

A non-myelo-ablative protocol without WBI was used by Fuchimoto et al (2000) in pigs, providing a model for BMT. Transplants were performed across full major histocompatibility antigen mismatches, as well as across minor histocompatibility antigen mismatches, using high doses of peripheral blood stem cell (PBSC) as a source of
10 hematopoietic cells. Tolerance was confirmed in these animals by skin grafting or transplantation of donor-matched kidney allografts.

The Brown Norway myelocytic leukemia (BNML) model for AML is described in detail by Martens et al (1990). The model is described in brief by Rozenmuller et al (1998). BNML is a leukemic cell-line which is injected i.v. into rats. Injection of 106
15 cells leads to death from leukemia in about 20 days. The model has been used to assess various treatment options.

Gowing et al (1998) examined the effects of ultraviolet-B irradiation to examine its effects of graft versus host disease (GVHD) using the BNML model and bone marrow transplantation treatment model. The idea was to spare the graft versus leukemia effect
20 while eliminating GVHD.

Rozenmuller et al (1998) examined the use of diphtheria toxin fused to murine GM-CSF for the treatment of AML. Since GM-CSF receptors are found on leukemic cells of more than 80% of patients with AML, the idea is to direct the diphtheria toxin to the leukemic cells and induce their death. They show that specific doses can be used to
25 effectively kill the leukemic cells without an effect on the hematopoietic stem cells.

Bone marrow failure occurs rather rarely and involves a variety of syndromes, inherited and acquired. Morley and Blake (1974) present a model for an acquired form which can occur from exposure to certain chemical. The model consists of treating mice with busulfan. Following a prolonged treatment, lasting several weeks it was found that
30 by 240 days 80% of the mice died from marrow failure. More specifically, the mice have shown persistent mild marrow hypoplasia turning into late severe marrow failure.

CLL, the most common leukemia in the Western world, typically presents as an accumulation of mature-appearing but biologically immature B-lymphocytes in patients

who are over 50 years of age. CLL tends to run a somewhat indolent course over several years. Occasional cases, however, evolve into an aggressive, diffuse large cell lymphoma which can be either widespread or localized and is characterized by weight loss, localized mass, fever, and dysproteinemia. The presence of large cell lymphoma in CLL has been
5 termed Richter's syndrome.

Phillips et al (1992) describe the NZB mouse model, which has a genetically regulated, age-dependent onset of a CLL like syndrome. Peripheral blood smears from old NZB mice show an increase in circulating lymphocytes and "smudged" or ruptured cells, often seen in human CLL. Continued passage of the cells in recipient mice
10 occasionally results in the development of a large cell lymphoma detectable in the spleen, lymph nodes, and liver. The histology of this lymphoma is quite distinct from that of the CLL-like cells, but the phenotype is that of an aneuploid CD5+ IgM+ cell. This apparently represents a continued transformation of the CLL-like clone similar to the development of Richter's syndrome in human CLL.

15 Naked 19-mer siRNA compounds having the alternating 2' O methylation motif as described above in Example 1, which specifically inhibit the genes described in the models above, are tested in the animal models which show that these siRNAs treat and/or prevent symptoms of the associated disorder and thus may be used to treat this disorder.

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WHAT IS CLAIMED IS:

1. A method of treating a bone marrow disorder in a subject in need thereof, which comprises systemically administering to the subject a naked siRNA which reduces or inhibits expression of a gene associated with the disorder in the bone marrow of the subject in an amount effective to treat the disorder.
2. The method according to claim 1 wherein the siRNA comprises a sufficient number of consecutive nucleotides having a sequence of sufficient homology to a nucleic acid sequence present within the gene to hybridize to the gene and reduce or inhibit expression of the gene in the subject.
3. The method according to claim 1 wherein the bone marrow disorder is selected from the group consisting of a leukemia, a lymphoma, a myeloproliferative disease, a myelodysplastic syndrome and a plasma cell disorder.
4. The method according to claim 3 wherein the leukemia is selected from the group consisting of Acute Lymphocytic Leukemia (ALL), Acute Myelocytic Leukemia (AML), Chronic Lymphocytic Leukemia (CLL), Chronic Myelocytic Leukemia (CML), Chronic Myelomonocytic Leukemia (CMML) and stem cell leukemia.
5. The method according to claim 3 wherein the lymphoma is selected from the group consisting of Anaplastic Large-Cell Lymphoma (ALCL), Hodgkin's lymphoma and non-Hodgkin's lymphoma.
6. The method according to claim 3 wherein the myelodysplastic syndrome is selected from the group consisting of Refractory anemia (RA), Refractory anemia with ringed sideroblasts (RARS), Refractory cytopenia with multilineage dysplasia (RCMD), Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS), Refractory anemia with excess blasts I and II; 5q- syndrome and myelodysplasia unclassifiable.
7. The method according to claim 3 wherein the myeloproliferative disorder is selected from Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Chronic Idiopathic Myelofibrosis (MF).
8. The method according to claim 3 wherein the plasma cell disease is a plasma cell neoplasm selected from the group consisting of multiple myeloma,

plasmacytoma, macroglobulinemia, monoclonal gammopathy of undetermined significance (MGUS).

9. The method according to claim 1 wherein the bone marrow disorder is a hemoglobinopathy selected from the group consisting of sickle-cell disease and a thalassemia.
10. The method according to claim 1 wherein the gene associated with the bone marrow disorder is an aberrantly expressed gene.
11. The method according to claim 10 wherein the aberrantly expressed gene comprises a chromosomal translocation.
12. The method according to claim 10 wherein the aberrantly expressed gene comprises an amplified gene.
13. The method according to claim 1 wherein the siRNA reduces or inhibits expression of a gene associated with a late stage bone marrow disorder.
14. The method according to claim 1 wherein the siRNA reduces or inhibits expression of a gene associated with an early stage bone marrow disorder.
15. The method according to claim 1 wherein the gene encodes a polypeptide associated with the bone marrow disorder.
16. The method according to claim 1 wherein the siRNA reduces or inhibits expression of the gene by inhibiting transcription of an mRNA which encodes a polypeptide associated with progression of the bone marrow disorder.
17. The method according to claim 1 wherein the siRNA reduces or inhibits expression of the gene by inhibiting transcription of an mRNA which encodes a polypeptide associated with etiology of the bone marrow disease.
18. The method according to claim 17 wherein the siRNA comprises a sense strand and an antisense strand which form an RNA duplex, and wherein the sense strand comprises ribonucleotides having a nucleotide sequence complementary to target sequence of about 18 to about 40 consecutive nucleotides in the mRNA transcribed from the gene associated with a bone marrow disorder.

19. The method according to any one of claims 1-18 wherein the siRNA molecule has structure

$$5' \quad (N)_x - Z \quad 3' \quad (\text{antisense strand})$$

$$3' \quad Z' - (N')_y \quad 5' \quad (\text{sense strand})$$

5 wherein each N and N' is a ribonucleotide which may be modified or unmodified in its sugar residue and (N)_x and (N')_y is an oligomer in which each consecutive N or N' is joined to the next N or N' by a covalent bond;

wherein each of x and y is an integer between 18 and 40;

10 wherein each of Z and Z' may be present or absent, but if present is dTdT and is covalently attached at the 3' terminus of the strand in which it is present; and

wherein the sequence of (N)_x comprises an antisense sequence relative to the mRNA transcribed from the gene associated with the bone marrow disorder.

20. The method according to claim 19 wherein $x = y$.

15 21. The method according to claim 20 wherein $x = y = 19$.

22. The method according to any one of claim 19-21 wherein the siRNA consists of ribonucleotides unmodified in their sugar residues.

23. The method according to claim 19 wherein the siRNA comprises at least one modified ribonucleotide.

20 24. The method according to claim 23 wherein the modified nucleotide comprises a modified sugar residue.

25. The method according to claim 24 wherein the modified nucleotide comprises a 2'-O-methyl modification.

25 26. The method according to any one of claims 23-25 wherein alternating nucleotides are modified in both the antisense and the sense strands.

27. The method according to claim 19 wherein at least one covalent bond joining the ribonucleotides is modified.

28. The method according to claim 19 wherein the siRNA comprises at least one phosphodiester bond.
29. The method according to claim 27 wherein all the covalent bonds joining the ribonucleotides are modified.
- 5 30. The method according to anyone of claims 1-29 wherein the oligonucleotide is administered parenterally.
31. The method according to claim 30 wherein parenteral administration comprises intravenous, intraarterial, intramuscular, intraperitoneal, or intranasal administration.
- 10 32. The method according to claim 30 wherein parenteral administration comprises intravenous administration.
33. Use of an siRNA for the preparation of a medicament in which the siRNA is naked siRNA for promoting recovery in a subject suffering from a bone marrow disorder.
- 15 34. Use according to claim 33 wherein the siRNA reduces or inhibits expression of a gene associated with the disorder.
35. Use according to any one of claims 33-34 wherein the siRNA has structure

$$5' \quad (N)_x - Z \quad 3' \quad (\text{antisense strand})$$

$$3' \quad Z' - (N')_y \quad 5' \quad (\text{sense strand})$$

- 20 wherein each N and N' is a ribonucleotide which may be modified or unmodified in its sugar residue and (N)_x and (N')_y is an oligomer in which each consecutive N or N' is joined to the next N or N' by a covalent bond;

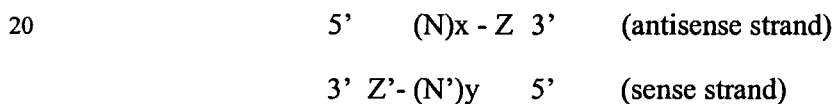
wherein each of x and y is an integer between 18 and 40;

- 25 wherein each of Z and Z' may be present or absent, but if present is dTdT and is covalently attached at the 3' terminus of the strand in which it is present;
and

wherein the sequence of (N)_x comprises an antisense sequence relative to the mRNA transcribed from the gene associated with the bone marrow disorder.

36. Use according to claim 35 wherein $x = y$.
37. Use according to claim 36 wherein $x = y = 19$.
38. Use according to any one of claims 33-37 wherein the siRNA consists of ribonucleotides unmodified in their sugar residues.
- 5 39. Use according to any one of claims 33-37 wherein the siRNA comprises at least one modified ribonucleotide.
40. Use according to claim 39 wherein the modified nucleotide comprises a modified sugar residue.
41. Use according to claim 40 wherein the modified nucleotide comprises a 2'-O-methyl modification.
- 10 42. Use according to any one of claims 40-41, wherein alternating nucleotides are modified in both the antisense and the sense strands.
43. Use according to claim 35 wherein at least one covalent bond joining the ribonucleotides is modified.
- 15 44. Use according to claim 43 wherein the siRNA comprises at least one phosphodiester bond.
45. A method for the delivery of siRNA to a bone marrow cell in a subject suffering from a bone marrow disorder, comprising systemically administering to the subject a naked siRNA which reduces or inhibits expression of a gene associated with the bone marrow disorder in an amount effective to treat the disorder.
- 20 46. The method according to claim 45 wherein the gene associated with the bone marrow disorder is an aberrantly expressed gene.
47. The method according to claim 46 wherein the aberrantly expressed gene comprises a chromosomal translocation.
- 25 48. The method according to claim 46 wherein the aberrantly expressed gene comprises an amplified gene.

49. The method according to claim 46 wherein the siRNA reduces or inhibits expression of a gene associated with a late stage bone marrow disorder.
50. The method according to claim 46 wherein the siRNA reduces or inhibits expression of a gene associated with an early stage bone marrow disorder.
- 5 51. The method according to claim 46 wherein the gene encodes a polypeptide associated with the bone marrow disorder.
52. The method according to claim 46 wherein the siRNA reduces or inhibits expression of the gene by inhibiting transcription of an mRNA which encodes a polypeptide associated with progression of the bone marrow disorder.
- 10 53. The method according to claim 46 wherein the siRNA reduces or inhibits expression of the gene by inhibiting transcription of an mRNA which encodes a polypeptide associated with etiology of the bone marrow disease.
54. The method according to claim 46 wherein the siRNA comprises a sense strand and an antisense strand which form an RNA duplex, and wherein the sense strand comprises ribonucleotides having a nucleotide sequence complementary to target sequence of about 18 to about 40 consecutive nucleotides in the mRNA transcribed from the gene associated with a bone marrow disorder.
- 15 55. The method according to any one of claims 45-54 wherein the siRNA molecule has structure



wherein each N and N' is a ribonucleotide which may be modified or unmodified in its sugar residue and (N)_x and (N')_y is an oligomer in which each consecutive N or N' is joined to the next N or N' by a covalent bond;

25 wherein each of x and y is an integer between 18 and 40;

wherein each of Z and Z' may be present or absent, but if present is dTdT and is covalently attached at the 3' terminus of the strand in which it is present; and

wherein the sequence of (N)_x comprises an antisense sequence relative to the mRNA transcribed from the gene associated with the bone marrow disorder.

56. The method according to claim 55 wherein $x = y$.
57. The method according to claim 56 wherein $x = y = 19$.
58. The method according to any one of claims 55-57 wherein the siRNA consists of ribonucleotides unmodified in their sugar residues.
59. The method according to claim 55 wherein the siRNA comprises at least one modified ribonucleotide.
60. The method according to claim 59 wherein the modified nucleotide comprises a modified sugar residue.
61. The method according to claim 60 wherein the modified nucleotide comprises a 2'-O-methyl modification.
62. The method according to any one of claims 59-61 wherein alternating nucleotides are modified in both the antisense and the sense strands.
63. The method according to claim 55 wherein at least one covalent bond joining the ribonucleotides is modified.
64. The method according to claim 55 wherein the siRNA comprises at least one phosphodiester bond.
65. The method according to claim 63 wherein all the covalent bonds joining the ribonucleotides are modified.
66. The method according to anyone of claims 55-65 wherein the oligonucleotide is administered parenterally.
67. The method according to claim 66 wherein parenteral administration comprises intravenous, intraarterial, intramuscular, intraperitoneal, or intranasal administration.
68. The method according to claim 67 wherein parenteral administration comprises intravenous administration.

FIGURE 1A

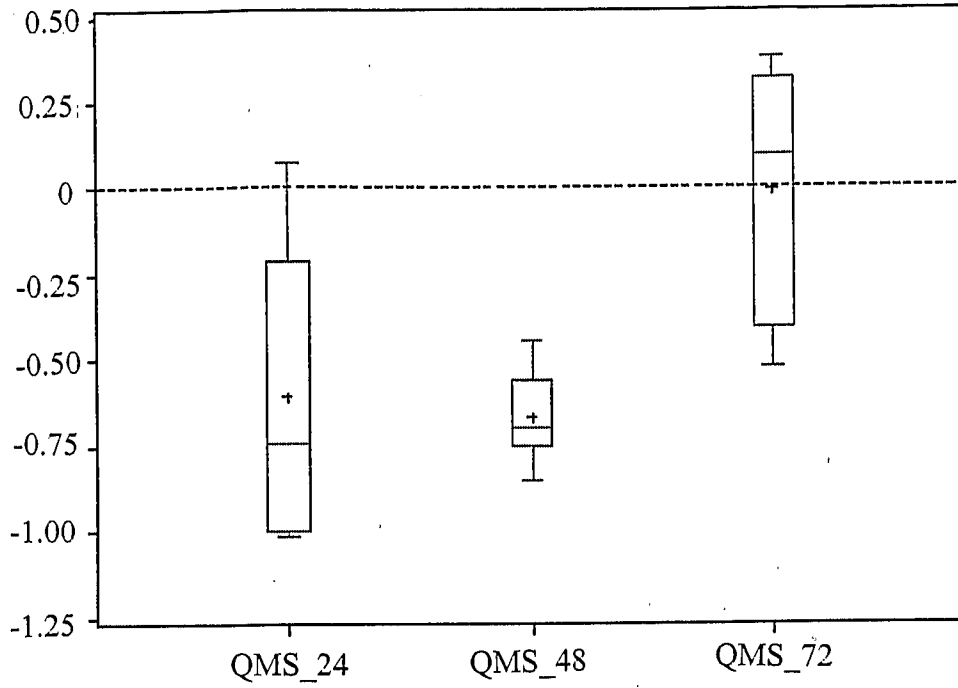


FIGURE 1B

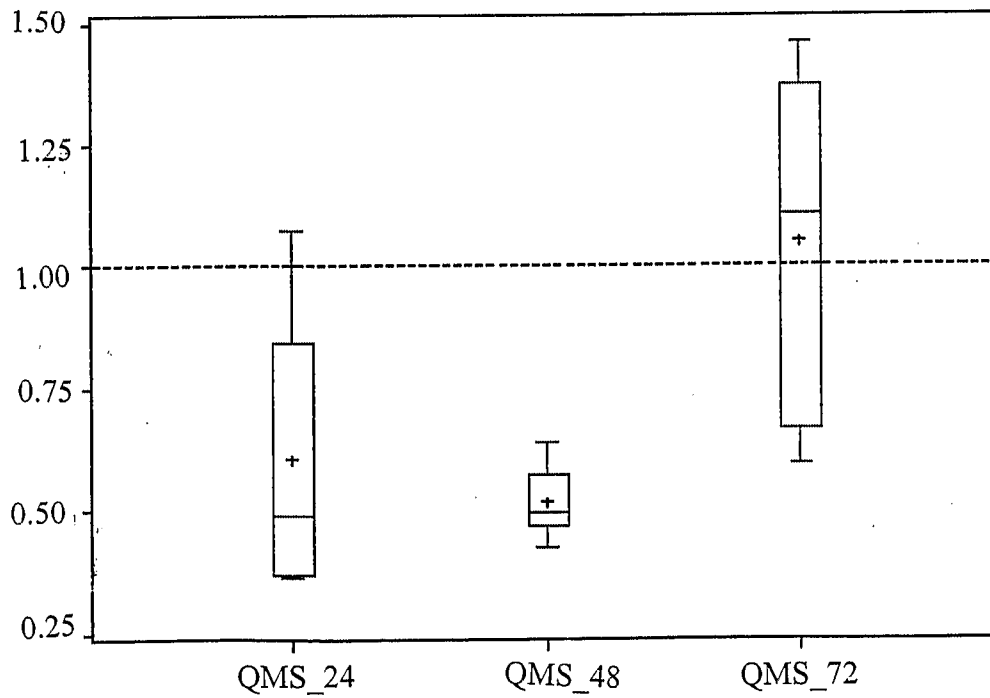


Figure 1C(b)

