

COMPOSITIONS AND METHODS FOR INHIBITING EXPRESSION OF PTP1B GENES

This invention relates to double-stranded ribonucleic acids (dsRNAs), and their use in mediating RNA interference to inhibit the expression of the PTP1B gene. Furthermore, the use of said dsRNAs to treat/prevent a wide range of diseases/disorders which are associated with the expression of the PTP1B gene, like type 2 diabetes, liver failure and obesity is part of the invention.

Type 2 diabetes is a polygenic disease that manifests insulin resistance, hyperinsulinemia, and hyperglycemia. The molecular mechanism underlying the insulin resistance is not well understood but appears to involve a defect in the post-insulin receptor (IR) signal transduction pathway. The IR is a receptor tyrosine kinase, and the binding of insulin to its receptor results in autophosphorylation of the IR and tyrosyl phosphorylation of IR substrate proteins. Protein tyrosine kinases and protein tyrosine phosphatases are important regulators of insulin signal transduction.

An essential regulatory role in signaling mediated by the insulin receptor has been established for PTP1B. PTP1B (also known as protein phosphatase 1B and PTPN1) is localized in the cytoplasmic face of the endoplasmic reticulum and is expressed ubiquitously, including liver, muscle and fat. Biochemical, genetic and pharmacological studies support a role for PTP1B as a negative regulator in both insulin and leptin signaling. PTP1B can associate with and dephosphorylate activated insulin receptor (IR) or insulin receptor substrates (IRS). Overexpression of PTP1B in cell cultures decreases insulin-stimulated phosphorylation of IR and/or IRS-1, whereas reduction in the level of PTP1B in cells and animals augments insulin-initiated signaling. In addition, analyses of quantitative trait loci and mutations in the gene encoding PTP1B in humans support the notion that aberrant expression of PTP1B can contribute to diabetes and obesity. This important role of PTP1B in diabetes and obesity was also demonstrated in animal models. Mice deficient in PTP1B expression have increased insulin sensitivity and low adiposity with resistance to weight gain on a high fat diet. In addition, these mice show increased basal metabolic rate and total energy expenditure. Interestingly, PTP1B was subsequently shown to bind and dephosphorylate JAK2, which is downstream of leptin

receptor. Thus, the resistance to diet-induced obesity observed in *PTP1B*^{-/-} mice is likely to be associated with increased energy expenditure owing to enhanced leptin-sensitivity.

Interestingly, liver-specific re-expression of PTP1B in *PTP1B*^{-/-} mice led to marked
5 attenuation of their enhanced insulin sensitivity in correlation with decreased insulin-stimulated tyrosyl phosphorylation of the insulin receptor and IRS-2-associated phosphatidylinositide 3-kinase activity, demonstrating that the liver is a major site of the peripheral action of PTP1B in regulating glucose homeostasis.

10 In recent years it has become apparent that cell death (apoptosis), firstly a morphologically defined process, is a highly controlled type of cell death that plays a critical role in embryonic development, deletion of autoreactive T-cells and adult tissue homeostasis. There is increasing evidence that derangement of the apoptotic program is the underlying cause of a series of diseases including liver diseases. Cell proliferation and cell death are governed by
15 stimulatory and inhibitory signals. Whereas trophic factors simultaneously stimulate mitosis and inhibit cell death, negative growth signals regulate the opposite of these biological effects. In the liver, trophic factors include endogenous growth factors such as EGF, bFGF, TGF- β , and IGFs that act through receptors belonging to the tyrosine kinase superfamily. Inhibitors of tyrosine kinases and PTPs can also modulate apoptosis in the liver. As the lack of PTP1B triggers
20 substantial changes in endogenous expression of pro-apoptotic genes in the liver the present dsRNAs targeting PTP1B can be used for treatment of liver failure.

Collectively, these biochemical, genetic and pharmacological studies provide strong proof-of-concept, validating the notion that inhibition of PTP1B could address diabetes, liver
25 failure and obesity and making PTP1B an exciting target for drug development.

Double-stranded ribonucleic acid (dsRNA) molecules have been shown to block gene expression in a highly conserved regulatory mechanism known as RNA interference (RNAi). The invention provides double-stranded ribonucleic acid (dsRNA) molecules able to selectively
30 and efficiently decrease the expression of PTP1B. The use of PTP1B RNAi provides a method for the therapeutic and/or prophylactic treatment of diseases/disorders which are associated with insulin and leptin signaling. Particular disease/disorder states include the therapeutic and/or prophylactic treatment of type 2 diabetes, obesity, liver failure, dislipidemia, diabetic

atherosclerosis, and hypertension, which method comprises administration of dsRNA targeting PTP1B to a human being or animal.

In one preferred embodiment the described dsRNA molecule is capable of inhibiting the expression of a PTP1B gene by at least 60 %, preferably by at least 70%, most preferably by at least 80%. The invention also provides compositions and methods for specifically targeting the liver with PTP1B dsRNA, for treating pathological conditions and diseases caused by the expression of the PTP1B gene including those described above.

In one embodiment, the invention provides double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a PTP1B gene, in particular the expression of the mammalian or human PTP1B gene. The dsRNA comprises at least two sequences that are complementary to each other. The dsRNA comprises a sense strand comprising a first sequence and an antisense strand may comprise a second sequence, see sequences provided in the sequence listing and also provision of specific dsRNA pairs in the appended Tables 1 and 4. In one embodiment the sense strand comprises a sequence which has an identity of at least 90% to at least a portion of an mRNA encoding PTP1B. Said sequence is located in a region of complementarity of the sense strand to the antisense strand, preferably within nucleotides 2-7 of the 5' terminus of the antisense strand. In one preferred embodiment the dsRNA targets particularly the human PTP1B gene, in yet another preferred embodiment the dsRNA targets the mouse (*Mus musculus*) and rat (*Rattus norvegicus*) PTP1B gene.

In one embodiment, the antisense strand comprises a nucleotide sequence which is substantially complementary to at least part of an mRNA encoding said PTP1B gene, and the region of complementarity is most preferably less than 30 nucleotides in length. Furthermore, it is preferred that the length of the herein described inventive dsRNA molecules (duplex length) is in the range of about 16 to 30 nucleotides, in particular in the range of about 18 to 28 nucleotides. Particularly useful in context of this invention are duplex lengths of about 19, 20, 21, 22, 23 or 24 nucleotides. Most preferred are duplex stretches of 19, 21 or 23 nucleotides. The dsRNA, upon contacting with a cell expressing a PTP1B gene, inhibits the expression of a PTP1B gene in vitro by at least 60%, preferably by at least 70%, most preferred by 80%.

Appended Table 13 relates to preferred molecules to be used as dsRNA in accordance with this invention. Also modified dsRNA molecules are provided herein and are in particular disclosed in appended Tables 1 and 4, providing illustrative examples of modified dsRNA

molecules of the present invention. As pointed out herein above, Table 1 provides for illustrative examples of modified dsRNAs of this invention (whereby the corresponding sense strand and antisense strand is provided in this Table). The relation of the unmodified preferred molecules shown in Table 13 to the modified dsRNAs of Table 1 is illustrated in Table 14. Yet, the illustrative modifications of these constituents of the inventive dsRNAs are provided herein as examples of modifications.

Tables 2 and 3 provide for selective biological, clinically and pharmaceutical relevant parameters of certain dsRNA molecules of this invention.

Most preferred dsRNA molecules are provided in the appended Table 13 and, inter alia and preferably, wherein the sense strand is selected from the group consisting of the nucleic acid sequences depicted in SEQ ID Nos 630, 632, 634, 638, 640, 644 and 652 and the antisense strand is selected from the group consisting of the nucleic acid sequences depicted in SEQ ID Nos 631, 633, 635, 639, 641, 645 and 653. Accordingly, the inventive dsRNA molecule may, inter alia, comprise the sequence pairs selected from the group consisting of SEQ ID NOs: 630/631, 632/633, 634/635, 638/639, 640/641, 644/645 and 652/653. In context of specific dsRNA molecules provided herein, pairs of SEQ ID NOs relate to corresponding sense and antisense strands sequences (5' to 3') as also shown in appended and included Tables.

In one embodiment said dsRNA molecules comprise an antisense strand with a 3' overhang of 1-5 nucleotides length, preferably of 1-2 nucleotides length. Preferably said overhang of the antisense strand comprises uracil or nucleotides which are complementary to the mRNA encoding PTP1B.

In another preferred embodiment, said dsRNA molecules comprise a sense strand with a 3' overhang of 1-5 nucleotides length, preferably of 1-2 nucleotides length. Preferably said overhang of the sense strand comprises uracil or nucleotides which are identical to the mRNA encoding PTP1B.

In another preferred embodiment, said dsRNA molecules comprise a sense strand with a 3' overhang of 1-5 nucleotides length, preferably of 1-2 nucleotides length, and an antisense strand with a 3' overhang of 1-5 nucleotides length, preferably of 1-2 nucleotides length. Preferably said overhang of the sense strand comprises uracil or nucleotides which are at least 90% identical to the mRNA encoding PTP1B and said overhang of the antisense strand

comprises uracil or nucleotides which are at least 90% complementary to the mRNA encoding PTP1B

The dsRNA molecules of the invention may be comprised of naturally occurring
5 nucleotides or may be comprised of at least one modified nucleotide, such as a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group. 2' modified nucleotides may have the additional advantage that certain immunostimulatory factors or cytokines are suppressed when the inventive dsRNA molecules are employed in vivo, for
10 example in a medical setting. Alternatively and non-limiting, the modified nucleotide may be chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. In one preferred embodiment the dsRNA molecules comprises at least
15 one of the following modified nucleotides: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group and a deoxythymidine. Preferred dsRNA molecules comprising modified nucleotides are given in Tables 1 and 4.

Most preferred dsRNA molecules are provided in the appended Tables 1 and 4 and, inter alia and preferably, wherein the sense strand is selected from the group consisting of the nucleic
20 acid sequences depicted in SEQ ID NOs: 3, 5, 7, 11, 13, 17, 25 and the antisense strand is selected from the group consisting of the nucleic acid sequences depicted in SEQ ID NOs: 4, 6, 8, 12, 14, 18, and 26. Accordingly, the inventive dsRNA molecule may, inter alia, comprise the sequence pairs selected from the group consisting of SEQ ID NOs: 3/4, 5/6, 7/8, 11/12, 13/14, 17/18, and 25/26. In context of specific dsRNA molecules provided herein, pairs of SEQ ID NOs
25 relate to corresponding sense and antisense strands sequences (5' to 3') as also shown in appended and included Tables.

In a preferred embodiment the inventive dsRNA molecules comprise modified nucleotides as detailed in the sequences given in Tables 1 and 4. In one preferred embodiment the inventive dsRNA molecule comprises sequence pairs selected from the group consisting of
30 SEQ ID NOs: 3/4, 5/6, 7/8, 11/12, 13/14, 17/18, and 25/26, and comprise modifications as detailed in Table 1.

In another embodiment the inventive dsRNAs comprise modified nucleotides on positions different from those disclosed in Tables 1 and 4. In one preferred embodiment two deoxythymidine nucleotides are found at the 3' of both strands of the dsRNA molecule.

In one embodiment the dsRNA molecules of the invention comprise of a sense and an antisense strand wherein both strands have a half-life of at least 5 hours. In one preferred embodiment the dsRNA molecules of the invention comprise of a sense and an antisense strand wherein both strands have a half-life of at least 5 hours in human serum. In another embodiment the dsRNA molecules of the invention are non-immunostimulatory, e.g. do not stimulate INF-alpha and TNF-alpha in vitro. In another embodiment the dsRNA molecules of the invention do stimulate INF-alpha and TNF-alpha in vitro to a very minor degree.

The invention also provides for cells comprising at least one of the dsRNAs of the invention. The cell is preferably a mammalian cell, such as a human cell. Furthermore, also tissues and/or non-human organisms comprising the herein defined dsRNA molecules are comprised in this invention, whereby said non-human organism is particularly useful for research purposes or as research tool, for example also in drug testing.

Furthermore, the invention relates to a method for inhibiting the expression of a PTP1B gene, in particular a mammalian or human PTP1B gene, in a cell, tissue or organism comprising the following steps:

- (a) introducing into the cell, tissue or organism a double-stranded ribonucleic acid (dsRNA) as defined herein;
- (b) maintaining said cell, tissue or organism produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a PTP1B gene, thereby inhibiting expression of a PTP1B gene in a given cell.

The invention also relates to pharmaceutical compositions comprising the inventive dsRNAs of this invention. These pharmaceutical compositions are particularly useful in the inhibition of the expression of a PTP1B gene in a cell, a tissue or an organism. The pharmaceutical composition comprising one or more of the dsRNA of the invention may also comprise (a) pharmaceutically acceptable carrier(s), diluent(s) and/or excipient(s).

In another embodiment, the invention provides methods for treating, preventing or managing type 2 diabetes, obesity, liver failure, dislipidemia, diabetic atherosclerosis, and

hypertension, which are associated with PTP1B, said method comprising administering to a subject in need of such treatment, prevention or management a therapeutically or prophylactically effective amount of one or more of the dsRNAs of the invention. Preferably, said subject is a mammal, most preferably a human patient.

5 In one embodiment, the invention provides a method for treating a subject having a pathological condition mediated by the expression of a PTP1B gene. Such conditions comprise disorders associated with diabetes, liver failure and obesity, as described above. In this embodiment, the dsRNA acts as a therapeutic agent for controlling the expression of a PTP1B gene. The method comprises administering a pharmaceutical composition of the invention to the
10 patient (e.g., human), such that expression of a PTP1B gene is silenced. Because of their high specificity, the dsRNAs of the invention specifically target mRNAs of a PTP1B gene. In one preferred embodiment the described dsRNAs specifically decrease PTP1B mRNA levels and do not directly affect the expression and / or mRNA levels of off-target genes in the cell.

In one preferred embodiment the described dsRNA decrease PTP1B mRNA levels in the
15 liver by at least 60%, preferably by at least 70%, most preferably by at least 80% in vivo. In another embodiment the described dsRNAs decrease PTP1B mRNA levels in vivo for at least 4 days. In another embodiment the described dsRNAs decrease PTP1B mRNA levels in vivo by at least 60% for at least 4 days.

Particularly useful with respect to therapeutic dsRNAs is the set of dsRNAs targeting
20 mouse and rat PTP1B which can be used to estimate toxicity, therapeutic efficacy and effective dosages and in vivo half-lives for the individual dsRNAs in an animal or cell culture model.

In another embodiment, the invention provides vectors for inhibiting the expression of a PTP1B gene in a cell, in particular PTP1B gene comprising a regulatory sequence operable linked to a nucleotide sequence that encodes at least one strand of one of the dsRNA of the
25 invention.

In another embodiment, the invention provides a cell comprising a vector for inhibiting the expression of a PTP1B gene in a cell. Said vector comprises a regulatory sequence operable linked to a nucleotide sequence that encodes at least one strand of one of the dsRNA of the invention. Yet, it is preferred that said vector comprises, besides said regulatory sequence a
30 sequence that encodes at least one "sense strand" of the inventive dsRNA and at least one "anti

sense strand” of said dsRNA. It is also envisaged that the claimed cell comprises two or more vectors comprising, besides said regulatory sequences, the herein defined sequence(s) that encode(s) at least one strand of one of the dsRNA of the invention.

In one embodiment, the method comprises administering a composition comprising a
5 dsRNA, wherein the dsRNA comprises a nucleotide sequence which is complementary to at least a part of an RNA transcript of a PTP1B gene of the mammal to be treated. As pointed out above, also vectors and cells comprising nucleic acid molecules that encode for at least one strand of the herein defined dsRNA molecules can be used as pharmaceutical compositions and may, therefore, also be employed in the herein disclosed methods of treating a subject in need of
10 medical intervention. It is also of note that these embodiments relating to pharmaceutical compositions and to corresponding methods of treating a (human) subject also relate to approaches like gene therapy approaches. PTP1B specific dsRNA molecules as provided herein or nucleic acid molecules encoding individual strands of these inventive dsRNA molecules may also be inserted into vectors and used as gene therapy vectors for human patients. Gene therapy
15 vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery
20 vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

In another aspect of the invention, PTP1B specific dsRNA molecules that modulate PTP1B gene expression activity are expressed from transcription units inserted into DNA or RNA vectors (see, e.g., Skillern, A., et al., International PCT Publication No. WO 00/22113).
25 These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be incorporated and inherited as a transgene integrated into the host genome. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, et al., Proc. Natl. Acad. Sci. USA (1995) 92:1292).

The individual strands of a dsRNA can be transcribed by promoters on two separate
30 expression vectors and co-transfected into a target cell. Alternatively each individual strand of the dsRNA can be transcribed by promoters both of which are located on the same expression

plasmid. In a preferred embodiment, a dsRNA is expressed as an inverted repeat joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

The recombinant dsRNA expression vectors are preferably DNA plasmids or viral vectors. dsRNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus (for a review, see Muzyczka, et al., *Curr. Topics Micro. Immunol.* (1992) 158:97-129)); adenovirus (see, for example, Berkner, et al., *BioTechniques* (1998) 6:616), Rosenfeld et al. (1991, *Science* 252:431-434), and Rosenfeld et al. (1992), *Cell* 68:143-155)); or alphavirus as well as others known in the art. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, in vitro and/or in vivo (see, e.g., Danos and Mulligan, *Proc. Natl. Acad. Sci. USA* (1998) 85:6460-6464). Recombinant retroviral vectors capable of transducing and expressing genes inserted into the genome of a cell can be produced by transfecting the recombinant retroviral genome into suitable packaging cell lines such as PA317 and Psi-CRIP (Comette et al., 1991, *Human Gene Therapy* 2:5-10; Cone et al., 1984, *Proc. Natl. Acad. Sci. USA* 81:6349). Recombinant adenoviral vectors can be used to infect a wide variety of cells and tissues in susceptible hosts (e.g., rat, hamster, dog, and chimpanzee) (Hsu et al., 1992, *J. Infectious Disease*, 166:769), and also have the advantage of not requiring mitotically active cells for infection.

The promoter driving dsRNA expression in either a DNA plasmid or viral vector of the invention may be a eukaryotic RNA polymerase I (e.g. ribosomal RNA promoter), RNA polymerase II (e.g. CMV early promoter or actin promoter or U1 snRNA promoter) or preferably RNA polymerase III promoter (e.g. U6 snRNA or 7SK RNA promoter) or a prokaryotic promoter, for example the T7 promoter, provided the expression plasmid also encodes T7 RNA polymerase required for transcription from a T7 promoter. The promoter can also direct transgene expression to the pancreas (see, e.g. the insulin regulatory sequence for pancreas (Bucchini et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:2511-2515)).

In addition, expression of the transgene can be precisely regulated, for example, by using an inducible regulatory sequence and expression systems such as a regulatory sequence that is sensitive to certain physiological regulators, e.g., circulating glucose levels, or hormones (Docherty et al., 1994, *FASEB J.* 8:20-24). Such inducible expression systems, suitable for the control of transgene expression in cells or in mammals include regulation by ecdysone, by estrogen, progesterone, tetracycline, chemical inducers of dimerization, and isopropyl-beta-D1 -

thiogalactopyranoside (EPTG). A person skilled in the art would be able to choose the appropriate regulatory/promoter sequence based on the intended use of the dsRNA transgene.

Preferably, recombinant vectors capable of expressing dsRNA molecules are delivered as described below, and persist in target cells. Alternatively, viral vectors can be used that provide
5 for transient expression of dsRNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the dsRNAs bind to target RNA and modulate its function or expression. Delivery of dsRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction
10 into a desired target cell.

dsRNA expression DNA plasmids are typically transfected into target cells as a complex with cationic lipid carriers (e.g. Oligofectamine) or non-cationic lipid-based carriers (e.g. Transit-TKOTM). Multiple lipid transfections for dsRNA-mediated knockdowns targeting different regions of a single PTP1B gene or multiple PTP1B genes over a period of a week or
15 more are also contemplated by the invention. Successful introduction of the vectors of the invention into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of ex vivo cells can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (e.g., antibiotics
20 and drugs), such as hygromycin B resistance.

The following detailed description discloses how to make and use the dsRNA and compositions containing dsRNA to inhibit the expression of a target PTP1B gene, as well as compositions and methods for treating diseases and disorders caused by the expression of said PTP1B gene.

25

DEFINITIONS

For convenience, the meaning of certain terms and phrases used in the specification, examples, and appended claims, are provided below. If there is an apparent discrepancy between the usage of a term in other parts of this specification and its definition provided in this section,
30 the definition in this section shall prevail.

"G," "C," "A", "U" and "T" or "dT" respectively, each generally stand for a nucleotide that contains guanine, cytosine, adenine, uracil and deoxythymidine as a base, respectively. However, the term "ribonucleotide" or "nucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety. Sequences comprising such replacement moieties are embodiments of the invention. As detailed below, the herein described dsRNA molecules may also comprise "overhangs", i.e. unpaired, overhanging nucleotides which are not directly involved in the RNA double helical structure normally formed by the herein defined pair of "sense strand" and "anti sense strand". Often, such an overhanging stretch comprises the deoxythymidine nucleotide, in most embodiments, 2 deoxythymidines in the 3' end. Such overhangs will be described and illustrated below.

The term „PTP1B“ as used herein relates in particular to the protein phosphatase 1B also known as and PTPN1 and said term relates to the corresponding gene, encoded mRNA, encoded protein/polypeptide as well as functional fragments of the same. Preferred is the human PTP1B gene. In other preferred embodiments the dsRNAs of the invention target the PTP1B gene of rat (Rattus norvegicus) and mouse (Mus musculus), in yet another preferred embodiment the dsRNAs of the invention target the human (H.sapiens) and cynomolgous monkey (Macaca fascicularis) PTP1B gene. The term "PTP1B gene/sequence" does not only relate to (the) wild-type sequence(s) but also to mutations and alterations which may be comprised in said gene/sequence. Accordingly, the present invention is not limited to the specific dsRNA molecules provided herein. The invention also relates to dsRNA molecules that comprise an antisense strand that is at least 85% complementary to the corresponding nucleotide stretch of an RNA transcript of a PTP1B gene that comprises such mutations/alterations.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a PTP1B gene, including mRNA that is a product of RNA processing of a primary transcription product.

As used herein, the term "strand comprising a sequence" refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature. However, as detailed herein, such a "strand comprising a sequence" may also comprise modifications, like modified nucleotides.

As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the

ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence. "Complementary" sequences, as used herein, may also include, or be formed entirely from, non-Watson-Crick base pairs and/or
5 base pairs formed from non-natural and modified nucleotides, in as far as the above requirements with respect to their ability to hybridize are fulfilled.

Sequences referred to as "fully complementary" comprise base-pairing of the oligonucleotide or polynucleotide comprising the first nucleotide sequence to the oligonucleotide or polynucleotide comprising the second nucleotide sequence over the entire length of the first
10 and second nucleotide sequence.

However, where a first sequence is referred to as "substantially complementary" with respect to a second sequence herein, the two sequences can be fully complementary, or they may form one or more, but preferably not more than 13 mismatched base pairs upon hybridization.

The terms "complementary", "fully complementary" and "substantially complementary"
15 herein may be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of a dsRNA and a target sequence, as will be understood from the context of their use.

The term "double-stranded RNA", "dsRNA molecule", or "dsRNA", as used herein, refers to a ribonucleic acid molecule, or complex of ribonucleic acid molecules, having a duplex
20 structure comprising two anti-parallel and substantially complementary nucleic acid strands. The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where the two strands are part of one larger molecule, and therefore are connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'end of the respective other strand forming the duplex structure, the connecting
25 RNA chain is referred to as a "hairpin loop". Where the two strands are connected covalently by means other than an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker". The RNA strands may have the same or a different number of nucleotides. In addition to the duplex structure, a dsRNA may comprise one or more nucleotide
30 overhangs. The nucleotides in said "overhangs" may comprise between 0 and 5 nucleotides, whereby "0" means no additional nucleotide(s) that form(s) an "overhang" and whereas "5"

means five additional nucleotides on the individual strands of the dsRNA duplex. These optional “overhangs” are located in the 3’ end of the individual strands. As will be detailed below, also dsRNA molecules which comprise only an “overhang” in one the two strands may be useful and even advantageous in context of this invention. The “overhang” comprises preferably between 0 and 2 nucleotides. Most preferably 2 “dT” (deoxythymidine) nucleotides are found at the 3’ end of both strands of the dsRNA. Also 2 “U”(uracil) nucleotides can be used as overhangs at the 3’ end of both strands of the dsRNA. Accordingly, a “nucleotide overhang” refers to the unpaired nucleotide or nucleotides that protrude from the duplex structure of a dsRNA when a 3'-end of one strand of the dsRNA extends beyond the 5'-end of the other strand, or vice versa. For example the antisense strand comprises 23 nucleotides and the sense strand comprises 21 nucleotides, forming a 2 nucleotide overhang at the 3’ end of the antisense strand. Preferably, the 2 nucleotide overhang is fully complementary to the mRNA of the target gene. “Blunt” or “blunt end” means that there are no unpaired nucleotides at that end of the dsRNA, i.e., no nucleotide overhang. A “blunt ended” dsRNA is a dsRNA that is double-stranded over its entire length, i.e., no nucleotide overhang at either end of the molecule.

The term “antisense strand” refers to the strand of a dsRNA which includes a region that is substantially complementary to a target sequence. As used herein, the term “region of complementarity” refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence. Where the region of complementarity is not fully complementary to the target sequence, the mismatches are most tolerated outside nucleotides 2-7 of the 5’ terminus of the antisense strand

The term “sense strand,” as used herein, refers to the strand of a dsRNA that includes a region that is substantially complementary to a region of the antisense strand. “Substantially complementary” means preferably at least 85% of the overlapping nucleotides in sense and antisense strand are complementary.

“Introducing into a cell”, when referring to a dsRNA, means facilitating uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of dsRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells in vitro; a dsRNA may also be "introduced into a cell", wherein the cell is part of a living organism. In such instance, introduction into the cell will include the delivery to the organism. For example, for in vivo delivery, dsRNA can be injected into a tissue site or administered systemically. It is, for example

envisaged that the dsRNA molecules of this invention be administered to a subject in need of medical intervention. Such an administration may comprise the injection of the dsRNA, the vector or an cell of this invention into a diseased side in said subject, for example into liver tissue/cells or into cancerous tissues/cells, like liver cancer tissue. However, also the injection in
 5 close proximity of the diseased tissue is envisaged. In vitro introduction into a cell includes methods known in the art such as electroporation and lipofection.

The terms “silence”, “inhibit the expression of” and “knock down”, in as far as they refer to a PTP1B gene, herein refer to the at least partial suppression of the expression of a PTP1B gene, as manifested by a reduction of the amount of mRNA transcribed from a PTP1B gene
 10 which may be isolated from a first cell or group of cells in which a PTP1B gene is transcribed and which has or have been treated such that the expression of a PTP1B gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition is usually expressed in terms of

$$15 \quad \frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} \bullet 100\%$$

Alternatively, the degree of inhibition may be given in terms of a reduction of a parameter that is functionally linked to the PTP1B gene transcription, e.g. the amount of protein encoded by a PTP1B gene which is secreted by a cell, or the number of cells displaying a certain phenotype.

20 As illustrated in the appended examples and in the appended Tables provided herein, the inventive dsRNA molecules are capable of inhibiting the expression of a human PTP1B by at least about 60%, preferably by at least 70%, most preferably by at least 80% in vitro assays, i.e. in vitro. The term “in vitro” as used herein includes but is not limited to cell culture assays. In another embodiment the inventive dsRNA molecules are capable of inhibiting the expression of
 25 a mouse or rat PTP1B by at least 60 %,preferably by at least 70%, most preferably by at least 80%. The person skilled in the art can readily determine such an inhibition rate and related effects, in particular in light of the assays provided herein.

The term “off target” as used herein refers to all non-target mRNAs of the transcriptome that are predicted by in silico methods to hybridize to the described dsRNAs based on sequence

complementarity. The dsRNAs of the present invention preferably do specifically inhibit the expression of PTP1B, i.e. do not inhibit the expression of any off-target.

The term “half-life” as used herein is a measure of stability of a compound or molecule and can be assessed by methods known to a person skilled in the art, especially in light of the
5 assays provided herein.

The term “non-immunostimulatory” as used herein refers to the absence of any induction of a immune response by the invented dsRNA molecules. Methods to determine immune responses are well know to a person skilled in the art, for example by assessing the release of cytokines, as described in the examples section.

10 The terms "treat", "treatment", and the like, mean in context of this invention to relief from or alleviation of a disorder related to PTP1B expression, like type 2 diabetes, obesity, liver failure, dislipidemia, diabetic atherosclerosis, and hypertension. The term “liver failure” as used herein refers to all conditions in which the liver fails to fulfill its function or is unable to meet the demand made on it. It may occur for example as a result of trauma, neoplastic invasion,
15 prolonged biliary obstruction, viral infections (e.g. hepatitis C), or chronic alcoholism.

As used herein, a “pharmaceutical composition” comprises a pharmacologically effective amount of a dsRNA and a pharmaceutically acceptable carrier. However, such a “pharmaceutical composition” may also comprise individual strands of such a dsRNA molecule or the herein described vector(s) comprising a regulatory sequence operably linked to a
20 nucleotide sequence that encodes at least one strand of a sense or an antisense strand comprised in the dsRNAs of this invention. It is also envisaged that cells, tissues or isolated organs that express or comprise the herein defined dsRNAs may be used as “pharmaceutical compositions”. As used herein, “pharmacologically effective amount,” “therapeutically effective amount” or simply “effective amount” refers to that amount of an RNA effective to produce the intended
25 pharmacological, therapeutic or preventive result.

The term “pharmaceutically acceptable carrier” refers to a carrier for administration of a therapeutic agent. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The term specifically excludes cell culture medium. For drugs administered orally, pharmaceutically acceptable carriers include, but are not
30 limited to pharmaceutically acceptable excipients such as inert diluents, disintegrating agents,

binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives as known to persons skilled in the art.

It is in particular envisaged that the pharmaceutically acceptable carrier allows for the systemic administration of the dsRNAs, vectors or cells of this invention. Whereas also the enteric administration is envisaged the parenteral administration and also transdermal or transmucosal (e.g. insufflation, buccal, vaginal, anal) administration as well as inhalation of the drug are feasible ways of administering to a patient in need of medical intervention the compounds of this invention. When parenteral administration is employed, this can comprise the direct injection of the compounds of this invention into the diseased tissue or at least in close proximity. However, also intravenous, intraarterial, subcutaneous, intramuscular, intraperitoneal, intradermal, intrathecal and other administrations of the compounds of this invention are within the skill of the artisan, for example the attending physician.

For intramuscular, subcutaneous and intravenous use, the pharmaceutical compositions of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. In a preferred embodiment, the carrier consists exclusively of an aqueous buffer. In this context, "exclusively" means no auxiliary agents or encapsulating substances are present which might affect or mediate uptake of dsRNA in the cells that express a PTP1B gene. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate. The pharmaceutical compositions useful according to the invention also include encapsulated formulations to protect the dsRNA against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in PCT publication WO 91/06309 which is incorporated by reference herein.

As used herein, a "transformed cell" is a cell into which at least one vector has been introduced from which a dsRNA molecule or at least one strand of such a dsRNA molecule may be expressed. Such a vector is preferably a vector comprising a regulatory sequence operably

linked to nucleotide sequence that encodes at least one of a sense strand or an antisense strand comprised in the dsRNAs of this invention.

It can be reasonably expected that shorter dsRNAs comprising one of the sequences of Table 1 and 4 minus only a few nucleotides on one or both ends may be similarly effective as compared to the dsRNAs described above. As pointed out above, in most embodiments of this invention, the dsRNA molecules provided herein comprise a duplex length (i.e. without “overhangs”) of about 16 to about 30 nucleotides. Particular useful dsRNA duplex lengths are about 19 to about 25 nucleotides. Most preferred are duplex structures with a length of 19 nucleotides. In the inventive dsRNA molecules, the antisense strand is at least partially complementary to the sense strand.

In one preferred embodiment the inventive dsRNA molecules comprise nucleotides 1-19 of the sequences given in Table 13.

The dsRNA of the invention can contain one or more mismatches to the target sequence. In a preferred embodiment, the dsRNA of the invention contains no more than 13 mismatches. If the antisense strand of the dsRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located within nucleotides 2-7 of the 5' terminus of the antisense strand. In another embodiment it is preferable that the area of mismatch not to be located within nucleotides 2-9 of the 5' terminus of the antisense strand. .

As mentioned above, at least one end/strand of the dsRNA may have a single-stranded nucleotide overhang of 1 to 5, preferably 1 or 2 nucleotides. dsRNAs having at least one nucleotide overhang have unexpectedly superior inhibitory properties than their blunt-ended counterparts. Moreover, the present inventors have discovered that the presence of only one nucleotide overhang strengthens the interference activity of the dsRNA, without affecting its overall stability. dsRNA having only one overhang has proven particularly effective in vivo, as well as in a variety of cells, cell culture mediums, blood, and serum. Preferably, the single-stranded overhang is located at the 3'-terminal end of the antisense strand or, alternatively, at the 3'-terminal end of the sense strand. The dsRNA may also have a blunt end, preferably located at the 5'-end of the antisense strand. Preferably, the antisense strand of the dsRNA has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

The dsRNA of the present invention may also be chemically modified to enhance stability. The nucleic acids of the invention may be synthesized and/or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry", Beaucage, S.L. et al. (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby
5 incorporated herein by reference. Chemical modifications may include, but are not limited to 2' modifications, introduction of non-natural bases, covalent attachment to a ligand, and replacement of phosphate linkages with thiophosphate linkages. In this embodiment, the integrity of the duplex structure is strengthened by at least one, and preferably two, chemical linkages. Chemical linking may be achieved by any of a variety of well-known techniques, for example by
10 introducing covalent, ionic or hydrogen bonds; hydrophobic interactions, van der Waals or stacking interactions; by means of metal-ion coordination, or through use of purine analogues. Preferably, the chemical groups that can be used to modify the dsRNA include, without limitation, methylene blue; bifunctional groups, preferably bis-(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; and psoralen. In one preferred embodiment, the
15 linker is a hexa-ethylene glycol linker. In this case, the dsRNA are produced by solid phase synthesis and the hexa-ethylene glycol linker is incorporated according to standard methods (e.g., Williams, D.J., and K.B. Hall, *Biochem.* (1996) 35:14665-14670). In a particular embodiment, the 5'-end of the antisense strand and the 3'-end of the sense strand are chemically linked via a hexaethylene glycol linker. In another embodiment, at least one nucleotide of the
20 dsRNA comprises a phosphorothioate or phosphorodithioate groups. The chemical bond at the ends of the dsRNA is preferably formed by triple-helix bonds.

In certain embodiments, a chemical bond may be formed by means of one or several bonding groups, wherein such bonding groups are preferably poly-(oxyphosphinicooxy-1,3-propandiol)- and/or polyethylene glycol chains. In other embodiments, a chemical bond may also
25 be formed by means of purine analogs introduced into the double-stranded structure instead of purines. In further embodiments, a chemical bond may be formed by azabenzene units introduced into the double-stranded structure. In still further embodiments, a chemical bond may be formed by branched nucleotide analogs instead of nucleotides introduced into the double-stranded structure. In certain embodiments, a chemical bond may be induced by ultraviolet light.

30 In yet another embodiment, the nucleotides at one or both of the two single strands may be modified to prevent or inhibit the activation of cellular enzymes, for example certain nucleases. Techniques for inhibiting the activation of cellular enzymes are known in the art

including, but not limited to, 2'-amino modifications, 2'-amino sugar modifications, 2'-F sugar modifications, 2'-F modifications, 2'-alkyl sugar modifications, uncharged backbone modifications, morpholino modifications, 2'-O-methyl modifications, and phosphoramidate (see, e.g., Wagner, *Nat. Med.* (1995) 1:1116-8). Thus, at least one 2'-hydroxyl group of the nucleotides on a dsRNA is replaced by a chemical group, preferably by a 2'-amino or a 2'-methyl group. Also, at least one nucleotide may be modified to form a locked nucleotide. Such locked nucleotide contains a methylene bridge that connects the 2'-oxygen of ribose with the 4'-carbon of ribose. Introduction of a locked nucleotide into an oligonucleotide improves the affinity for complementary sequences and increases the melting temperature by several degrees.

Modifications of dsRNA molecules provided herein may positively influence their stability in vivo as well as in vitro and also improve their delivery to the (diseased) target side. Furthermore, such structural and chemical modifications may positively influence physiological reactions towards the dsRNA molecules upon administration, e.g. the cytokine release which is preferably suppressed. Such chemical and structural modifications are known in the art and are, inter alia, illustrated in Nawrot (2006) *Current Topics in Med Chem*, 6, 913-925.

Conjugating a ligand to a dsRNA can enhance its cellular absorption as well as targeting to a particular tissue. In certain instances, a hydrophobic ligand is conjugated to the dsRNA to facilitate direct permeation of the cellular membrane. Alternatively, the ligand conjugated to the dsRNA is a substrate for receptor-mediated endocytosis. These approaches have been used to facilitate cell permeation of antisense oligonucleotides. For example, cholesterol has been conjugated to various antisense oligonucleotides resulting in compounds that are substantially more active compared to their non-conjugated analogs. See M. Manoharan *Antisense & Nucleic Acid Drug Development* 2002, 12, 103. Other lipophilic compounds that have been conjugated to oligonucleotides include 1-pyrene butyric acid, 1,3-bis-O-(hexadecyl)glycerol, and menthol. One example of a ligand for receptor-mediated endocytosis is folic acid. Folic acid enters the cell by folate-receptor-mediated endocytosis. dsRNA compounds bearing folic acid would be efficiently transported into the cell via the folate-receptor-mediated endocytosis. Attachment of folic acid to the 3'-terminus of an oligonucleotide results in increased cellular uptake of the oligonucleotide (Li, S.; Deshmukh, H. M.; Huang, L. *Pharm. Res.* 1998, 15, 1540). Other ligands that have been conjugated to oligonucleotides include polyethylene glycols, carbohydrate clusters, cross-linking agents, porphyrin conjugates, and delivery peptides.

In certain instances, conjugation of a cationic ligand to oligonucleotides often results in improved resistance to nucleases. Representative examples of cationic ligands are propylammonium and dimethylpropylammonium. Interestingly, antisense oligonucleotides were reported to retain their high binding affinity to mRNA when the cationic ligand was dispersed
5 throughout the oligonucleotide. See M. Manoharan *Antisense & Nucleic Acid Drug Development* 2002, 12, 103 and references therein.

The ligand-conjugated dsRNA of the invention may be synthesized by the use of a dsRNA that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the dsRNA. This reactive oligonucleotide may be reacted directly with
10 commercially-available ligands, ligands that are synthesized bearing any of a variety of protecting groups, or ligands that have a linking moiety attached thereto. The methods of the invention facilitate the synthesis of ligand-conjugated dsRNA by the use of, in some preferred embodiments, nucleoside monomers that have been appropriately conjugated with ligands and that may further be attached to a solid-support material. Such ligand-nucleoside conjugates,
15 optionally attached to a solid-support material, are prepared according to some preferred embodiments of the methods of the invention via reaction of a selected serum-binding ligand with a linking moiety located on the 5' position of a nucleoside or oligonucleotide. In certain instances, an dsRNA bearing an aralkyl ligand attached to the 3'-terminus of the dsRNA is prepared by first covalently attaching a monomer building block to a controlled-pore-glass
20 support via a long-chain aminoalkyl group. Then, nucleotides are bonded via standard solid-phase synthesis techniques to the monomer building-block bound to the solid support. The monomer building block may be a nucleoside or other organic compound that is compatible with solid-phase synthesis.

The dsRNA used in the conjugates of the invention may be conveniently and routinely
25 made through the well-known technique of solid-phase synthesis. It is also known to use similar techniques to prepare other oligonucleotides, such as the phosphorothioates and alkylated derivatives.

Teachings regarding the synthesis of particular modified oligonucleotides may be found in the following U.S. patents: U.S. Pat. No. 5,218,105, drawn to polyamine conjugated
30 oligonucleotides; U.S. Pat. Nos. 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Pat. No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Pat. No. 5,539,082, drawn to peptide nucleic acids; U.S. Pat.

No. 5,554,746, drawn to oligonucleotides having β -lactam backbones; U.S. Pat. No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Pat. No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Pat. No. 5,587,361
5 drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Pat. No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Pat. No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Pat. No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Pat. No. 5,608,046, both drawn to conjugated 4'-
10 desmethyl nucleoside analogs; U.S. Pat. No. 5,610,289, drawn to backbone-modified oligonucleotide analogs; U.S. Pat. No. 6,262,241 drawn to, inter alia, methods of synthesizing 2'-fluoro-oligonucleotides.

In the ligand-conjugated dsRNA and ligand-molecule bearing sequence-specific linked nucleosides of the invention, the oligonucleotides and oligonucleosides may be assembled on a
15 suitable DNA synthesizer utilizing standard nucleotide or nucleoside precursors, or nucleotide or nucleoside conjugate precursors that already bear the linking moiety, ligand-nucleotide or nucleoside-conjugate precursors that already bear the ligand molecule, or non-nucleoside ligand-bearing building blocks.

When using nucleotide-conjugate precursors that already bear a linking moiety, the
20 synthesis of the sequence-specific linked nucleosides is typically completed, and the ligand molecule is then reacted with the linking moiety to form the ligand-conjugated oligonucleotide. Oligonucleotide conjugates bearing a variety of molecules such as steroids, vitamins, lipids and reporter molecules, has previously been described (see Manoharan et al., PCT Application WO 93/07883). In a preferred embodiment, the oligonucleotides or linked nucleosides of the
25 invention are synthesized by an automated synthesizer using phosphoramidites derived from ligand-nucleoside conjugates in addition to commercially available phosphoramidites.

The incorporation of a 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-allyl, 2'-O-aminoalkyl or 2'-deoxy-2'-fluoro group in nucleosides of an oligonucleotide confers enhanced hybridization properties to the oligonucleotide. Further, oligonucleotides containing phosphorothioate
30 backbones have enhanced nuclease stability. Thus, functionalized, linked nucleosides of the invention can be augmented to include either or both a phosphorothioate backbone or a 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-aminoalkyl, 2'-O-allyl or 2'-deoxy-2'-fluoro group.

In some preferred embodiments, functionalized nucleoside sequences of the invention possessing an amino group at the 5'-terminus are prepared using a DNA synthesizer, and then reacted with an active ester derivative of a selected ligand. Active ester derivatives are well known to those skilled in the art. Representative active esters include N-hydrosuccinimide esters, tetrafluorophenolic esters, pentafluorophenolic esters and pentachlorophenolic esters. The reaction of the amino group and the active ester produces an oligonucleotide in which the selected ligand is attached to the 5'-position through a linking group. The amino group at the 5'-terminus can be prepared utilizing a 5'-Amino-Modifier C6 reagent. In a preferred embodiment, ligand molecules may be conjugated to oligonucleotides at the 5'-position by the use of a ligand-nucleoside phosphoramidite wherein the ligand is linked to the 5'-hydroxy group directly or indirectly via a linker. Such ligand-nucleoside phosphoramidites are typically used at the end of an automated synthesis procedure to provide a ligand-conjugated oligonucleotide bearing the ligand at the 5'-terminus.

In one preferred embodiment of the methods of the invention, the preparation of ligand conjugated oligonucleotides commences with the selection of appropriate precursor molecules upon which to construct the ligand molecule. Typically, the precursor is an appropriately-protected derivative of the commonly-used nucleosides. For example, the synthetic precursors for the synthesis of the ligand-conjugated oligonucleotides of the invention include, but are not limited to, 2'-aminoalkoxy-5'-ODMT-nucleosides, 2'-6-aminoalkylamino-5'-ODMT-nucleosides, 5'-6-aminoalkoxy-2'-deoxy-nucleosides, 5'-6-aminoalkoxy-2'-protected-nucleosides, 3'-6-aminoalkoxy-5'-ODMT-nucleosides, and 3'-aminoalkylamino-5'-ODMT-nucleosides that may be protected in the nucleobase portion of the molecule. Methods for the synthesis of such amino-linked protected nucleoside precursors are known to those of ordinary skill in the art.

In many cases, protecting groups are used during the preparation of the compounds of the invention. As used herein, the term "protected" means that the indicated moiety has a protecting group appended thereon. In some preferred embodiments of the invention, compounds contain one or more protecting groups. A wide variety of protecting groups can be employed in the methods of the invention. In general, protecting groups render chemical functionalities inert to specific reaction conditions, and can be appended to and removed from such functionalities in a molecule without substantially damaging the remainder of the molecule.

Representative hydroxyl protecting groups, as well as other representative protecting groups, are disclosed in Greene and Wuts, *Protective Groups in Organic Synthesis*, Chapter 2,

2d ed., John Wiley & Sons, New York, 1991, and *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y, 1991.

Amino-protecting groups stable to acid treatment are selectively removed with base treatment, and are used to make reactive amino groups selectively available for substitution.

- 5 Examples of such groups are the Fmoc (E. Atherton and R. C. Sheppard in *The Peptides*, S. Udenfriend, J. Meienhofer, Eds., Academic Press, Orlando, 1987, volume 9, p.1) and various substituted sulfonyl ethyl carbamates exemplified by the Nsc group (Samukov et al., *Tetrahedron Lett.*, 1994, 35:7821).

- Additional amino-protecting groups include, but are not limited to, carbamate protecting
10 groups, such as 2-trimethylsilyl ethoxycarbonyl (Teoc), 1-methyl-1-(4-biphenyl)ethoxycarbonyl (Bpoc), t-butoxycarbonyl (BOC), allyloxycarbonyl (Alloc), 9-fluorenylmethyloxycarbonyl (Fmoc), and benzyloxycarbonyl (Cbz); amide protecting groups, such as formyl, acetyl, trihaloacetyl, benzoyl, and nitrophenylacetyl; sulfonamide protecting groups, such as 2-nitrobenzenesulfonyl; and imine and cyclic imide protecting groups, such as
15 phthalimido and dithiasuccinoyl. Equivalents of these amino-protecting groups are also encompassed by the compounds and methods of the invention.

- Many solid supports are commercially available and one of ordinary skill in the art can readily select a solid support to be used in the solid-phase synthesis steps. In certain embodiments, a universal support is used. A universal support allows for preparation of
20 oligonucleotides having unusual or modified nucleotides located at the 3'-terminus of the oligonucleotide. For further details about universal supports see Scott et al., *Innovations and Perspectives in solid-phase Synthesis, 3rd International Symposium*, 1994, Ed. Roger Epton, Mayflower Worldwide, 115-124]. In addition, it has been reported that the oligonucleotide can be cleaved from the universal support under milder reaction conditions when oligonucleotide is
25 bonded to the solid support via a *syn*-1,2-acetoxyposphate group which more readily undergoes basic hydrolysis. See Guzaev, A. I.; Manoharan, M. *J. Am. Chem. Soc.* 2003, 125, 2380.

- The nucleosides are linked by phosphorus-containing or non-phosphorus-containing covalent internucleoside linkages. For the purposes of identification, such conjugated nucleosides can be characterized as ligand-bearing nucleosides or ligand-nucleoside conjugates.
30 The linked nucleosides having an aralkyl ligand conjugated to a nucleoside within their sequence

will demonstrate enhanced dsRNA activity when compared to like dsRNA compounds that are not conjugated.

The aralkyl-ligand-conjugated oligonucleotides of the invention also include conjugates of oligonucleotides and linked nucleosides wherein the ligand is attached directly to the nucleoside or nucleotide without the intermediacy of a linker group. The ligand may preferably be attached, via linking groups, at a carboxyl, amino or oxo group of the ligand. Typical linking groups may be ester, amide or carbamate groups.

Specific examples of preferred modified oligonucleotides envisioned for use in the ligand-conjugated oligonucleotides of the invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined here, oligonucleotides having modified backbones or internucleoside linkages include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of the invention, modified oligonucleotides that do not have a phosphorus atom in their intersugar backbone can also be considered to be oligonucleosides.

Specific oligonucleotide chemical modifications are described below. It is not necessary for all positions in a given compound to be uniformly modified. Conversely, more than one modifications may be incorporated in a single dsRNA compound or even in a single nucleotide thereof.

Preferred modified internucleoside linkages or backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free-acid forms are also included.

Representative United States Patents relating to the preparation of the above phosphorus-atom-containing linkages include, but are not limited to, U.S. Pat. Nos. 4,469,863; 5,023,243;

5,264,423; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233 and 5,466,677, each of which is herein incorporated by reference.

Preferred modified internucleoside linkages or backbones that do not include a phosphorus atom therein (i.e., oligonucleosides) have backbones that are formed by short chain alkyl or cycloalkyl intersugar linkages, mixed heteroatom and alkyl or cycloalkyl intersugar linkages, or one or more short chain heteroatomic or heterocyclic intersugar linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents relating to the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,214,134; 5,216,141; 5,264,562; 5,466,677; 5,470,967; 5,489,677; 5,602,240 and 5,663,312, each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleoside units are replaced with novel groups. The nucleobase units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligonucleotide, an oligonucleotide mimetic, that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide-containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to atoms of the amide portion of the backbone. Teaching of PNA compounds can be found for example in U.S. Pat. No. 5,539,082.

Some preferred embodiments of the invention employ oligonucleotides with phosphorothioate linkages and oligonucleosides with heteroatom backbones, and in particular --CH₂--NH--O--CH₂--, --CH₂--N(CH₃)--O--CH₂-- [known as a methylene (methylimino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂--, and --O--N(CH₃)--CH₂--CH₂-- [wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S.

Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

The oligonucleotides employed in the ligand-conjugated oligonucleotides of the invention may additionally or alternatively comprise nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C), and uracil (U). Modified nucleobases include other synthetic and natural nucleobases, such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie, International Edition*, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligonucleotides of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-Methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C. (Id., pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-methoxyethyl sugar modifications.

Representative United States patents relating to the preparation of certain of the above-noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos 5,134,066; 5,459,255; 5,552,540; 5,594,121 and 5,596,091 all of which are hereby incorporated by reference.

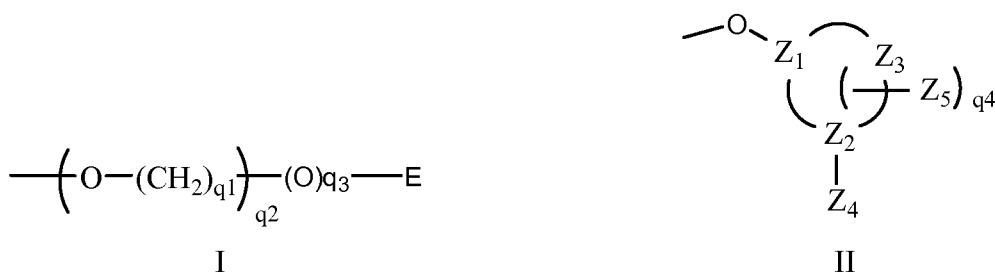
In certain embodiments, the oligonucleotides employed in the ligand-conjugated oligonucleotides of the invention may additionally or alternatively comprise one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂ CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. a preferred modification includes 2'-methoxyethoxy [2'-O--CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE], i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in U.S. Pat. No. 6,127,533, filed on Jan. 30, 1998, the contents of which are incorporated by reference.

Other preferred modifications include 2'-methoxy (2'-O--CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides.

As used herein, the term "sugar substituent group" or "2'-substituent group" includes groups attached to the 2'-position of the ribofuranosyl moiety with or without an oxygen atom. Sugar substituent groups include, but are not limited to, fluoro, O-alkyl, O-alkylamino, O-alkylalkoxy, protected O-alkylamino, O-alkylaminoalkyl, O-alkyl imidazole and polyethers of the formula (O-alkyl)_m, wherein m is 1 to about 10. Preferred among these polyethers are linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and, inter alia, those which are disclosed by Delgado et. al. (*Critical Reviews in Therapeutic Drug Carrier Systems* 1992, 9:249), which is hereby incorporated by reference in its entirety. Further sugar modifications are disclosed by Cook (*Anti-fibrosis Drug Design*, 1991, 6:585-607). Fluoro,

O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl amino substitution is described in U.S. Patent 6,166,197, entitled "Oligomeric Compounds having Pyrimidine Nucleotide(s) with 2' and 5' Substitutions," hereby incorporated by reference in its entirety.

Additional sugar substituent groups amenable to the invention include 2'-SR and 2'-NR₂ groups, wherein each R is, independently, hydrogen, a protecting group or substituted or unsubstituted alkyl, alkenyl, or alkynyl. 2'-SR Nucleosides are disclosed in U.S. Pat. No. 5,670,633, hereby incorporated by reference in its entirety. The incorporation of 2'-SR monomer synthons is disclosed by Hamm et al. (*J. Org. Chem.*, 1997, 62:3415-3420). 2'-NR nucleosides are disclosed by Goettingen, M., *J. Org. Chem.*, 1996, 61, 6273-6281; and Polushin et al., *Tetrahedron Lett.*, 1996, 37, 3227-3230. Further representative 2'-substituent groups amenable to the invention include those having one of formula I or II:



wherein,

E is C₁-C₁₀ alkyl, N(Q₃)(Q₄) or N=C(Q₃)(Q₄); each Q₃ and Q₄ is, independently, H, C₁-C₁₀ alkyl, dialkylaminoalkyl, a nitrogen protecting group, a tethered or untethered conjugate group, a linker to a solid support; or Q₃ and Q₄, together, form a nitrogen protecting group or a ring structure optionally including at least one additional heteroatom selected from N and O;

q_1 is an integer from 1 to 10;

q_2 is an integer from 1 to 10;

20 q_3 is 0 or 1;

q_4 is 0, 1 or 2;

each Z₁, Z₂ and Z₃ is, independently, C₄-C₇ cycloalkyl, C₅-C₁₄ aryl or C₃-C₁₅ heterocyclyl, wherein the heteroatom in said heterocyclyl group is selected from oxygen, nitrogen and sulfur;

Z₄ is OM₁, SM₁, or N(M₁)₂; each M₁ is, independently, H, C₁-C₈ alkyl, C₁-C₈ haloalkyl, C(=NH)N(H)M₂, C(=O)N(H)M₂ or OC(=O)N(H)M₂; M₂ is H or C₁-C₈ alkyl; and

Z₅ is C₁-C₁₀ alkyl, C₁-C₁₀ haloalkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, C₆-C₁₄ aryl, N(Q₃)(Q₄), OQ₃, halo, SQ₃ or CN.

5 Representative 2'-O-sugar substituent groups of formula I are disclosed in U.S. Pat. No. 6,172,209, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety. Representative cyclic 2'-O-sugar substituent groups of formula II are disclosed in U.S. Patent 6,271,358, entitled "RNA Targeted 2'-Modified Oligonucleotides that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

10 Sugars having O-substitutions on the ribosyl ring are also amenable to the invention. Representative substitutions for ring O include, but are not limited to, S, CH₂, CHF, and CF₂.

 Oligonucleotides may also have sugar mimetics, such as cyclobutyl moieties, in place of the pentofuranosyl sugar. Representative United States patents relating to the preparation of such modified sugars include, but are not limited to, U.S. Pat. Nos. 5,359,044; 5,466,786; 5,519,134; 15 5,591,722; 5,597,909; 5,646,265 and 5,700,920, all of which are hereby incorporated by reference.

 Additional modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide. For example, one additional modification of the ligand-conjugated oligonucleotides of the invention involves chemically 20 linking to the oligonucleotide one or more additional non-ligand moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties, such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 25 660, 306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 111; Kabanov et al., *FEBS Lett.*, 1990, 259, 327; Svinarchuk et al., *Biochimie*, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan 30 et al., *Tetrahedron Lett.*, 1995, 36, 3651; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777), a

polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923).

The invention also includes compositions employing oligonucleotides that are substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (Cook et al., U.S. Pat. No. 5,587,361) and those having substantially chirally pure (Sp or Rp) alkylphosphonate, phosphoramidate or phosphotriester linkages (Cook, U.S. Pat. Nos. 5,212,295 and 5,521,302).

In certain instances, the oligonucleotide may be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to oligonucleotides in order to enhance the activity, cellular distribution or cellular uptake of the oligonucleotide, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86:6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4:1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660:306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3:2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20:533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10:111; Kabanov et al., *FEBS Lett.*, 1990, 259:327; Svinarchuk et al., *Biochimie*, 1993, 75:49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651; Shea et al., *Nucl. Acids Res.*, 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14:969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277:923). Typical conjugation protocols involve the synthesis of oligonucleotides bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction may be performed either with the oligonucleotide still bound to the solid support or following

cleavage of the oligonucleotide in solution phase. Purification of the oligonucleotide conjugate by HPLC typically affords the pure conjugate. The use of a cholesterol conjugate is particularly preferred since such a moiety can increase targeting to tissues in the liver, a site of PTP1B protein production.

- 5 Alternatively, the molecule being conjugated may be converted into a building block, such as a phosphoramidite, via an alcohol group present in the molecule or by attachment of a linker bearing an alcohol group that may be phosphorylated.

10 Importantly, each of these approaches may be used for the synthesis of ligand conjugated oligonucleotides. Amino linked oligonucleotides may be coupled directly with ligand via the use of coupling reagents or following activation of the ligand as an NHS or pentfluorophenolate ester. Ligand phosphoramidites may be synthesized via the attachment of an aminohexanol linker to one of the carboxyl groups followed by phosphitylation of the terminal alcohol functionality. Other linkers, such as cysteamine, may also be utilized for conjugation to a chloroacetyl linker present on a synthesized oligonucleotide.

15 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are
20 incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The above provided embodiments and items of the present invention are now illustrated with the following, non-limiting examples.

25 **Description of figures and appended Tables:**

Figure 1- Effect of dsRNA targeting PTP1B (“PTP1B dsRNA”) on PTP1B liver mRNA levels in mice two days after i.v. injection of PTP1B dsRNA at 6 mg/kg in a LNP01 (1:14) liposome formulation. Luciferase dsRNA/LNP01 (“Luc”) and PBS (“Saline”) are controls. Results are group averages from 3 individual animals.

Figure 2 – Duration of effect study for the effect of PTP1B dsRNA on PTP1B liver mRNA levels in mice after i.v. injection of PTP1B dsRNA (Seq ID 479/480) at 6 mg/kg in a LNP01 (1:14) liposome formulation. PTP1B liver mRNA levels after 1, 2, 3, 4, 6 and 9 days post-injection. Luciferase dsRNA /LNP01 (“Luc”) and untreated animals are controls. Results are group averages from 4 individual animals.

Figure 3– Effect of PTP1B dsRNA comprising SEQ ID pair 5/6 on silencing off-target sequences. Expression of renilla luciferase protein after transfection of COS7 cells expressing dual-luciferase constructs, representative for either 19 mer target site of PTP1B mRNA (“on”) or *in silico* predicted off-target sequences (“off 1” to “off 13”; with “off 1” – “off 9” being antisense strand off- targets and “off 10” to “off 13” being sense strand off -targets), with 50 nM PTP1B dsRNA. Perfect matching off-target dsRNAs are controls.

Figure 4– Effect of PTP1B dsRNA comprising SEQ ID pair 13/14 on silencing off-target sequences. Expression of renilla luciferase protein after transfection of COS7 cells expressing dual-luciferase constructs, representative for either 19 mer target site of PTP1B mRNA (“on”) or *in silico* predicted off-target sequences (“off 1” to “off 13”; with “off 1” – “off 9” being antisense strand off- targets and “off 10” to “off 13” being sense strand off -targets), with 50 nM PTP1B dsRNA. Perfect matching off-target dsRNAs are controls.

Figure 5– Effect of PTP1B dsRNA comprising SEQ ID pair 11/12 on silencing off-target sequences. Expression of renilla luciferase protein after transfection of COS7 cells expressing dual-luciferase constructs, representative for either 19 mer target site of PTP1B mRNA (“on”) or *in silico* predicted off-target sequences (“off 1” to “off 17”; with “off 1” – “off 13” being antisense strand off- targets and “off 14” to “off 17” being sense strand off -targets), with 50 nM PTP1B dsRNA. Perfect matching off-target dsRNAs are controls.

Figure 6 – Activity of dsRNAs in insuling signaling. HepG2 cells were transfected for 48 hr with 5 nM dsRNA, starved for 24 hr, and treated with insulin for 30 minutes prior to PathScan ELISA assay. * Significant ($p < 0.05$) increase compared to mock. “A”: PTP1B dsRNA comprising SEQ ID pair 25/26, “B”: PTP1B dsRNA comprising SEQ ID pair 11/12, “C”: PTP1B dsRNA comprising SEQ ID pair 17/18, “D”: PTP1B dsRNA comprising SEQ ID pair 5/6, “E”: PTP1B dsRNA comprising SEQ ID pair 7/8, “F”: PTP1B dsRNA comprising SEQ ID pair 3/4, “G”: PTP1B dsRNA comprising SEQ ID pair 13/14.

Table 1 – dsRNA targeting human PTP1B gene. Letters in capitals represent RNA nucleotides, lower case letters “c”, “g”, “a” and “u” represent 2’ O-methyl-modified nucleotides, “s” represents phosphorothioate and “dT” deoxythymidine.

Table 2 - Characterization of dsRNAs targeting human PTP1B: Activity testing for dose response in HepG2 and HeLaS3 cells. IC 50: 50 % inhibitory concentration.

Table 3 - Characterization of dsRNAs targeting human PTP1B: Stability and Cytokine Induction. $t_{1/2}$: half-life of a strand as defined in examples, PBMC: Human peripheral blood mononuclear cells.

Table 4 - dsRNAs targeting mouse and rat PTP1B genes. Letters in capitals represent RNA nucleotides, lower case letters “c”, “g”, “a” and “u” represent 2’ O-methyl-modified nucleotides, “s” represents phosphorothioate and “dT” deoxythymidine. “f” represents 2’ fluoro modification of the preceding nucleotide.

Table 5 - Characterization of dsRNA targeting mouse and rat PTP1B genes: Stability and Cytokine Induction. $t_{1/2}$: half-life of a strand as defined in examples, PBMC: Human peripheral blood mononuclear cells.

Table 6 - Selected off-targets of dsRNAs targeting human PTP1B comprising sequence ID pair 5/6.

Table 7 - Selected off-targets of dsRNAs targeting human PTP1B comprising sequence ID pair 13/14.

Table 8 - Selected off-targets of dsRNAs targeting human PTP1B comprising sequence ID pair 11/12.

Table 9 - Sequences of bDNA probes for determination of human PTP1B; LE= label extender, CE= capture extender, BL= blocking probe.

Table 10 - Sequences of bDNA probes for determination of human GAPDH; LE= label extender, CE= capture extender, BL= blocking probe.

Table 11 - Sequences of bDNA probes for determination of mouse/rat PTP1B; LE= label extender, CE= capture extender, BL= blocking probe.

Table 12 - Sequences of bDNA probes for determination of mouse/rat GAPDH; LE= label extender, CE= capture extender, BL= blocking probe.

Table 13 – dsRNA targeting human PTP1B gene. Letters in capitals represent RNA nucleotides.

5 **Table 14** - dsRNA targeting human PTP1B gene without modifications and their modified counterparts. Letters in capitals represent RNA nucleotides, lower case letters “c”, “g”, “a” and “u” represent 2’ O-methyl-modified nucleotides, “s” represents phosphorothioate and “dT” deoxythymidine.

10

EXAMPLES

Identification of dsRNAs for therapeutic use

dsRNA design was carried out to identify dsRNAs specifically targeting human PTP1B for therapeutic use. First, the known mRNA sequence of human (Homo sapiens) PTP1B
15 (NM_002827.2 listed as SEQ ID NO. 620) was downloaded from NCBI Genbank.

mRNAs of rhesus monkey (Macaca mulatta) PTP1B (XM_001096053.1, XM_001096168.1, XM_001096290.1, and XM_001096412.1) were further used to generate a representative consensus mRNA sequence (SEQ ID NO. 621).

This sequence was examined together with the human PTP1B mRNA sequence (SEQ ID
20 NO. 620) by computer analysis to identify homologous sequences of 19 nucleotides that yield RNA interference (RNAi) agents cross-reactive to both sequences.

In identifying RNAi agents, the selection was limited to 19mer sequences having at least 2 mismatches to any other sequence in the human RefSeq database (release 25), which we assumed to represent the comprehensive human transcriptome, by using the fastA algorithm.

25 The cynomolgous monkey (Macaca fascicularis) PTP1B gene was sequenced (see SEQ ID NO.622) and examined for target regions of RNAi agents.

dsRNAs cross-reactive to human as well as cynomolgous monkey PTP1B were defined as most preferable for therapeutic use. All sequences containing 4 or more consecutive G's (poly-G sequences) were excluded from the synthesis.

5 The sequences thus identified formed the basis for the synthesis of the RNAi agents in appended Table 1.

Identification of dsRNAs for *in vivo* proof of concept studies

10 dsRNA design was carried out to identify dsRNAs targeting mouse (*Mus musculus*) and rat (*Rattus norvegicus*) for *in vivo* proof-of-concept experiments. First, the transcripts for mouse PTP1B (NM_011201.3, SEQ ID NO. 623) and rat PTP1B (NM_012637.2, SEQ ID NO. 624) were examined by computer analysis to identify homologous sequences of 19 nucleotides that yield RNAi agents cross-reactive between these sequences.

15 In identifying RNAi agents, the selection was limited to 19mer sequences having at least 2 mismatches in the antisense strand to any other sequence in the mouse and rat RefSeq database (release 25), which we assumed to represent the comprehensive mouse and rat transcriptome, by using the fastA algorithm.

All sequences containing 4 or more consecutive G's (poly-G sequences) were excluded from the synthesis. The sequences thus identified formed the basis for the synthesis of the RNAi agents in appended Table 4.

dsRNA synthesis

20 Where the source of a reagent is not specifically given herein, such reagent may be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

25 Single-stranded RNAs were produced by solid phase synthesis on a scale of 1 μ mole using an Expedite 8909 synthesizer (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) and controlled pore glass (CPG, 500Å, Proligo Biochemie GmbH, Hamburg, Germany) as solid support. RNA and RNA containing 2'-O-methyl nucleotides were generated by solid phase synthesis employing the corresponding phosphoramidites and 2'-O-methyl phosphoramidites, respectively (Proligo Biochemie GmbH, Hamburg, Germany). These building blocks were incorporated at selected sites within the sequence of the

oligoribonucleotide chain using standard nucleoside phosphoramidite chemistry such as described in Current protocols in nucleic acid chemistry, Beaucage, S.L. et al. (Edrs.), John Wiley & Sons, Inc., New York, NY, USA. Phosphorothioate linkages were introduced by replacement of the iodine oxidizer solution with a solution of the Beaucage reagent (Chruachem Ltd, Glasgow, UK) in acetonitrile (1%). Further ancillary reagents were obtained from Mallinckrodt Baker (Griesheim, Germany).

Deprotection and purification of the crude oligoribonucleotides by anion exchange HPLC were carried out according to established procedures. Yields and concentrations were determined by UV absorption of a solution of the respective RNA at a wavelength of 260 nm using a spectral photometer (DU 640B, Beckman Coulter GmbH, Unterschleißheim, Germany). Double stranded RNA was generated by mixing an equimolar solution of complementary strands in annealing buffer (20 mM sodium phosphate, pH 6.8; 100 mM sodium chloride), heated in a water bath at 85 - 90°C for 3 minutes and cooled to room temperature over a period of 3 - 4 hours. The annealed RNA solution was stored at -20 °C until use.

Activity testing

The activity of the PTP1B-dsRNAs for therapeutic use described above was tested in HepG2 and HeLa cells. Cells in culture were used for quantitation of PTP1B mRNA by branched DNA in total mRNA derived from cells incubated with PTP1B-specific dsRNAs.

HepG2 cells were obtained from American Type Culture Collection (Rockville, Md., cat. No. HB-8065) and cultured in MEM (Gibco Invitrogen, Invitrogen GmbH, Karlsruhe, Germany, cat. No. 21090-022) supplemented to contain 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany, cat. No. S0115), 2 mM L-Glutamin (Biochrom AG, Berlin, Germany, cat. No. K0238), Penicillin 100 U/ml, Streptomycin 100 mg/ml (Biochrom AG, Berlin, Germany, cat. No. A2213), 1.times non-essential amino acids (NEA) (Biochrom AG, Berlin, Germany, cat. No. K0293) and 1 mM sodium pyruvate (Biochrom AG, Berlin, Germany, cat. No. L0473) at 37.degree. C. in an atmosphere with 5% CO.sub.2 in a humidified incubator (Heraeus HERAccl, Kendro Laboratory Products, Langenselbold, Germany).

HeLaS3 cells were obtained from *American Type Culture Collection* (Rockville, Md., cat. No. CCL-2.2) and cultured in Ham's F12 (Biochrom AG, Berlin, Germany, cat. No. FG 0815) supplemented to contain 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany, cat. No.

S0115), Penicillin 100 U/ml, Streptomycin 100 mg/ml (Biochrom AG, Berlin, Germany, cat. No. A2213) at 37°C in an atmosphere with 5% CO₂ in a humidified incubator (Heraeus HERAcell, Kendro Laboratory Products, Langenselbold, Germany).

5 Cell seeding and transfection of dsRNA were performed at the same time. For transfection with dsRNA, HepG2 cells were seeded at a density of 2.0.times.10.sup.4 cells/well in 96-well plates and HeLaS3 cells at a density of 1.5.times.10.sup.4 cells/well. Transfection of dsRNA was carried out with lipofectamine 2000 (Invitrogen GmbH, Karlsruhe, Germany, cat.No. 11668-019) as described by the manufacturer. In a first single dose experiment dsRNAs were
10 transfected at a concentration of 30 nM in HepG2 cells. Each datapoint was determined in quadruplicate. Two independent experiments were performed. In a second single dose experiment most active dsRNAs showing a PTP1BmRNA knockdown of 50% or more were reanalyzed at 30nM in HeLaS3 cells. Most effective dsRNAs showing a mRNA knockdown of more than 60% from the first single dose screen at 30nM in HepG2 cells were further
15 characterized by dose response curves. For dose response curves, transfections were performed in HeLaS3 cells as described for the single dose screen above, but with the following concentrations of dsRNA (nM): 24, 6, 1.5, 0.375, 0.0938, 0.0234, 0.0059, 0.0015, 0.0004 and 0.0001 nM . After transfection cells were incubated for 24 h at 37°C and 5 % CO₂ in a humidified incubator (Heraeus GmbH, Hanau, Germany). For measurement of PTP1B mRNA
20 cells were harvested and lysed at 53°C following procedures recommended by the manufacturer of the Quantigene Explore Kit (Genospectra, Fremont, Calif., USA, cat. No. QG-000-02) for bDNA quantitation of mRNA. Afterwards, 50 µl of the lysates were incubated with probesets specific to human PTP1B and human GAPDH (sequence of probesets see appended Tables 9 and 10) and processed according to the manufacturer's protocol for QuantiGene.
25 Chemoluminescence was measured in a Victor2-Light (Perkin Elmer, Wiesbaden, Germany) as RLUs (relative light units) and values obtained with the human PTP1B probeset were normalized to the respective human GAPDH values for each well. Unrelated control dsRNAs were used as a negative control. Activity of dsRNAs targeting mouse and rat PTP1B were measured accordingly in MH7777A cells using a concentration of 20nM for dsRNAs, bDNA probesets
30 specific for determination of rat GAPDH and mouse/rat PTP1B are shown in appended Tables 11 and 12.

Inhibition data are given in appended Tables 2 and 4.

Stability of dsRNAs

Stability of dsRNAs was determined in in vitro assays with either human serum or plasma from cynomolgous monkey for dsRNAs targeting human PTP1B and with mouse serum for dsRNAs targeting mouse/rat PTB1B by measuring the half-life of each single strand.

5 Measurements were carried out in triplicates for each time point, using 3µl 50µM dsRNA sample mixed with 30µl human serum or cynomolgous plasma (Sigma Aldrich). Mixtures were incubated for either 0min, 30min, 1h, 3h, 6h, 24h, or 48h at 37°C. As control for unspecific degradation dsRNA was incubated with 30µl 1x PBS pH 6.8 for 48h. Reactions were stopped by the addition of 4µl proteinase K (20mg/ml), 25µl of “Tissue and Cell Lysis Solution” (Epicentre)
 10 and 38µl Millipore water for 30 min at 65°C. Samples were afterwards spin filtered through a 0.2 µm 96 well filter plate at 1400 rpm for 8 min, washed with 55µl Millipore water twice and spin filtered again.

For separation of single strands and analysis of remaining full length product (FLP), samples were run through an ion exchange Dionex Summit HPLC under denaturing conditions
 15 using as eluent A 20mM Na₃PO₄ in 10% ACN pH=11 and for eluent B 1 M NaBr in eluent A.

The following gradient was applied:

Time	%A	%B
-1.0 min	75	25
1.00 min	75	25
19.0 min	38	62
19.5 min	0	100
21.5 min	0	100
22.0 min	75	25
24.0 min	75	25

20 For every injection, the chromatograms were integrated automatically by the Dionex Chromeleon 6.60 HPLC software, and were adjusted manually if necessary. All peak areas were corrected to the internal standard (IS) peak and normalized to the incubation at t=0 min. The area
 25 under the peak and resulting remaining FLP was calculated for each single strand and triplicate separately. Half-life (t_{1/2}) of a strand was defined by the average time point [h] for triplicates at which half of the FLP was degraded. Results are given in appended Tables 3 and 5.

Cytokine induction

Potential cytokine induction of dsRNAs was determined by measuring the release of INF-a and TNF-a in an *in vitro* PBMC assay.

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coat blood of two donors by Ficoll centrifugation at the day of transfection. Cells were transfected in quadruplicates with dsRNA and cultured for 24h at 37°C at a final concentration of 130nM in Opti-MEM, using either Gene Porter 2 (GP2) or DOTAP. dsRNA sequences that were known to induce INF-a and TNF-a in this assay, as well as a CpG oligo, were used as positive controls. Chemical conjugated dsRNA or CpG oligonucleotides that did not need a transfection reagent for cytokine induction, were incubated at a concentration of 500nM in culture medium. At the end of incubation, the quadruplicate culture supernatant were pooled.

INF-a and TNF-a was then measured in these pooled supernatants by standard sandwich ELISA with two data points per pool. The degree of cytokine induction was expressed relative to positive controls using a score from 0 to 5, with 5 indicating maximum induction. Results are given in appended Tables 3 and 5.

In vitro off-target analysis of dsRNA targeting human PTP1B

The psiCHECK™- vector (Promega) contains two reporter genes for monitoring RNAi activity: a synthetic version of the Renilla luciferase (hRluc) gene and a synthetic firefly luciferase gene (hluc+). The firefly luciferase gene permits normalization of changes in Renilla luciferase expression to firefly luciferase expression. Renilla and firefly luciferase activities were measured using the Dual-Glo® Luciferase Assay System (Promega). To use the psiCHECK™ vectors for analyzing off-target effects of the inventive dsRNAs, the predicted off-target sequence was cloned into the multiple cloning region located 3' to the synthetic Renilla luciferase gene and its translational stop codon. After cloning, the vector is transfected into a mammalian cell line, and subsequently cotransfected with dsRNAs targeting PTP1B. If the dsRNA effectively initiates the RNAi process on the target RNA of the predicted off-target, the fused Renilla target gene mRNA sequence will be degraded, resulting in reduced Renilla luciferase activity.

In silico off-target prediction

The human genome was searched by computer analysis for sequences homologous to the inventive dsRNAs. Homologous sequences that displayed less than 5 mismatches with the inventive dsRNAs were defined as a possible off-targets. Off-targets selected for *in vitro* off-target analysis are given in appended Tables 6, 7 and 8.

Generation of psiCHECK vectors containing predicted off-target sequences

The strategy for analyzing off target effects for an dsRNA lead candidate includes the cloning of the predicted off target sites into the psiCHECK2 Vector system (Dual Glo®-system, Promega, Braunschweig, Germany cat. No C8021) via XhoI and NotI restriction sites. Therefore, the off target site is extended with 10 nucleotides upstream and downstream of the dsRNA target site. Additionally, a NheI restriction site is integrated to prove insertion of the fragment by restriction analysis. The single-stranded oligonucleotides were annealed according to a standard protocol (e.g. protocol by Metabion) in a Mastercycler (Eppendorf) and then cloned into psiCHECK (Promega) previously digested with XhoI and NotI. Successful insertion was verified by restriction analysis with NheI and subsequent sequencing of the positive clones. The selected primer (Seq ID No. 625) for sequencing binds at position 1401 of vector psiCHECK. After clonal production the plasmids were analyzed by sequencing and then used in cell culture experiments.

Analysis of dsRNA off-target effects

Cell culture:

Cos7 cells were obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany, cat. No. ACC-60) and cultured in DMEM (Biochrom AG, Berlin, Germany, cat. No. F0435) supplemented to contain 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany, cat. No. S0115), Penicillin 100 U/ml, and Streptomycin 100 µg/ml (Biochrom AG, Berlin, Germany, cat. No. A2213) and 2 mM L-Glutamine (Biochrom AG, Berlin, Germany, cat. No. K0283) as well as 12 µg/ml Natrium-bicarbonat at 37°C in an atmosphere with 5% CO₂ in a humidified incubator (Heraeus HERAccl, Kendro Laboratory Products, Langenselbold, Germany).

Transfection and Luciferase quantification:

For transfection with plasmids, Cos-7 cells were seeded at a density of 2.25×10^4 cells/well in 96-well plates and transfected directly. Transfection of plasmids was carried out with lipofectamine 2000 (Invitrogen GmbH, Karlsruhe, Germany, cat. No. 11668-019) as described by the manufacturer at a concentration of 50 ng/well. 4 hours after transfection, the medium was discarded and fresh medium was added. Now the dsRNAs were transfected in a concentration at 50 nM using lipofectamine 2000 as described above. 24h after dsRNA transfection the cells were lysed using Luciferase reagent described by the manufacturer (Dual-Glo™ Luciferase Assay system, Promega, Mannheim, Germany, cat. No. E2980) and Firefly and Renilla Luciferase were quantified according to the manufacturer's protocol. Renilla Luciferase protein levels were normalized to Firefly Luciferase levels. For each dsRNA twelve individual data points were collected in three independent experiments. A dsRNA unrelated to all target sites was used as a control to determine the relative Renilla Luciferase protein levels in dsRNA treated cells.

Results are given in figures 3, 4 and 5.

In vivo effects of dsRNA targeting PTP1B (mice and rat)**PTP1B mRNA measurement in mouse liver tissue:**

PTP1B mRNA measurements were done from liver tissue using QuantiGene 1.0 branched DNA (bDNA) Assay Kit (Panomics, Fremont, Calif., USA, Cat-No: QG0004).

At necropsy 1-2g liver tissue was snap frozen in liquid nitrogen. Frozen tissue was powdered with mortar and pestle on dry ice. 15-25 mg of tissue was transferred to a chilled 1.5 ml reaction tube, 1ml 1:3 Lysis Mixture prediluted in MilliQ water and 3.3 µl Proteinase K (50 µg/µl) was added and tissue was lysed by several seconds ultrasound sonication at 30-50% power (HD2070, Bandelin, Berlin, Germany). Lysates were stored at -80°C until analysis. For mRNA analysis lysate was thawed and Proteinase K digested for 15min at 1000 rpm and 65°C (Thermomixer comfort, Eppendorf, Hamburg, Germany). PTP1B and GAPDH mRNA levels were determined using QuantiGene 1.0 bDNA Assay Kit reagents and according to the manufacturer's recommendations. PTP1B expression was analysed using 20 µl lysate and

mouse/rat PTP1B probeset and GAPDH expression was analysed using 40µl lysate and rattus norvegicus probesets shown to crossreact with mice (sequences of probesets see above). Chemiluminescence signal at end of assay was measured in a Victor 2 Light luminescence counter (Perkin Elmer, Wiesbaden, Germany) as relative light units (RLU). PTP1B signal was divided by same lysate GAPDH signal and values depicted as PTP1B expression normalized to GAPDH.

dsRNAs were formulated in LNP01 as described previously (Akinc, A. et al., Nature Biotech 2008, 26(5):561-9.)

Results are shown in figures 1 and 2.

10 **Activity of dsRNAs in insuling signaling**

HepG2 cells were transfected for 48 hr with 5 nM dsRNA, starved for 24 hr, and treated with insulin for 30 minutes prior to PathScan ELISA assay. Overview of procedure:

Day 1: HepG2 Cells P8 (ATCC MEME, 10% ATCC FBS, 1x l-gln) reverse transfected with 5nM dsRNA and DharmaFECT1 transfection reagent

15 **Day 3:** Cells were washed with dPBS 1X and starve media was added for an overnight incubation (ATCC MEME, 1x l-gln, 2% stripped serum)

Day 4: Insulin Stimulation and then lyse. Insulin from Lynn at 689 µM concentration (Invitrogen 12585-014). Mix insulin into MEME (nothing added) at proper concentration (Example: 10 ml at 5µM use 72.6 µl insulin and 9.9 ml MEME)

20 –Wash cells 1X with MEME before adding MEME containing insulin

–Incubate cells with insulin for 30 minutes at 37°C

–Wash cells 1X with ice-cold dPBS

–Remove dPBS and add 100 ul ice-cold lysis buffer (1X CST #9803 with 1 mM PMSF) to each well and incubate on ice for 5 min

25 –Shake cell plates in freezer for 10 minutes to ensure lysis before storing at -80°C

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Cell Signaling Technologies PathScan Phospho-Akt1 ELISA:

–Initially performed dilution curve with HepG2 lysate to determine approx. protein to load (2-5 µg)

–Diluted 10µl lysate and added to plate ELISA plate. Followed CST protocol.

- 5 –Ran a microBCA assay with 3µl protein to normalize ELISA results to protein added in each well.

The following controls were used in this experiment:

–Ctrl-10: Risc-free control from Dharmacon (D-001220-01)

–Ctrl-11: universal control synthesized by Dharmacon

- 10 –Mock: Transfection reagent but no dsRNA

Results are shown in figure 6.

Claims

1. A double-stranded ribonucleic acid molecule capable of inhibiting the expression of PTP1B gene in vitro by at least 60 %, preferably by at least 70% and most preferably by at least 80%
5
2. The double-stranded ribonucleic acid molecule of claim 1, wherein said double-stranded ribonucleic acid molecule comprises a sense strand and an antisense strand, the antisense strand being at least partially complementary to the sense strand, whereby the sense strand comprises a sequence, which has an identity of at least 90 % to at least a portion of an mRNA encoding PTP1B, wherein said sequence is (i) located in the region of complementarity of said sense strand to said antisense strand; and (ii) wherein said sequence is less than 30 nucleotides in length.
10
3. The double-stranded ribonucleic acid molecule of claims 1 to 2, wherein said sense strand comprises nucleotide acid sequences depicted in SEQ ID Nos: 630, 632, 634, 638, 640, 644 and 652 and said antisense strand comprises nucleic acid sequences depicted in SEQ ID Nos: 631, 633, 635, 639, 641, 645 and 653 wherein said double-stranded ribonucleic acid molecule comprises the sequence pairs selected from the group consisting of SEQ ID NOs: 630/631, 632/633, 634/635, 638/639, 640/641, 644/645 and 652/653.
15 20
4. The double-stranded ribonucleic acid molecule of claim 3, wherein the antisense strand further comprises a 3' overhang of 1-5 nucleotides in length, preferably of 1-2 nucleotides in length.
25
5. The double-stranded ribonucleic acid molecule of claim 4, wherein the overhang of the antisense strand comprises uracil or nucleotides which are complementary to the mRNA encoding PTP1B.
30
6. The double-stranded ribonucleic acid molecule of any of claims 3 to 5, wherein the sense strand further comprises a 3' overhang of 1-5 nucleotides in length, preferably of 1-2 nucleotides in length.

7. The double-stranded ribonucleic acid molecule of any of claim 6 wherein the overhang of the sense strand comprises uracil or nucleotides which are identical to the mRNA encoding PTP1B.
- 5 8. The double-stranded ribonucleic acid molecule of any one of claims 1 to 7, wherein said double-stranded ribonucleic acid molecule comprises at least one modified nucleotide.
- 10 9. The double-stranded ribonucleic acid molecule of claim 8, wherein said modified nucleotide is selected from the group consisting of a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group, a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.
- 15 10. The double-stranded ribonucleic acid molecule of any one of claims 8 and 9, wherein said modified nucleotide is a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a deoxythymidine.
- 20 11. The double-stranded ribonucleic acid molecule of claims 3 to 10, wherein said sense strand and / or said antisense strand comprise an overhang of 1-2 deoxythymidines.
- 25 12. The double-stranded ribonucleic acid molecule of any one of claims 1 to 11, wherein said sense strand is selected from the group consisting of the nucleic acid sequences depicted in SEQ ID Nos: 3, 5, 7, 11, 13, 17, and 25 and said antisense strand is selected from the group consisting of the nucleic acid sequences depicted in SEQ ID Nos: 4, 6, 8, 12, 14, 18 and 26 wherein said double-stranded ribonucleic acid molecule comprises the sequence pairs selected from the group consisting of SEQ ID NOs: 3/4, 5/6, 7/8, 11/12, 13/14, 17/18, and 25/26.
- 30 13. A nucleic acid sequence encoding a sense strand and/or an antisense strand comprised in the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12.

14. A vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes at least one of a sense strand or an antisense strand comprised in the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12 or comprising the nucleic acid sequence of claim 13.
15. A cell, tissue or non-human organism comprising the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12, the nucleic acid molecule of claim 13 or the vector of claim 14.
16. A pharmaceutical composition comprising the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12, the nucleic acid molecule of claim 13, the vector of claim 14 or the cell or tissue of claim 15.
17. The pharmaceutical composition of claim 16, further comprising a pharmaceutically acceptable carrier, stabilizer and/or diluent.
18. A method for inhibiting the expression of PTP1B gene in a cell, a tissue or an organism comprising the following steps:
- (a) introducing into the cell, tissue or organism the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12, the nucleic acid molecule of claim 13,, the vector of claim 14; and
- (b) maintaining the cell, tissue or organism produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a PTP1B gene, thereby inhibiting expression of a PTP1B gene in the cell.
19. A method of treating, preventing or managing pathological conditions and diseases caused by the expression of the PTP1B gene disease comprising administering to a subject in need of such treatment, prevention or management a therapeutically or prophylactically effective amount of a the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12, a nucleic acid molecule of claim 13, a vector of claim 14 and/or a pharmaceutical composition as defined in claims 16 or 18.

20. The method of claim 19, wherein said subject is a human.
21. A double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12, a nucleic acid molecule of claim 13, a vector of claim 14 and/or a pharmaceutical composition as defined in claims 16 or 18 for use in treating type 2 diabetes, liver failure, obesity, dislipidemia, diabetic arteriosclerosis or hypertension.
22. Use of a the double-stranded ribonucleic acid molecule as defined in any one of claims 1 to 12, a nucleic acid molecule of claim 13, a vector of claim 14 and/or a cell or tissue of claim 15 for the preparation of a pharmaceutical composition for the treatment of type 2 diabetes, liver failure, obesity, dislipidemia, diabetic arteriosclerosis or hypertension.