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(54) **Title:** CELLULAR TARGETS OF THIAZOLIDINEDIONES

(57) **Abstract:** A method for identifying a lead candidate compound that is effective in treating or preventing an insulin-resistance disease or disorder in a subject comprises: screening one or more candidate compounds in a binding assay, the binding assay comprising the steps: (i) providing an mTOT protein; (ii) contacting the mTOT protein with a candidate compound; and (iii) detecting if the candidate compound specifically binds to the mTOT protein or inhibits the specific binding of a thiazolidinedione compound to the mTOT protein. The candidate compound is identified as a lead candidate compound if the candidate compound specifically binds to the mTOT protein or inhibits the binding of the thiazolidinedione compound to the mTOT protein.



CELLULAR TARGETS OF THIAZOLIDINEDIONES**CROSS-REFERENCE TO PRIOR APPLICATIONS**

[0001] This application claims the benefit of U.S. Provisional Application No. 61/441,260, filed February 9, 2011, and U.S. Provisional Application No. 61/583,996, filed January 6, 2012, and are hereby incorporated by reference into this application in their entireties.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED**ELECTRONICALLY**

[0002] Incorporated by reference in its entirety is a computer-readable sequence listing submitted concurrently herewith and identified as follows: One 77.9 KB ASCII (Text) file named "223259-323110_Seq_Listing_ST25," created on February 9, 2012, at 2:44 pm.

TECHNICAL FIELD

[0003] The present invention relates to assays for identifying compounds and chemically active agents that possess PPAR γ -sparing and/or insulin sensitizing activity for the treatment of metabolic diseases, for example, metabolic syndrome, obesity, diabetes mellitus, and neurodegenerative diseases.

BACKGROUND

[0004] Peroxisome Proliferator Activated Receptors (PPARs) are members of the nuclear hormone receptor super family, which are ligand-activated transcription factors regulating gene expression. PPARs have been implicated in autoimmune diseases and other diseases, i.e. diabetes mellitus, cardiovascular disease, gastrointestinal disease, and Alzheimer's disease.

[0005] Over the past several decades, scientists have postulated that PPAR γ is the generally accepted site of action for insulin sensitizing thiazolidinedione compounds (TZDs).

[0006] PPAR γ is a key regulator of adipocyte differentiation and lipid metabolism. PPAR γ is also found in other cell types including fibroblasts, myocytes, breast cells, human bone-marrow precursors, and macrophages/monocytes. In addition, PPAR γ has been shown in macrophage foam cells in atherosclerotic plaques.

[0007] TZDs, developed originally for the treatment of type-2 diabetes, generally exhibit high-affinity as PPAR γ ligands. The finding that thiazolidinediones might mediate their therapeutic effects through direct interactions with PPAR γ helped to establish the concept that PPAR γ is a key regulator of glucose and lipid homeostasis. However, compounds that involve the activation of PPAR γ also trigger sodium reabsorption and other unpleasant side

effects that can negatively influence the health of the patient being administered the compound.

[0008] TZDs that have reduced binding and/or activation of PPAR γ ligands, demonstrated beneficial biological properties such as increased insulin sensitivity, reduced blood glucose, reduced blood pressure, increased HDL cholesterol, and preservation of beta cells in the pancreas, without the negative side effects observed with PPAR γ -activating TZDs. Thus there is a need for screening assays to identify TZDs or compounds mimicking TZDs that are PPAR γ sparing and which have a therapeutic effect on metabolic diseases or disorders, such as, diabetes mellitus, cardiovascular disease, gastrointestinal disease, and Alzheimer's disease.

SUMMARY

[0009] This section provides a general summary of the disclosure, and is not a comprehensive disclosure of its full scope or all of its features.

[0010] In one aspect, the present invention provides screening assays for the identification of candidate compounds that are capable of binding to a membrane Target of Thiazolidinedione (mTOT protein) protein, or a fusion protein comprising the same. As used herein, a "mTOT protein" of the present invention includes: a BP44 protein, a BRP44-Like protein, a BP44-BRP44-Like heterodimer and homologs, orthologs, fusions, functional fragments, derivatives and analogs thereof.

[0011] In one illustrative assay, the method includes: a) combining an mTOT protein, with one or more candidate compounds under conditions to allow specific binding between the mTOT protein and the one or more candidate compounds, and b) detecting specific binding, thereby identifying one or more lead candidate compounds which specifically binds the mTOT protein. In another aspect, the screening method includes verifying that the candidate compound which binds to the mTOT protein is a therapeutic active agent.

[0012] In a further aspect, a screening assay for identifying an insulin sensitizing therapeutic agent effective in treating or preventing an insulin-resistance disease or disorder in a subject, the method comprises: screening one or more candidate compounds in a binding assay, the binding assay comprising the steps: (i) providing an mTOT protein;(ii) contacting the mTOT protein with a candidate compound; and (iii) detecting if the candidate compound specifically binds to the mTOT protein or inhibits the specific binding of a thiazolidinedione compound to the mTOT protein, wherein the candidate compound is identified as a lead candidate compound if the candidate compound specifically binds to the mTOT protein or inhibits the binding of the thiazolidinedione compound to the mTOT protein. In some

aspects, the mTOT protein is provided on the surface of a reaction substrate, for example, as used in high-throughput assays wherein samples are spotted or imprinted on the surface of a substantially planar substrate. Alternatively, the mTOT protein can be provided in a reaction receptacle, for example, a tube, a well of a microtiter plate, a pore or sample well of a microfluidic device, or an indentation on the surface of a substrate operable to receive additional reagents or fluids. In some aspects, the mTOT protein is contacted with labeled binding partner in a reaction receptacle, for example, a tube, a well of a microtiter plate, an indentation on the surface of a substrate.

[0013] Optionally, the screening method can include repeating steps (i) - (iii) in a high throughput screen. In some embodiments, the method further includes assaying the lead candidate compound in an activity assay to determine whether the lead candidate compound is an insulin-sensitizing therapeutic agent.

[0014] In another aspect, a method for screening a plurality of candidate compounds to identify a lead candidate compound effective against an insulin resistance disease or disorder includes:

- (a) providing a candidate compound and at least one mTOT protein;
 - (b) incubating the at least one mTOT protein with a competitor compound in the presence of the candidate compound to produce a test combination;
 - (c) incubating the at least one mTOT protein with said competitor compound in the absence of the candidate compound to produce a corresponding control combination;
 - (d) measuring the amount of the competitor compound bound to the mTOT protein in the test combination and in the control combination; and
 - (e) selecting as a lead candidate compound any candidate compound that causes a measurable decrease in the amount of competitor compound bound to the mTOT protein measured in step (d) in the test combination relative to the control combination. In some embodiments, the method further includes repeating steps (b)-(d) in a high throughput screen.
- [0015]** In another aspect, the present invention provides screening assays that are capable of identifying a lead candidate compound. In some embodiments, further validation of the lead candidate compound as a therapeutic active agent can be achieved by performing an activity assay that demonstrates the lead candidate compound's ability to mimic the insulin sensitizing activity of a thiazolidinedione compound, for example, a PPAR γ -sparring thiazolidinedione compound. In some aspects, the activity assay enables the determination whether the lead candidate compound has insulin sensitizing activity or anti-diabetic, anti-obesity, metabolic protective, or neuroprotective activity.

[0016] Further areas of applicability will become apparent from the description provided herein. The description and specific examples in this summary are intended for purposes of illustration only and are not intended to limit the scope of the present disclosure.

DRAWINGS

[0017] The drawings described herein are for illustrative purposes only of selected embodiments and not all possible implementations, and are not intended to limit the scope of the present disclosure.

[0018] Figure 1 represents a chemical structure of mitoglitazone in accordance with the embodiments of the present invention.

[0019] Figure 2 depicts a photograph of a Western Blot of brown adipose cell lysate after incubation of the cells with varying concentrations of mitoglitazone and probing for BP44 protein.

[0020] Figures 3A & 3B depict an alignment of amino acid sequences of BR44 derived from different organisms.

[0021] Figures 4A& 4B depict consensus sequences between BP44, BRP44-Like protein and homology with a UPF0041 domain sequence.

[0022] Figure 5 depicts the sequencing results of BP44 protein used in the transfection assays.. The optimized BP44-His₆ gene was excised from the DNA2.0 cloning and ligated into the BamHI/NotI sites of the expression vector pcDNA3.1. Correct clones were selected by restriction analysis and the sequence was confirmed by DNA sequence analysis by ACGT, Inc.

[0023] Figure 6 depicts the sequencing results of BRP44-Like protein used in the transfection assays. The optimized MTOT-Like-His₆ gene was excised from the DNA2.0 cloning vector pJ221 with BamHI and NotI and ligated into the BamHI/NotI sites of the expression vector pcDNA3.1. Correct clones were selected by restriction analysis and the sequence was confirmed by DNA sequence analysis by ACGT, Inc.

[0024] Figure 7 depicts a Western blot indicating the relative sizes of the cloned BP44, BP44-His₆, BRP44-Like and BRP44-Like His₆ sequences cloned and expressed in HEK293 cells using antibodies to BP44, BRP44-Like and Hex-His.

[0025] Figure 8 depicts the chemical structure of a thiazolidinedione labeled with a 3H radionuclide for use in various competitive binding assays and a non-thiazolidinedione compound also known to bind to mTOT proteins.

[0026] Figure 9 depicts an autoradiography film indicate the specifically labeled native mTOT (~14 kDa) from the wild type HEK293 P2 fraction. The mTOT (~14 kDa) and mTOT

6-His(~15.6 kDa) indicated by the two arrows in the P2 fraction from the transiently transfected HEK 293 cells show the increased size of the expressed his-tagged protein is specifically crosslinked. FIG. 9B shows the chemical structure of the photoaffinity crosslinker, ¹²⁵I-MSDC-1101. FIG. 9C Autoradiography film showing the crosslinking of rat liver P2 membranes. The arrow indicates the specifically crosslinked mTOT protein in the control DMSO treated membranes (lane 1), and the displacement of the crosslinker with either 25 μM MSDC-160 (lane 2) or 25 μM UK5099 (lane 3).

DETAILED DESCRIPTION

[0027] The following description of technology is merely exemplary in nature of the subject matter, manufacture and use of one or more inventions, and is not intended to limit the scope, application, or uses of any specific invention claimed in this application or in such other applications as may be filed claiming priority to this application, or patents issuing therefrom. The following definitions and non-limiting guidelines must be considered in reviewing the description of the technology set forth herein.

[0028] The headings (such as "Introduction" and "Summary") and sub-headings used herein are intended only for general organization of topics within the present invention, and are not intended to limit the disclosure of the present invention or any aspect thereof. In particular, subject matter disclosed in the "Introduction" may include novel technology and may not constitute a recitation of prior art. Subject matter disclosed in the "Summary" is not an exhaustive or complete disclosure of the entire scope of the technology or any embodiments thereof. Classification or discussion of a material within a section of this specification as having a particular utility is made for convenience, and no inference should be drawn that the material must necessarily or solely function in accordance with its classification herein when it is used in any given composition.

[0029] The citation of references herein does not constitute an admission that those references are prior art or have any relevance to the patentability of the technology disclosed herein. Any discussion of the content of references cited in the Introduction is intended merely to provide a general summary of assertions made by the authors of the references, and does not constitute an admission as to the accuracy of the content of such references. All references cited in the "Description" section of this specification are hereby incorporated by reference in their entirety.

[0030] The description and specific examples, while indicating embodiments of the technology, are intended for purposes of illustration only and are not intended to limit the scope of the technology. Moreover, recitation of multiple embodiments having stated

features is not intended to exclude other embodiments having additional features, or other embodiments incorporating different combinations of the stated features. Specific examples are provided for illustrative purposes of how to make and use the compositions and methods of this technology and, unless explicitly stated otherwise, are not intended to be a representation that given embodiments of this technology have, or have not, been made or tested.

[0031] As used herein, the words "preferred" and "preferably" refer to embodiments of the technology that afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

[0032] As referred to herein, all compositional percentages are by weight of the total composition, unless otherwise specified. As used herein, the word "include" and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the materials, compositions, devices, and methods of this technology. Similarly, the terms "can" and "may" and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present invention that do not contain those elements or features.

[0033] Disclosure of values and ranges of values for specific parameters (such as temperatures, molecular weights, weight percentages, etc.) are not exclusive of other values and ranges of values useful herein. It is envisioned that two or more specific exemplified values for a given parameter may define endpoints for a range of values that may be claimed for the parameter. For example, if Parameter X is exemplified herein to have value A and also exemplified to have value Z, it is envisioned that parameter X may have a range of values from about A to about Z. Similarly, it is envisioned that disclosure of two or more ranges of values for a parameter (whether such ranges are nested, overlapping or distinct) subsume all possible combination of ranges for the value that might be claimed using endpoints of the disclosed ranges. For example, if parameter X is exemplified herein to have values in the range of 1 – 10, or 2 – 9, or 3 – 8, it is also envisioned that Parameter X may have other ranges of values including 1 – 9, 1 – 8, 1 – 3, 1 - 2, 2 – 10, 2 – 8, 2 – 3, 3 – 10, and 3 – 9.

[0034] Although the open-ended term "comprising" as a synonym of terms such as including, containing, or having, is used herein to describe and claim the present invention, the invention, or embodiments thereof, may alternatively be described using more limiting terms such as "consisting of" or "consisting essentially of" the recited ingredients.

[0035] The following publications provide conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, peptide synthesis in solution, solid phase peptide synthesis, and biochemistry. Such procedures are described, for example, in the following texts that are incorporated by reference herein in their entireties:

[0036] 1. Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;

[0037] 2. *DNA Cloning: A Practical Approach*, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;

[0038] 3. *Oligonucleotide Synthesis: A Practical Approach* (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp 1-22; Atkinson et al., pp 35-81; Sproat et al., pp 83-115; and Wu et al., pp 135-151;

[0039] 4. *Animal Cell Culture: Practical Approach*, Third Edition (John R. W. Masters, ed., 2000), ISBN 0199637970, whole of text;

[0040] 5. J. F. Ramalho Ortigao, "The Chemistry of Peptide Synthesis" In: Knowledge database of Access to Virtual Laboratory website (Interactiva, Germany);

[0041] 6. Merrifield, R. B. (1963). *J. Am. Chem. Soc.* 85, 2149-2154.

[0042] 7. Barany, G. and Merrifield, R. B. (1979) in *The Peptides* (Gross, E. and Meienhofer, J. eds.), vol. 2, pp. 1-284, Academic Press, New York.

[0043] 8. Bodanszky, M. & Bodanszky, A. (1984) *The Practice of Peptide Synthesis*, Springer-Verlag, Heidelberg; and

[0044] 9. Energy Transfer Assays for the Discovery of Inhibitors of Estrogen Receptor-Coactivator Binding. Jillian R. Gunther, Yuhong Du, Eric Rhoden, Iestyn Lewis, Brian Revennaugh, Terry W. Moore, Sung Hoon Kim, Raymond Dingledine, Haian Fu, and John A. Katzenellenbogen *J Biomol Screen*, 2009; 14: 181 - 193.

[0045] The present invention provides assays for screening or identifying candidate compounds including, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides, small organic molecules, genes, gene products, drugs and other active molecules capable of binding to an mTOT protein. Identification of a candidate compound which specifically binds to an mTOT protein is indicative that the candidate compound is a potential therapeutic active agent which may be useful in the treatment of metabolic and

insulin resistance diseases or disorders. The term "insulin resistance" refers to a state in which a normal amount of insulin produces a subnormal biologic response relative to the biological response in a subject that does not have insulin resistance.

[0046] An "insulin resistance disorder," as discussed herein, refers to any disease or condition that is caused by or contributed to by insulin resistance. Examples of insulin resistance disease or disorders include: diabetes mellitus, obesity (obesity can include individuals having a body mass index (BMI) of at least 25 or greater. Obesity may or may not be associated with insulin resistance), weight gain, metabolic syndrome, insulin-resistance syndromes, syndrome X, insulin resistance, high blood pressure, hypertension, high blood cholesterol, dyslipidemia, hyperlipidemia, dyslipidemia, hyperglycemia, hyperinsulinemia and/or hyperproinsulinemia, impaired glucose tolerance, delayed insulin release, diabetic complications, including cognitive functions in dementia, retinopathy, peripheral neuropathy, nephropathy, glomerulonephritis, glomerulosclerosis, nephrotic syndrome, hypertensive nephrosclerosis some types of cancer (such as endometrial, breast, prostate, and colon), complications of pregnancy, poor female reproductive health (such as menstrual irregularities, infertility, irregular ovulation, polycystic ovarian syndrome (PCOS)), lipodystrophy, cholesterol related disorders, such as gallstones, cholecystitis and cholelithiasis, gout, obstructive sleep apnea and respiratory problems, osteoarthritis, and bone loss, e.g. osteoporosis.), metabolic or inflammation mediated diseases (e.g., dyslipidemia, central obesity, rheumatoid arthritis, lupus, myasthenia gravis, vasculitis, Chronic Obstructive Pulmonary Disease (COPD), or inflammatory bowel disease), cardiovascular disease (e.g. atherosclerosis, arteriosclerosis, angina pectoris, coronary artery disease, congestive heart failure, stroke, or myocardial infarction) as well as neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Candidate compounds that may be identifiable using the assays provided herein may also be useful for treating or preventing metabolic diseases such as diabetes or obesity. Moreover, candidate compounds may also be useful in co-therapies directed to the treatment of any of these diseases.

[0047] In an illustrative embodiment, a screening method to identify a lead candidate compound of the present invention includes the steps:

a) combining an mTOT protein with one or more candidate compounds under conditions operable to allow specific binding between the mTOT protein and the one or more candidate compounds, and

b) detecting specific binding, thereby identifying one or more candidate compounds which specifically bind to the mTOT protein.

[0048] The new screening methods described herein are predicated, at least in part, on the unexpected discovery that mitoglitazone a PPAR γ -sparing thiazolidinedione compound, being effective in increasing the sensitivity of insulin, specifically binds to and has an affinity for an mTOT protein, for example, Brain Protein 44 (BP44), Brain R Protein 44-like (BRP44-Like) protein and a heterodimer of these two proteins (BP44-BRP44-Like).

[0049] **Mitoglitazones**

[0050] Mitoglitazone is a PPAR γ -sparing thiazolidinedione shown to effectively stimulate brown adipose tissue ("BAT") stores, and is useful for treating obesity and other metabolic diseases such as diabetes mellitus. A formula for mitoglitazone is shown in Figure 1.

[0051] **I. mTOT PROTEINS**

[0052] As used herein, an mTOT protein includes any mitochondrial protein that is capable of specifically binding to a thiazolidinedione compound. In some embodiments, an mTOT protein is any mitochondrial protein that is capable of specifically binding to a thiazolidinedione compound, for example mitoglitazone, pioglitazone, rosiglitazone and troglitazone. In some embodiments, an mTOT protein is any mitochondrial protein that is capable of specifically binding to PPAR γ -sparing thiazolidinedione compounds, for example mitoglitazone. In some embodiments, an mTOT protein is any mitochondrial protein that is capable of specifically binding to a non-PPAR γ -sparing thiazolidinedione compound, for example rosiglitazone, troglitazone or pioglitazone. In some embodiments, an mTOT protein is any mitochondrial protein that is capable of specifically binding to only PPAR γ -sparing thiazolidinedione compounds, for example mitoglitazone, but not capable of binding to non-PPAR γ -sparing thiazolidinedione compounds. Illustrative examples of mTOT proteins include BP44 protein, BRP44-Like protein and heterodimer proteins comprising both BP44 and BRP44-Like proteins. In some embodiments, the mTOT protein is a prokaryotic protein, or a eukaryotic protein, for example a drosophila protein, a yeast protein, a mammalian protein, a human protein. In some embodiments, the mTOT protein is a human mitochondrial protein, for example, a human BP44 protein, a human BRP44-Like protein or a heterodimer protein comprising both human BP44 and human BRP44-Like proteins. mTOT proteins for use in the screening assays described herein, also include orthologs, homologs, functional fragments of mTOT proteins, or fusion proteins comprising the mTOT proteins exemplified herein. As used herein mTOT protein or mTOT protein-Like refer to BP44 and BRP44-Like proteins respectively.

[0053] **A. Brain Protein 44 (BP44)**

[0054] Brain protein 44 is also known as CGI-129, dJ68L15.3, DKFZp564B167,

MGC125752, MGC125753, BRP44, has been previously isolated from brain tissue and other tissue sources. As used herein, BP44, BRP44, BP44 protein and BP44 peptide are synonyms and are used interchangeably. The term BP44 protein refers to a protein that is a target for the PPAR γ -sparing thiazolidinediones (e.g., mitoglitazone). BP44 protein can range in size from about 200 amino acids to about 50 amino acids, or from about 150 amino acids to about 50 amino acids or from about 125 amino acids to about 50 amino acids, or from about 200 amino acids to about 75 amino acids, or from about 200 amino acids to about 100 amino acids or from about 200 amino acids to about 125 amino acids. BP44 can include illustrative embodiments wherein BP44 include proteins having 127 or 105 amino acids. In some illustrative examples of BP44 proteins, the BP44 proteins share a putative conserved domain called the UPF0041 domain as set forth at least in part as shown in SEQ ID NO:41. In some embodiments, the theoretical pI of the BP44 protein is 9.67 and has a calculated molecular weight of 12,347 Daltons.

[0055] As used herein, accession numbers accompanying examples of BP44 and BRP44-Like proteins are derived from the National Center For Biotechnology Information - NCBI (USA). The BP44 protein (protein and polypeptide both refer to a polymer of amino acids and are used interchangeably herein) used in the various assays of the present invention can include natural or chemically synthesized BP44 protein, full length, substantially full-length, homologs, orthologs, functionally equivalent form of the BP44 protein, or mutant forms of BP44. Exemplary BP44 proteins are provided in Tables 1, 3 and 4 and shown in Figures 3A, 3B, 4A and 4B. As used herein, orthologs, homologs, functional fragments, or mutant forms of BP44 refer to proteins having at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 92%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least 100%, identity to, or homologous with the BP44 proteins provided in or encoded by any illustrative BP44 proteins or nucleic acids described in Tables 1-4 and shown in Figures 3A, 3B, 4A and 4B. Alternatively, the orthologs, homologs, functional fragments, or mutant forms of BP44 can be a truncated polypeptide or a polypeptide with one or more internal deletions. In some embodiments, BP44 protein useful in the present invention is derived from a source, exemplified, but not limited to, those proteins having amino acid sequences provided in Tables 1, 3 or 4 and shown in Figures 3A, 3B, 4A and 4B. In some embodiments, the BP44 protein is a human BP44 protein described herein having the amino acid sequence set forth in any one or more of SEQ ID NOs: 1-4, functional fragments or variants of SEQ ID NOs: 1-4, allelic variants of SEQ ID NOs: 1-4, species variants of SEQ ID NOs: 1-4, or homologs of SEQ ID NOs: 1-4. In one

embodiment, the BP44 protein is a human BP44 protein described herein linked to a His6 tag, having the amino acid sequence set forth in any one or more of SEQ ID NO: 75.

[0056] In some embodiments, BP44 proteins useful in the present invention can include proteins that may be encoded by the polynucleotides of SEQ ID NOs: 5 - 7, and 74 and BP44 proteins encoded by a nucleic acid sequence that hybridizes to the BP44 coding region of a nucleic acid sequence in SEQ ID NOs: 5 - 7 or complementary sequences thereof under "stringent hybridization conditions" as is defined herein and commonly used in the art of molecular biology, for example, in: Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridisation with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993).

[0057] As used herein, a "homolog" of a first gene (for example, a human BRP44 encoding gene as disclosed in SEQ ID NOs: 5 -7) generally refers to a second, different gene that is substantially identical to the first gene, or that encodes a gene product that is substantially identical (i.e. having a % identity greater than 90%) to the gene product encoded by the first gene. An "ortholog" of a first gene refers to a second gene from a different organism that is substantially identical to the first gene, or that encodes a gene product that is substantially identical or substantially identical to the gene product encoded by the first gene.

[0058] In some embodiments, BP44 proteins that find utility in the assays of the present invention can include proteins or polypeptides having at least 50%, or at least 55%, or at least 60%, or at least 65%, or at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 92%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least 100%, identity to, or homologous with the BP44 protein having the amino acid sequence set forth in any one of SEQ ID NOs: 1-4 and 8-41, or any full-length BP44 protein described herein. In another illustrative example, BP44 proteins comprising truncated forms of BP44 having at least 50%, or at least 55%, or at least 60%, or at least 65%, or at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90% sequence identity or homology to any one of SEQ ID NOs: 1-4, 8-41 and 75 are contemplated herein. In some embodiments, useful truncated forms comprise functional regions in their amino acid sequence when compared to the full length BP44 protein since they can be functionally identified using the mitochondrial membrane competitive binding crosslinking assay described in Example 1, or can be proven to be functional by directly binding to an unlabeled or labeled mitoglitazone or rosiglitazone compound or analog in vitro or in vivo as exemplified, for example, in the Examples described herein. In some embodiments, an mTOT protein can also include a functional region of the BP44 protein. In

some embodiments, an mTOT protein can include a mitochondrial protein having a conserved domain called the UPF0041 domain. In some embodiments, illustrative examples of proteins containing a conserved domain called the UPF0041 domain are provided in a family of proteins called pfam03650, which are part of Super Family Accession No: cl04196. A representative UPF0041 domain is provided in SEQ ID NO: 41 or a protein which contains the consensus sequence of FIG. 4B.

[0059] Exemplary nucleic acids encoding illustrative BP44 proteins are provided in Table 2 and in Example 3.

[0060] In some embodiments, an illustrative BP44 protein includes an amino acid sequence derived from non-human organisms as described in Table 3 and FIG. 3A & 3B. In some embodiments, BP44 proteins include homolog or ortholog proteins of BP44 that belong to the protein family UPF0041 having an identifier: pfam03650 illustratively shown in Table 4. In some embodiments, illustrative BP44 proteins can include proteins which includes within their amino acid sequence, a domain of pfam03650 having an amino acid sequence comprising:

KTVHFWAPTLKWGLVFAGLNDIKRPVEKVSGAQNLSELLATALIWTRWSFVIKPKNY
LLASVNFFLGCTAGYHLTRIA (SEQ ID NO: 41) or an amino sequences comprising at least 70 %, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99 % sequence identity to SEQ ID NO: 41. In some embodiments, the BP44 protein is a protein having a consensus amino acid sequence: $FW_xPx_3WGLx_2Ax_3DMx_4(E/D)x_2Sx_6Lx_8Rx_6P(K/R)Nx_2LxAx_7Ax_3Qx_2Rx_9Rx_{(9-21)}Ax_A$ wherein x is any of the known 20 amino acids.

[0061] TABLE 1. Amino acid sequence of human BP44 protein.

SEQ ID NO:	BP44 Human Protein Sequence
1	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FVGA
2	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FVGAAGASQ LFRIWRYNQE LKAKAHK

SEQ ID NO:	BP44 Human Protein Sequence
3	MSAAGARGLRATYHRLLDKVELMLPEKLRPLYNHPAGPRTVFFWAPIM KWGLVCAGLADM ARPAEKLSTAQSAVLMATGFIWLRYSLVIIIPKNWSLFAVNFFVGAAGAS QLFRIWRYNQE LKAKAHK
4	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATDI TKN

[0062] TABLE 2. Exemplary nucleotide sequence encoding human BP44 protein.

SEQ ID NO:	BP44 Nucleotide Sequence
5	1 acagcgggca gggaaagccg cgggaagggt actccaggcg agaggcggac gcgagtcgtc 61 gtggcaggaa aagtgactag ctccccttcg ttgtcagcca gggacgagaa cacagccacg 121 ctcccacccg gctgccaacg atccctcggc ggcgatgtcg gccgccggtg cccgaggcct 181 gcggggccacc taccaccggc tctcgataa agtggagctg atgtgcccc agaaattgag 241 gccgtgttac aaccatccag caggtcccag aacagttttc ttctgggctc caattatgaa 301 atggggggtg gtgtgtgctg gattggctga tatggccaga cctgcagaaa aacttagcac 361 agctcaatct gctgttttga tggctacagg gtttatttgg tcaagatact cacttgtaat 421 tattccaaaa aattggagtc tgttgctgt taatttctt gtgggggcag caggagcctc 481 tcagctttt cgtatttga gatataacca agaactaaaa gctaaagcac acaaataaaa 541 gagttcctga tcacctgaac aatctagatg tggacaaaac cattgggacc tagtttatta 601 ttggttatt gataaagcaa agctaactgt gtgttagaa ggcactgtaa ctggtagcta 661 gttcttgatt caatagaaaa atgcagcaaa ctttaataa cagtctctet acatgactta 721 aggaacttat ctatggatat tagtaacatt ttctacat ttgtccgtaa taaaccatac 781 ttgctcgtaa aaaaaaaaaa aaaaaaaaa

SEQ ID NO:	BP44 Nucleotide Sequence
6	1 ctcagcgct cgcggcggg gccccgctc acccaggtat cgactccga gccgggacgg 61 gtctccagc cggaggacc ttctctcac gtccacaac agccaggac gagaacacag 121 ccacgtccc accgggtgc caacgatcc tggcggcga tgcggccgc cgtgcccga 181 ggctgcggg ccacctacca cgggtctctc gataaagtgg agctgatgct gcccgagaaa 241 ttgaggccgt tgtacaacca tccagcaggt ccagaacag tttctctg ggctccaatt 301 atgaaatggg ggttggtgtg tgctggattg gctgatatgg ccagacctgc agaaaaactt 361 agcacagctc aatctgctgt ttgatggct acagggttta ttggtcaag atactcactt 421 gtaattattc caaaaaattg gagtctgtt gctgtaatt tcttgtggg ggcagcagga 481 gcctctcagc tttctgtat ttgagatat aaccaagaac taaaagctaa agcacacaaa 541 taaaagagtt cctgatcacc tgaacaatct agatgtggac aaaaccattg ggacctagtt 601 tattatttgg ttattgataa agcaaagcta actgtgtgt tagaaggcac tgtaactggt 661 agctagttct tgattcaata gaaaaatgca gcaaacttt aataacagtc tctctacatg 721 acttaaggaa ctatctatg gatattagta acattttct accattgtc cgtaataaac 781 catacttgct cgtaaaaaaa aaaaaaaaaa aaa
7	1 ctcagcgct cgcggcggg gccccgctc acccaggtat cgactccga gccgggacgg 61 gtctccagc cggaggacc ttctctcac gtccacaac agccaggac gagaacacag 121 ccacgtccc accgggtgc caacgatcc tggcggcga tgcggccgc cgtgcccga 181 ggctgcggg ccacctacca cgggtctctc gataaagtgg agctgatgct gcccgagaaa 241 ttgaggccgt tgtacaacca tccagcaggt ccagaacag tttctctg ggctccaatt 301 atgaaatggg ggttggtgtg tgctggattg gctgatatgg ccagacctgc agaaaaactt 361 agcacagctc aatctgctgt ttgatggct acagggttta ttggtcaag atactcactt 421 gtaattattc caaaaaattg gagtctgtt gctgtaatt tcttgtggg ggcagcagga 481 gcctctcagc tttctgtat ttgagatat aaccaagaac taaaagctaa agcacacaaa 541 taaaagagtt cctgatcacc tgaacaatct agatgtggac aaaaccattg ggacctagtt 601 tattatttgg ttattgataa agcaaagcta actgtgtgt tagaaggcac tgtaactggt 661 agctagttct tgattcaata gaaaaatgca gcaaacttt aataacagtc tctctacatg 721 acttaaggaa ctatctatg gatattagta acattttct accattgtc cgtaataaac 781 catacttgct cgtaaaaaaa aaaaaaaaaa aaa

[0063] Table 3. Additional Exemplary Protein Sequences of BP44.

SEQ ID NO:	Species	Accession No.	Amino Acid Sequence
8	<i>P. troglodytes</i>	XP 001174837	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYNQE LKAKAHK
9	<i>Equus caballus</i>	XP 001493863	MSAAGARGLR ATYHRVLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYNQE LKAKASK
10	<i>Canis familiaris</i>	XP 537209	MSAAGARGLR ATYHRVLDKV ELLPEKLRP LYNHPAGPKT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYNQE LGTKANK
11	<i>Bos taurus</i>	NP 001180050	MSAAGVRGLR ATYHRVLDKV ELLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGTAGASQ LFRIWRYNQE LKAKANK
12	<i>Mus musculus</i>	NP 081706	MAAAGARGLR ATYHRLMDKV ELLPPKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSTVLMATGF IWSRYSLVII PKNWSLFAVN FFVGSAGASQ LFRIWRYNQE LKSKGIQ

SEQ ID NO:	Species	Accession No.	Amino Acid Sequence
13	<i>Rattus norvegicus</i>	NP 001071111	MAAAGARGLR ATYHRLMDKV ELLPPKKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSTVLMATGF IWSRYSLVII PKNWSLFAVN FFVGSAGASQ LFRIWKYNQE LKSKGIQ
14	<i>Danio rerio</i>	AAH71315	MAMVGLRASY HRILDRMEHM LPAKLRPFYN HPAGPKTVFF WAPMFKWGLV LAGLADMARP AEKLSTSQSA VLTATGLIWS RYSLVIIIPKN WNLFAVNFFV GSAGGSQLYR IWMHNREQKA KEKEAQA
15	<i>M. mulatta</i>	XP 001103255	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGTAGASQ LFRIWRYNQE LKAKAHK
16	<i>Callithrix jacchus</i>	XP 002760466	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSTVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYKQE LKAEEHK
17	<i>Sus scrofa</i>	XP 001928682	MAAAGSTRAP SPPKPGRTRA RARLILSLSD YPEHPEISNS PLPRPPSNRR RPRRRKRPRL LPLLVLTYTLP SKPSREITTT LGTEAGGGRP VGGLGAGHRS VSAPSTFSGC GPAGSWKEVR QSYGKGTRRK RRARGVEAGE SDEVPFIVSQ GRARGHSPLT PTAHDHSSAE MSAAGARGLR ATYHRVLDKV ELLPPEKLRP LYNHPAGPRT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGTAGASQ LFRIWRYNQE LKAKANK

SEQ ID NO:	Species	Accession No.	Amino Acid Sequence
18	<i>Ailuropoda melanoleuca</i>	XP 002928153	MSAAGARGLR ATYHRVLDKV ELLLPEKLRP LYNHPAGPKT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYNQE LKAKANK
19	<i>Pongo abelii</i>	NP 001126868	MSAAGARGLR ATYHRLLDKV ELMLPEKLRP LYNHPAGPRT VFFWAPIMKR GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYNQE LKAKAHK
20	<i>Monodelphis domestica</i>	XP 001371657	MAAHGVRSR ATYHRFLDKV ELMLPEKLRP LYNHPAGPKT VFFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGL IWSRYSLVII PKNWSLFAVN FFVGAAGGSQ LFRIWRYQRE LKSKELQK
21	<i>Oryctolagus cuniculus</i>	XP 002712406	MSAASTRGLR AAYHRLLDKV ELMLPEKLRP LCNHLAGPRT VLFWAPIMKW GLVCAGLADM ARPAEKLSTA QSAVLMATGF IWSRYSLVII PKNWSLFAVN FFVGAAGASQ LFRIWRYNQE LKTKANQ
22	<i>Taeniopygia guttata</i>	XP 002192887	MAAAVAGLRA SYHRLLDRIE LMLPPRFRPF YNHPAGPKTV FFWAPVMKWG LVCAGLADMA RPAEKLSTGQ SAVLTATGLI WSRYSLVIIP KNWSLFAVNF FVGCAGGSQ FRIWRYNQEL KAQKQVQ
23	<i>Xenopus laevis</i>	NP 001079531	MAAAVGLRAS YHRALGRIEM MLPPKLRPIY NHPAGPKTVF FWAPIMKWGL VFAGLADMTR PADKLSTGQS AVLTATGLIW SRYSLVIIPK NWSLFAVNFF VGCAGGSQ FRIWRYNQELK AIDVQQEVKP

[0064] Table 4. Exemplary Functional Orthologs of BP44.

SEQ ID NO:	Species	Accession No	Amino Acid Sequence
24	<i>Saccharomyces cerevisiae</i> S288c (YHR162W)	NP 012032	MSTSSVRFAF RRFWQSETGP KTVHFWAPTL KWGLVFAGFS DMKRPVEKIS GAQNLSLLST ALIWTRWSFV IKPRNILLAS VNSFLCLTAG YQLGRIANYR IRNGDSISQL CSYILSGADE SKKEITTGR
25	<i>Saccharomyces cerevisiae</i> S288c (Fmp43p)	NP 011759	MSASAFNFAF RRFWNSETGP KTVHFWAPTL KWGLVFAGLN DIKRPVEKVS GAQNLSLLAT ALIWTRWSFV IKPKNYLLAS VNFFLGCTAG YHLTRIANFR IRNGDSFKQV IHYIHKGETP AAVAAKQTAS TSMNKG VIGT NPPITH
26	<i>Saccharomyces cerevisiae</i>	P53157	MSQPVQRAAA RSFLQKYINK ETLKYIFTTH FWGPVSNFGI PIAAIYDLKK DPTLISGPMT FALVTYSGVF MKYALSVSPK NYLLFGCHLI NETAQLAQGY RFLKYTYFTT DEEKKALDKE WKEKEKTGKQ
27	<i>Saccharomyces cerevisiae</i>	P38857	MSTSSVRFAF RRFWQSETGP KTVHFWAPTL KWGLVFAGFS DMKRPVEKIS GAQNLSLLST ALIWTRWSFV IKPRNILLAS VNSFLCLTAG YQLGRIANYR IRNGDSISQL CSYILSGADE SKKEITTGR
28	<i>Caenorhabditis elegans</i> (F53F10.3)	NP 491234	MGPHRLLYQA LCKAGDKFVY PVLPAFAKPA WNHAAGPKTV FFWAPTIKWT LIGAGLADLA RPADKLSLYQ NSALFATGAI WTRYCLVITP INYYLSSVNF FVMCTGLAQL CRIAHYRYQN PDWETKEIME THN
29	<i>Arabidopsis thaliana</i> (AT4G22310)	NP 193962	MATSKLQAIW NHPAGPKTIH FWAPTFKWGI SIANIADFAK PPEKLSYPQQ IAVTCTGVIW SRYSMVINPK NWNLF SVNVA MAGTGIYQLA RKIKHDYATE AEP AVASE

SEQ ID NO:	Species	Accession No	Amino Acid Sequence
30	<i>Drosophila melanogaster</i> (CG9396)	NP 649912	MSATPPTTPA PTAAASAGKG LHSRAYNGLI KACDKYVPPK MRPLWMHPAG PKTIFFWAPI VKWSLVIAGL SDLTRPADKI SPNGCLALGA TNLIWTRYSL VIIPKNYSLF AVNLFVSLTQ LFQLGRYYNY QWEQSRLEKN GEQCPAIEAS
31	<i>Drosophila melanogaster</i> (CG9399), isoform A	NP 649913	MSSTAVQPPP PVPPPPPSAV PSAGKGIHSK LYNMGIGAAD KFVPAKLRPL WMHPAGPKTI FFWAPVFKWG LVAAGLSDLA RPADTISVSG CAALAATGII WSRYSLVIIP KNYSLFAVNL FVGITQVVQL ARAYHYHQSQ EKLKQEQQQP AVQN
32	<i>Drosophila melanogaster</i> CG9399, isoform C	NP 001163571	MSSTAVQPPP PVPPPPPSAV PSAGKGIHSK LYNMGIGAAD KFVPAKLRPL WMHPAGPKTI FFWAPVFKWG LVAAGLSDLA RPADTISVSG CAALAATGII WSRYSLVIIP KNYSLFAVNL FVGITQVVQL ARAYHYHQSQ EKLKQEQQQP AVQN
33	<i>Drosophila melanogaster</i> CG9399, isoform B	NP 731376	MSSTAVQPPP PVPPPPPSAV PSAGKGIHSK LYNMGIGAAD KFVPAKLRPL WMHPAGPKTI FFWAPVFKWG LVAAGLSDLA RPADTISVSG CAALAATGII WSRYSLVIIP KNYSLFAVNL FVGITQVVQL ARAYHYHQSQ EKLKQEQQQP AVQN
34	<i>Oryza sativa Japonica</i> (Os07g0449100)	NP 001059546	MASKLQAFWN HPAGPKTIHF WAPTFKWGIS IANVADFAKP PEKISYPQQV AVACTGVIWS RYSMVITPKN WNLFSNVAM AGTGLYQLSR KIRKDYFSDE KDAAASLEG
35	<i>Neurospora crassa</i> (OR74A)	XP 330591	MAPMNNFFRA ARPVFRNTFF TSNAARNGGK RFQSTASSEQ AQESFFKRMW NSPIGFKTVH FWAPVMKWGL VLAGISDFAR PAEKLSLTQN AALTATGLIW TRWCLIIKPK NYLLAAVNFF LGIVGVVQVS RILMWQQSQK NKTLPAAAE VKENVKEEVK KVVKA

SEQ ID NO:	Species	Accession No	Amino Acid Sequence
36	<i>Chlamydomonas reinhardtii</i>	XP 001702478	MTIAQTLAAF WNHPAGPKTI HFWAPTFKWG ISLANIADIN RPADKISLPQ QCAITATGVI WSRYSTQITP VNYNLLAVNA FMAVTGGYQL FRKIT
37	<i>Schistosoma mansoni</i>	XP 002570126	MSAVYRALIG AVDKKVPARL KPLWNHPAGP KTIFFWAPTF KWLLVIAGLA DINRPVENVS LYQSTALAAT GLIWSRYSLV IIPKNYNLLS VNAFVALTGL YQLARIARYE SSK
38	<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21	XP 566510	MSAPLGNAAA QQTTSKFQAF LNHPAGPKTI FFWAPIAKWG LVLAGVKDLS RPAEKLSVSQNALAATGFI WVRYSFVITP VNYSLA AVNF FVGSTGVAQL YRVWDYRRKN PGAVSSA
39	<i>Schizosaccharomyces pombe</i>	Q09896	MFRAGFKRFW NHPAGPKTVH FWAPAMKWT LVLGIGDYAR SPEYLSIRQY AALCATGAIW TRWSLIVRPK NYFNATVNFF LAIVGAVQVS RILVYQRQQK RITAQSEQRT ELARSLAA
40	<i>Caenorhabditis elegans</i> (R07E5.13)	Q21828	MSRVISKVTT YFKQHSTA EW KHYFLSTHFW GPVANWGLPL AALGDLKKNP DMISGPM TSA LLIYSSVFMR FAWHVQPRNL LLFACHFANF SAQGAQLGRF VNHNYLHYVE DPVHHKLMMK KEVLEHEHDA ERVSLFLWYP FFEFLGNFRK NKLNKNKFMI

[0065] In some embodiments, the BP44 protein useful for the assays described herein can be obtained commercially from translatable DNA form from Abnova (BRP44 (Accession No. NP 056230, 1-127 amino acids, full-length recombinant protein with GST tag) Cat. No. H00025874-P01, Abnova, Taipei City, Taiwan)). In addition, human BP44 can be recombinantly produced using Invitrogen's Gene Clone IOH41193 (Invitrogen Corp, Carlsbad, CA USA) using the manufacturer's instructions.

[0066] In some embodiments, the BP44 protein useful for the assays described herein can be obtained commercially from Abnova (BRP44 (Accession No. NP 056230, 1-127 amino acids, full-length recombinant protein with GST tag) Cat. No. H00025874-P01). Antibodies to human and mouse BP44 are commercially available from Novus (Clone (H00025874-

M12, Novus, Littleton CO, USA) and Abnova (Clone H00025874-M11, Abnova, Walnut, CA, USA).

[0067] In some embodiments, the BP44 protein for use in the assays described herein, can include proteins and polypeptides having protein sequences comprising at least 70 %, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99 % sequence identity to SEQ ID NOs: 1-4, 8-41 and 75.

[0068] **B. BRP44-Like proteins**

[0069] BRP44-Like proteins can be used as a binding target in the assays described herein alone and/or with BP44, or as part of a heterodimer with BP44. In some embodiments, representative BRP44-Like proteins have an amino acid sequence comprising or consisting of those provided in Table 5.

[0070] Table 5. Exemplary Amino Acid Sequences of BRP44 Proteins.

SEQ ID NO:	Species	NCBI Accession No	Amino Acid Sequence
42	Human	NP 057182	MAGALVRKAA DYVRSKDFRD YLMSTHFWGP VANWGLPIAA INDMKKSPEI ISGRMTFALC CYSLTFMRFA YKVQPRNWLL FACHATNEVA QLIQGGRLIK HEMTKTASA
43	<i>Mus Musculus</i>	XP 003086084	MAGALVRKAA DYVRSKDFRD YLMSTHFWGP VANWGLPIAA INDMKKSPEI ISGRMTFALC CYSLTFMRFA YKVQPRNWLL FACHVTNEVA QLIQGGRLIN YEMSKRPSA
44	<i>Pan troglodytes</i>	XP 001137474	MAGALVRKAA DYVRSKDFRD YLMSTHFWGP VANWGLPIAA INDMKKSPEI ISGRMTFALC CYSLTFMRFA YKVQPRNWLL FACHATNEVA QLIQGGRLIK HEMTKKASA
45	<i>Canis lupus familiaris,</i>	XP 851085	MAGALVRKTA DYVRSKDFRD YLMSTHFWGP VANWGLPIAA INDMKKSPEI ISGRMTFALC CYSLTFMRFA YKVQPRNWLL FACHATNEVA QLIQGGRLIK HEMSKKASA
46	<i>Drosophila melanogaster</i>	NP 650762	MSIRRAMSTT ASKEWRDYFM STHFWGPVAN WGIPVAALAD TQKSPKFISG KMTLALTLYS CIFMRFA YKV QPRNWLLFAC HATNATAQSI QGLRFLHYN GSKEQQA

SEQ ID NO:	Species	NCBI Accession No	Amino Acid Sequence
47	Human	CAI19655	MKKSPEIISG RMTFALCCYS LTFMRFA YKV QPRNWLLFAC HATNEVAQLI QGGRLIKHEM TKTASA
48	<i>Bos taurus</i>	NP 001070510	MAGALVRKAA DYVRSKDFRD YLMSTHFWGP VANWGLPIAA INDMKKSPEI ISGRMTFALC CYSLTFMRFA YKVQPRNWLL FACHATNEVA QLIQGGRLIR HEMSKKASA
49	<i>Gallus gallus</i>	CAG32383	MAGALARKAA DYVRSKEFRD YLMSTHFWGP VANWGLPVAA INDMKKSPEI ISGRMTFALC CYSLTFMRFA YKVQPRNWLL FACHLTNEVA QLIQGGRLIK YRLEKKN
50	<i>Aedes aegypti</i>	XP 001656098	MAAAMGRKLI DSLKSKEFRE YLMSTHFWGP VANWGIPIAA LADIKKDPKI ISGTMTTALC LYSLVFMRF A WKVTPRNMLL FGCHITNFTA QSIQGARCLE YNYLGGNKRA QQQQQQPSDS PAEQQKH
51	<i>Schistosoma japonicum</i>	AAW26690	MKVLNYLRRK EFRDYITSTH FWGPLANWGL PLAALGDLKN NPEKISGKMT TALMFYSLAF MRFAYLVQPR NMLLFACHLA NETAQSFQMV RYCNYWYMKS ESERDEIRKK FTL

[0071] Representative nucleic acids encoding BPR44-Like proteins are provided in Table 6.

[0072] Table6. Exemplary nucleotide sequences encoding human BRP44-Like proteins.

SEQ ID NO:	BPR44-Like Nucleotide Sequence
52	NM_016098.2 1 gtcgtgaggc gggccttcgg gctggctcgc cgtcggctgc cgggggggtg gccgggggtg 61 cattggctct gggaagcggc agcagaggca gggaccactc ggggtctggt gtcggcacag 121 ccatggcggg cgcgttggtg cggaaagcgg cggactatgt ccgaagcaag gattccggg 181 actacctcat gactacgcac ttctggggcc cagtagccaa ctggggtctt cccattgctg 241 ccatcaatga tatgaaaag tctccagaga ttatcagtgg gcggatgaca ttgccctct 301 gttgctattc ttgacattc atgagatttg cctacaaggt acagcctcgg aactggcttc 361 tgtttgcatt ccacgcaaca aatgaagtag ccagctcat ccaggaggag cggttatca 421 aacacgagat gactaaaacg gcatctgcat aacaatggaa aaggaagaac aaggtcttga 481 agggacagca ttgccagctg ctgctgagtc acagatttca ttataaatag cctccctaag 541 gaaatacac tgaatgctat ttctactaac cattctattt ttatagaaat agctgagagt 601 ttctaaacca actctctgct gcctacaag tattaaatat ttacttctt tccataaaga 661 gtagtcaaaa atatgcaatt aatttaataa ttctgatga tggttttatc tgcagtaata 721 tgtatatcat ctattagaat ttacttaatg aaaaactgaa gagaacaaaa ttgtaacca 781 ctagcactta agtactctg attcttaaca ttgtctttaa tgaccacaag acaaccaaca 841 gctggccacg tacttaaaat ttgtcccca ctgttataaa atgttacctg tgtattcca 901 tgcagtgtat atattgagat gctgtaactt aatggcaata aatgatttaa atatttgta 961

SEQ ID NO:	BPR44-Like Nucleotide Sequence
53	1 cgccggtttg caggatctca ccgtctctgt gcagccgccc gcggggcaaa gggacaggcg 61 aggggtgaca ggagccccgac ctgcccacca cgccccgctg agaccccgga agcctgccc 121 ggacccccgg gcagaggagt gtcacctcg cagcccctag caaccgccag tcgtccgggc 181 cgccggggccc tttagcagc gcagccccgc ggcgcgcacc gccccgaggg caggcggggg 241 gtgtgtcgac cgcggcagcc aatccccggac gcgtctgctg gtctagcgcg ttctcattgg 301 tggagcgccg agcggctgta aggtctctcc gactgtctg aggcggggcg tggggtgaga 361 gcgccgaggg ctcagccga gtccacagcg gtgtcatctg tctaggtagc ggcttcaccg 421 ccaacgccac ggccatggct ggagcgctgg tgcgcaaagc ggcggactat gtccggagca 481 aggacttccg ggactatctc atgagtaagc acttctgggg cccagttgcc aactggggtc 541 tccccattgc tgctatcaat gacatgaaga aatctccaga gattatcagt gggcggatga 601 ctttgccect ctgttctat ttctgacat tcatgagatt tgcctacaag gtacaacctc 661 gaaactggct ttgtttgca tgccatgtaa caaacgaagt agctcagtc attcaggag 721 gacgacttat caactacgag atgagtaagc ggccatctgc atagcagtac aaggaccagc 781 tcttgaaaga gacagtgtc cagccactgc tgcagccaca gatcatgtca gcatgagtag 841 tcgtgtgaa gggaaaacac agaattgctat ctaaatgacc atgccaacat tattgaatag 901 ccgagagtcc ctaaacccac ttctgtctgc ctatcaatg ctaaacctta ttgtcttca 961 tcaagagtag tcaaaaatat gcaactaatt taataattt gaatgatggt ttatctata 1021 gcaatctgta gtaatatgta tattatctat tgggatttgt gtaataaaaa atctaaggga 1081 acaaaaattt ataactacaa gcacttaagt actcaaaatt ctgactttt tctttaatga 1141 caatagtaaa ccctcagttg gtcacatgtc tacacataat ttccagtgat aacaagtatc 1201 ggtgttttcc atatgtaact cagatctgta acttaatggc aataaatggt ttaaatattt 1261 gctatttga

[0073] The BRP44-Like protein used in the various assays of the present invention can include natural or chemically synthesized BRP44-Like protein, full length, substantially full-length, homologs, orthologs, functionally equivalent form of the BRP44-Like protein, mutant forms of BRP44-Like or fusion constructs comprising BRP44-Like protein and a second protein, for example a different mTOT protein or a protein tag, for example His₆. Exemplary BRP44-Like proteins are provided in Table 5 and in SEQ ID NO: 99. As used herein, exemplary forms of BRP44-Like proteins refer to proteins having at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 92%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least 99%, identity to, or homologous with the BRP44-Like proteins provided in Table 5, or SEQ ID

NO: 99 or encoded by any examples described in Table 6. Alternatively, the mutant BRP44-Like protein can be a truncated polypeptide or a polypeptide with one or more internal deletions. In some embodiments, BRP44-Like protein useful in the present invention is derived from a human source, exemplified, but not limited to those proteins having amino acid sequences provided in Table 1. In some embodiments, the BRP44-Like protein is a human BRP44-Like protein described herein having the amino acid sequence set forth in any one or more of SEQ ID NOs: 42 and 47, functional fragments or variants of SEQ ID NOs: 42 and 47 allelic variants of SEQ ID NOs: 42 and 47, species variants of SEQ ID NOs: 42 and 47, or homologs or orthologs of SEQ ID NOs: 42 and 47, or a fusion construct encoded by the nucleotide sequence of SEQ ID NO: 79. In some embodiments, BRP44-Like protein (NCBI Accession - NM 016098) is commercially available from OriGene Catalog. No. TP301461 (OriGene, Rockville, MD, USA).

[0074] In some embodiments, BRP44-Like proteins useful in the present invention can include proteins that may be encoded by the polynucleotides of SEQ ID NOs: 52, 53, and 79 or their complementary sequence thereof and BRP44-Like proteins encoded by a nucleic acid sequence that hybridizes to the BRP44-Like coding region of a nucleic acid sequence in SEQ ID NOs: 52, 53, and 79 or complementary sequences thereof under “stringent hybridization conditions” which can include, 50% formamide, 5X SCC and 1% SDS, incubating at 42°C and wash in 0.2X SSC and 0.1% SDS at 65°C.

[0075] In some embodiments, BRP44-Like proteins that find utility in the assays of the present invention can include proteins or polypeptides having at least 50%, or at least 55%, or at least 60%, or at least 65%, or at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 92%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or at least 100%, identity to, or homologous with the BRP44-Like protein having the amino acid sequence set forth in any one of SEQ ID NOs: 42 - 51, and 99 or any full-length BRP44-Like protein described herein. In another illustrative example, BRP44-Like proteins comprising truncated forms of BRP44-Like having at least 50%, or at least 55%, or at least 60%, or at least 65%, or at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90% sequence identity or homology to any one of SEQ ID NOs: 42-51 and 99 are contemplated herein. In some embodiments, useful truncated forms comprise functional regions in their amino acid sequence when compared to the full length BRP44-Like protein since they can be functionally identified using the mitochondrial membrane competitive binding crosslinking assay described in Example 1, or can be proven to be functional by directly binding to an unlabeled or labeled mitoglitazone molecule or

analog in vitro or in vivo. In some embodiments, functional regions of the BRP44-Like protein can include proteins having a conserved domain called the UPF0041 domain which are provided in a family of proteins called pfam03650, which is part of Super Family Accession No: cl04196. A representative UPF0041 domain is provided in SEQ ID NO: 41.

[0076] The sequence of a BP44 protein or a BRP44-Like protein can also be modified by amino acid substitutions, replacements, insertions, deletions, truncations and other modifications. Typically such modifications can be used to prepare mimics of biologically-occurring polypeptides or to generate suitable targets for screening. For example, certain amino acids can be substituted for other amino acids in a polypeptide without appreciable loss of physiological activity (e.g., activities associated with mitoglitazone action intra and inter cellular, for example, anti-diabetic activity, reduction of blood glucose in an animal model and the like). Changes to the amino acid sequence can be conservative changes. The following eight groups each contain amino acids that are regarded conservative substitutions for one another: 1) Alanine (A) and Glycine (G); 2) Aspartic acid (D) and Glutamic acid (E); 3) Asparagine (N) and Glutamine (Q); 4) Arginine (R) and Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M) and Valine (V); 6) Phenylalanine (F), Tyrosine (Y) and Tryptophan (W); 7) Serine (S) and Threonine (T); and 8) Cysteine (C) and Methionine (M) (see, e.g., Creighton, *Proteins*, W.H. Freeman and Co., New York (1984)).

[0077] In some embodiments, conservative substitution tables providing functionally similar amino acids are well known in the art. For example, one exemplary guideline to select conservative substitutions includes (original residue followed by exemplary substitution): ala/gly or ser; arg/lys; asn/gln or his; asp/glu; cys/ser; gln/asn; gly/asp; gly/ala or pro; his/asn or gln; ile/leu or val; leu/ile or val; lys/arg or gln or glu; met/leu or tyr or ile; phe/met or leu or tyr; ser/thr; thr/ser; trp/tyr; tyr/trp or phe; val/ile or leu. An alternative exemplary guideline uses the following six groups, each containing amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (I); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); (see also, e.g., Creighton, *Proteins*, W.H. Freeman and Company (1984); Schultz and Schimer, *Principles of Protein Structure*, Springer-Verlag (1979)). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, for some purposes, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions that alter,

add or delete a single amino acid or a small percentage of amino acids in an encoded sequence can also be considered "conservatively modified variations".

[0078] As used herein, "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window can comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0079] The terms "identical" or "percent identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same (i.e., 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% percent identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Sequences are "substantially identical" to each other if they are at least 60%, at least 70%, at least 80% or at least 90% identical. These definitions also refer to the complement of a test sequence. Optionally, the identity exists over a region that is at least about 50 nucleotides in length, or more typically over a region that is 100 to 500 or 1000 or more nucleotides in length.

[0080] The terms "similarity" or "percent similarity," in the context of two or more polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of amino acid residues that are either the same or similar as defined by a conservative amino acid substitutions (i.e., 60% similarity, optionally 65%, 70%, 75%, 80%, 85%, 90%, or 95% similar over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Sequences are "substantially similar" to each other if they are at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, or at least 80% similar to each other. Optionally, this similarity exists over a region that is at least about 50 amino acids in length, or more typically over a region that is at least about 100 to 500 or 1000 or more

amino acids in length.

[0081] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities or similarities for the test sequences relative to the reference sequence, based on the program parameters.

[0082] A "comparison window," as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482 (1970)), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443 (1970)), by the search for similarity method of Pearson and Lipman (Proc. Natl. Acad. Sci. USA 85:2444 ((1988))), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see, e.g., Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)).

[0083] One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (J. Mol. Evol. 35:351-360 (1987)). The method used is similar to the method described by Higgins and Sharp (CABIOS 5:151-153 (1989)). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise

alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package (e.g., version 7.0 (Devereaux et al., Nucl. Acids Res. 12:387-95 (1984))).

[0084] Another example of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (Nuc. Acids Res. 25:3389-402 (1977)), and Altschul et al. (J. Mol. Biol. 215:403-10 (1990)), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length (W) in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (E) of 50, expectation (B) of 10, M=5, N=-4, and a comparison of both strands.

[0085] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-87). One

measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, typically less than about 0.01, and more typically less than about 0.001.

[0086] In designing modified BP44 or BRP44-Like proteins or polypeptides for the use in the present assay methods, the hydropathic index of amino acids can be considered. Amino acid substitutions can also be made on the basis of hydrophilicity.

[0087] In some embodiments, the BP44 protein, BRP44-Like protein and BP44-BRP44-Like heterodimer can also be made or expressed as a fusion protein comprising a BP44 protein, BP44-Like protein and BP44-BRP44-Like heterodimer or a ortholog, homolog and functional fragment thereof joined at its N- or C-terminus to a second polypeptide or other molecular entity. The second adjoining polypeptide can be, for example, an epitope, a selectable protein, an enzyme, polyethylene glycol (PEG) and the like. For example, the second polypeptide can be beta-galactosidase, a hexaHis tag, a fluorescence protein tag, for example, a green fluorescent protein (GFP), FLAG, influenza A hemagglutinin, c-Myc, or the like. These constructs may be used for TR-FRET fluorescence transfer assays described below in greater detail. In some embodiments, the BP44 protein, BRP44-Like protein and BP44-BRP44-Like heterodimer can include a C-terminal tag comprising a hexaHis (His₆-) cleavable sequence.

[0088] A wide range of additional host/vector systems suitable for expressing a BP44 protein or fusion protein of the present invention are available publicly, and described, for example, in Sambrook et al (In: Molecular cloning, A laboratory manual, second edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

[0089] C. Recombinant Expression mTOT Proteins and Fusion Constructs Thereof

[0090] Methods for recombinantly producing mTOT proteins for use in the various assays of the present invention are provided herein. In some embodiments, a BP44-BRP44-Like heterodimer refers to a single protein that has fused at least a partial sequence of BP44 as provided in any one of SEQ ID NO: 1-4 and 8-41 with at least a partial sequence of BRP44-Like protein as provided in any one of SEQ ID NO: 42-51. In some embodiments, the N-terminal portion of the heterodimer is BP44 and the C-terminal portion is BRP44-Like and vice versa. Each portion may represent a full-length sequence or a partial sequence of each

of BP44 and BRP44-Like proteins described herein. In some embodiments, the mTOT protein BP44-BRP44-Like heterodimer, may comprise a linker consisting of one to twenty amino acids, separating the two protein portions. In some embodiments, an mTOT protein or fusion proteins thereof (for example, an mTOT protein fused with a reporter protein, for example, a 6X His tag, a myc tag, a glutathione enzyme tag, a chloramphenicol acetyltransferase (CAT) protein or a fluorescence protein tag e.g. a green fluorescence protein GFP, Enhanced Green Fluorescent Protein (EGFP), a red fluorescence protein and the like) can be produced as a recombinant protein.

[0091] To facilitate the production of a recombinant mTOT protein or fusion protein thereof, a nucleic acid encoding the recombinant mTOT protein or fusion protein thereof is preferably isolated or synthesized. In some embodiments, a nucleic acid encoding an mTOT protein can comprise one or more nucleotide sequences provided in one of SEQ ID NOs: 5-7, 52-53, 74, 79 or a complement thereof. Typically the nucleic acid encoding the mTOT protein or combination of mTOT proteins is/are isolated using a known method, such as, for example, amplification (e.g., using PCR or splice overlap extension) or isolated from nucleic acid from an organism using one or more restriction enzymes or isolated from a library of nucleic acids. Methods of PCR are known in the art and described, for example, in Dieffenbach (ed) and Dveksler (ed) (In: PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratories, NY, 1995). Generally, for PCR techniques, two non-complementary nucleic acid primer molecules comprising at least about 20 nucleotides in length, and more preferably at least 25 nucleotides in length are hybridized to different strands of a nucleic acid template molecule, and specific nucleic acid molecule copies of the template are amplified enzymatically. Preferably, the primers hybridize to nucleic acid sequences adjacent to polynucleotides encoding an mTOT protein of the invention, thereby facilitating amplification of the nucleic acid that encodes the mTOT protein. In illustrative examples, PCR primers for BP44 are commercially available from RealTimePrimers.com, Catalog No. VHPS-894 (RealTimePrimers LLC, Elkins Park, PA USA). Following amplification, the amplified nucleic acid is isolated using a method known in the art and, preferably cloned into a suitable vector. PCR primers can also be selected on the basis of nucleic acid sequences 5' upstream and 3' downstream of the coding sequence of an mTOT protein genes which are obtainable from public gene databases, such as NCBI, and are known to those skilled in the art. cDNA clones of exemplary mTOT proteins are also commercially available and can be transfected into an expression host of interest. In an illustrative embodiment, *Homo sapiens* BRP44-Like (NM_016098.1) cDNA clones (available as non-tagged or tagged (Myc-DDK

tag or GFP-tagged)) (Catalog No. SC127044, RC201461 and RG201461) OriGene, Rockville, MD, USA) are available as transfection-ready DNA for transfection into a host, such as a eukaryotic cell line, e.g. a human cell-line (HEK-293 cell line).

[0092] Methods for such isolation will be apparent to the ordinary skilled artisan and/or described in Ausubel et. al. (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987), Sambrook et. al. (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001).

[0093] In an illustrative example, a nucleic acid (e.g., genomic DNA, cDNA, or RNA that is then reverse transcribed to form cDNA) from a cell, brain tissue or organism capable of expressing an mTOT protein of the present invention can be isolated using a method known in the art and cloned into a suitable vector. The vector can be then introduced into a suitable organism, for example, a eukaryotic cell or prokaryotic cell, for example, a bacterial cell.

Using a nucleic acid probe from a known mTOT protein encoding gene, a cell comprising the nucleic acid of interest is isolated using methods known in the art and described, for example, in Ausubel et al (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987), Sambrook et al (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001).

[0094] Other methods for the production of a nucleic acid encoding an mTOT protein will be apparent to the skilled artisan and are encompassed by the present invention.

[0095] For expressing an mTOT protein by recombinant means, an mTOT protein-encoding nucleotide sequence, for example, SEQ ID NOs: 5-7, 52-53, 74, 79, or a complement thereof, is operably linked to a promoter or other regulatory sequence capable of regulating expression in a cell-free system or cellular system. For example, a nucleic acid comprising a sequence that encodes a BP44 protein is operably linked with a suitable promoter and is expressed in a suitable cell for a time and under conditions sufficient for expression of the BP44 protein to occur. Nucleic acids encoding BP44 proteins, homologs or a functional fragments thereof are readily derived from the publicly available amino acids and corresponding nucleic acid sequences, set forth in any one of SEQ ID NOs: 1-4, 8-51 and 75.

[0096] As used herein, the term "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a genomic gene, including the TATA box or initiator element, which is required for accurate transcription initiation, with or without additional regulatory elements (e.g., upstream activating sequences, transcription factor binding sites, enhancers and silencers) that alter expression of a nucleic acid (e.g., a transgene), e.g., in response to a developmental and/or external stimulus, or in a tissue

specific manner. In the present context, the term "promoter" is also used to describe a recombinant, synthetic or fusion nucleic acid, or derivative, which confers, activates or enhances the expression of a nucleic acid (e.g., a transgene and/or a selectable marker gene and/or a detectable marker gene) to which it is operably linked. Preferred promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or alter the spatial expression and/or temporal expression of said nucleic acid.

[0097] As used herein, the term "in operable connection with" "in connection with" or "operably linked to" means positioning a promoter relative to a nucleic acid (e.g., a transgene) such that expression of the nucleic acid is controlled by the promoter. For example, a promoter is generally positioned 5' (upstream) to the nucleic acid, the expression of which it controls. To construct heterologous promoter/nucleic acid combinations (e.g., promoter/transgene and/or promoter/selectable marker gene combinations), it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the nucleic acid it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function.

[0098] Should it be preferred that an mTOT protein, or a functional fragment or fusion protein of the present invention be expressed in vitro, a suitable promoter can include, but is not limited to, a T3 or a T7 bacteriophage promoter. Typical expression vectors for in vitro expression or cell-free expression are readily known and have been described and include, but are not limited to the TNT T7 and TNT T3 systems (Promega), the pEXP1-DEST and pEXP2-DEST vectors (Invitrogen, Carlsbad, CA USA) and pINVITRO plasmids from (InvivoGen, San Diego, CA USA).

[0099] Typical promoters suitable for expression of mTOT proteins in bacterial cells include, but are not limited to, the lacZ promoter, the lpp promoter, temperature-sensitive lamda L or .lamda R promoters, T7 promoter, T3 promoter, SP6 promoter or semi-artificial promoters such as the IPTG-inducible Tac promoter or lacUV5 promoter. A number of other gene construct systems for expressing the nucleic acid fragment of the invention in bacterial cells are well-known in the art and are described for example, in Ausubel et al (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987), U.S. Pat. No. 5,763,239 (Diversa Corporation) and Sambrook et al (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001).

[0100] Numerous expression vectors for expression of recombinant polypeptides in

bacterial cells and efficient ribosome binding sites have been described, and include, for example, PKC30, pKK173-3, pET-3; the pCR vector suite (Invitrogen, Carlsbad, CA USA), pGEM-T Easy vectors (Promega, Madison, WI USA), the pL expression vector suite (Invitrogen, Carlsbad, CA USA) the pBAD/TOPO or pBAD/thio--TOPO series of vectors containing an arabinose-inducible promoter (Invitrogen, Carlsbad, CA USA), the latter of which is designed to also produce fusion proteins with a Trx loop for conformational constraint of the expressed protein; the pFLEX series of expression vectors (Pfizer, Grotton, CT, USA); the pQE series of expression vectors (QIAGEN, Valencia, CA, USA), or the pL series of expression vectors (Invitrogen, Carlsbad, CA USA), amongst others.

[0101] Typical promoters suitable for expression in viruses of eukaryotic cells and eukaryotic cells include the SV40 late promoter, SV40 early promoter and cytomegalovirus (CMV) promoter, CMV IE (cytomegalovirus immediate early) promoter amongst others. Preferred vectors for expression in mammalian cells (e.g., 293, COS, CHO, 10T cells, 293T cells) include, but are not limited to, the pcDNA vector suite supplied by Invitrogen, in particular pcDNA 3.1 myc-His-tag comprising the CMV promoter and encoding a C-terminal 6x His and MYC tag; and the retrovirus vector pSRalpha-tkneo.

[0102] Methods for introducing an isolated nucleic acid molecule or a gene construct comprising same into a cell for expression are well known to those skilled in the art. The technique used for a given organism can depend on known successful techniques suitable for introducing heterogenous nucleic acids for the particular host organism of interest. Means for introducing recombinant DNA into cells include microinjection, transfection mediated by DEAE-dextran, transfection mediated by liposomes such as by using lipofectamine (Gibco/Invitrogen, Carlsbad, CA USA) and/or cellfectin (Gibco/Invitrogen, Carlsbad, CA USA), PEG-mediated DNA uptake, electroporation and microparticle bombardment such as by using DNA-coated tungsten or gold particles (Agracetus Inc., WI, USA) amongst others.

[0103] A variety of eukaryotic cell expression systems can be used in the methods according to the present invention. A suitable eukaryotic cell expression system is one in which expression of the BP44 protein in a suitable genetic background causes toxicity. One model eukaryotic organism, yeast, provides a well-established system for genetic and chemical screening. Many genes can be studied in yeast because they are non-essential under certain growth conditions. In addition, homologs and orthologs of yeast genes can be studied in yeast because such homologs and orthologs often have overlapping functions with the yeast genes, allowing deletion or inactivation of the yeast gene.

[0104] Suitable yeast strains which can be used in the context of the present invention

include, for example, *Saccharomyces cerevisiae*, *Saccharomyces uvae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Pichia pastoris*, *Pichia methanolica*, *Pichia kluyveri*, *Yarrowia lipolytica*, *Candida* species such as *Candida utilis* or *Candida cacaoi*, *Geotrichum* species such as *Geotrichum fermentans*, and the like. In a typical embodiment, the yeast strain can be *Saccharomyces cerevisiae*.

[0105] Other suitable eukaryotic cell expression systems can include, for example, rat, mouse, *Drosophila*, or *C. elegans*. The eukaryotic cell expression system can be another non-human, animal, insect or lower model system. A suitable eukaryotic cell expression system also can be human cells or cells isolated from such a non-human eukaryotic organism, such as, for example, a human cell line, rat, mouse, guinea pig, hamster, dog, horse, pig, *Drosophila*, Chinese Hamster Ovary (CHO) or *C. elegans* cells cultured in vitro. The eukaryotic cell expression system can be genetically engineered to express an mTOT protein and fusion constructs thereof, or may constitutively express these proteins independently without any further manipulation. For example, *Drosophila* can be genetically engineered to express a BP44 protein that causes over expression in at least some cells in a suitable genetic background. Alternatively, the system can express an endogenous mTOT protein that causes over expression of it in a suitable genetic background. Suitable expression vectors containing mTOT protein DNA/cDNA/RNA for expression of these proteins in vitro are readily available, for example, *Homo sapiens* BRP44-Like, mRNA (cDNA clone MGC:4871 IMAGE:3452973 complete CDS expressed in pCMV-SPORT6 expression vector in *E.coli* DH10B), from Invitrogen, (Catalog No. 3452973, Invitrogen, Carlsbad, CA USA).

[0106] The eukaryotic cell of the expression system (e.g., a yeast strain) can optionally include alleles of, or mutations in, genes that facilitate uptake or increase permeability of a candidate agent(s) when employed in a screening assay. The eukaryotic cell of the cell expression systems (e.g., a yeast strain) also can optionally include alleles of, or mutations in, genes that reduce or prevent metabolism of a candidate agent(s). For example, a yeast strain can include mutations in one or more of the yeast genes *erg6*, *pdr1* and/or *pdr3*, which affect membrane efflux pumps and may increase permeability of candidate agents.

[0107] In some embodiments, the genetic background of the eukaryotic cell expression system is one in which expression of the mTOT protein causes some activity that can be measured and at least partially quantified. In some embodiments, a cell having a "suitable genetic background" refers to a cell having a genetic makeup in which the mTOT protein is over expressed for the purposes of evaluating the effects of a candidate compound when the

cell is used in a screening assay. In some embodiments, a useful assay using the discovery outlined herein involves detecting, analyzing or quantifying the effects of compounds on metabolic and/or cellular function in intact cells. It is anticipated that the over expression (greater than 50% normal levels) of the mTOT protein (i.e. BP44 protein, and/or BRP44-Like protein and/or BP44-BRP44-Like heterodimer) or fusion constructs comprising an mTOT protein in various cells will result in change in metabolism and/or cell function that can be measured. Candidate compounds may be added to incubations of such cells and those that interfere with the function of the over expressed mTOT protein will then be selected for evaluation of anti-diabetic, anti-obesity, metabolic protective, or neuroprotective activity in one or more activity assays.

[0108] Following production/expression/synthesis, an mTOT protein or fusion protein comprising same, can be purified using any known methods in the art. Such purification preferably provides an mTOT protein, substantially free of non-specific protein, acids, lipids, nucleic acids, carbohydrates, and the like. Antibodies and other affinity ligands are particularly preferred for producing isolated protein. Antibodies specific for human and mouse BP44 protein are known and described above. Preferably, the mTOT protein will be in a preparation wherein more than about 80%, 85%, or more than 90% (e.g. 95%, 96%, 97%, 98% or 99%) of the protein in the preparation is an mTOT protein of the present invention or fusion protein comprising same.

[0109] Standard methods of protein purification can be employed to obtain an isolated mTOT protein of the present invention, including, but not limited to, various high-pressure (or high-performance) liquid chromatography (HPLC) and non-HPLC peptide isolation protocols, such as size exclusion chromatography, ion exchange chromatography, phase separation methods, electrophoretic separations, precipitation methods, salting in/out methods, immunochromatography, and/or other methods.

[0110] In an illustrative embodiment, a method for isolating an mTOT protein employs reversed-phase HPLC using an alkylated silica column such as C₄-, C₈- or C₁₈-silica. A gradient mobile phase of increasing organic content is generally used to achieve purification, for example, acetonitrile in an aqueous buffer, usually containing a small amount of trifluoroacetic acid. Ion-exchange chromatography can also be used to separate a peptide based on its charge.

[0111] Alternatively, affinity purification is useful for isolating a fusion protein comprising a label attached to BP44 protein. Methods for isolating fusion proteins using affinity chromatography are known in the art and described, for example, in Scopes (In: Protein

purification: principles and practice, Third Edition, Springer Verlag, 1994). For example, an antibody or compound that binds to the label (in the case of a polyhistidine tag this may be, for example, nickel-NTA) is preferably immobilized on a solid support. A sample comprising a BP44 fusion protein is then contacted to the immobilized antibody or compound for a time and under conditions sufficient for binding to occur. Following washing to remove any unbound or non-specifically bound protein, the fusion protein is eluted.

[0112] The degree of purity of the peptide compound may be determined by various methods, including identification of a major large peak on HPLC. A peptide compound that produces a single peak that is at least 95% of the input material on an HPLC column is preferred. Even more preferable is a polypeptide that produces a single peak that is at least 97%, at least 98%, at least 99% or even 99.5% of the input material on an HPLC column.

[0113] To ensure that the mTOT proteins are obtained using any of the techniques described above is a functional mTOT protein for use in compositions and assays and screening methods of the present invention, analysis of the composition of the mTOT protein can be determined by any of a variety of analytical methods known in the art. Such composition analysis may be conducted using high resolution mass spectrometry to determine the molecular weight of the mTOT protein. Alternatively, hydrolyzing the protein in aqueous acid, and separating, identifying and quantifying the components of the mixture using HPLC, or an amino acid analyzer can confirm the amino acid content of an mTOT protein. Protein sequencers, which sequentially degrade the protein and/or peptide and identify the amino acids in order, may also be used to determine the sequence of the mTOT protein. Since some of the mTOT proteins may contain amino and/or carboxy terminal capping groups, it may be necessary to remove the capping group or the capped amino acid residue prior to a sequence analysis. Thin-layer chromatographic methods may also be used to authenticate one or more constituent groups or residues of a desired peptide.

[0114] II. SCREENING ASSAYS FOR IDENTIFYING COMPOUNDS CAPABLE OF ANTI-DIABETIC, ANTI-OBESITY AND NEUROPROTECTIVE ACTIVITY

[0115] The screening assays of the present invention are aimed at identifying compounds that are capable of binding to at least one mTOT protein.

[0116] The present invention utilizes the finding that BP44 proteins and in some embodiments, mTOT proteins are specific targets of PPAR γ sparring thiazolidinedione compounds, for example, mitoglitazone. Compounds that bind to an mTOT protein are postulated to play a role in insulin sensitization, particularly at sites involving the mitochondria. Thus, in some embodiments, the present invention provides a method for

screening one or more candidate compounds to identify therapeutic agents effective against one or more insulin resistance or metabolic diseases, the method comprises: screening one or more candidate compounds in a binding assay, the binding assay comprising the steps:

- (i) providing an mTOT protein;
- (ii) contacting the mTOT protein with a candidate compound; and
- (iii) detecting if the candidate compound specifically binds to the mTOT protein or inhibits the specific binding of a thiazolidinedione compound to the mTOT protein, wherein the candidate compound is identified as a lead candidate compound if the candidate compound specifically binds to the mTOT protein or inhibits the binding of the thiazolidinedione compound to the mTOT protein.

[0117] In some embodiments, the mTOT protein or proteins used in the various screening assays can include: mTOT protein having an amino acid sequence as provided in any one of SEQ ID NOs: 1-4, and 8-51, or an mTOT protein having an amino acid sequence as provided in any one of SEQ ID NOs: 1-4, 42, and 47, or an amino acid sequence having at least 85% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an amino acid sequence having at least 90% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an amino acid sequence having at least 95% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an mTOT protein has an amino acid sequence having at least 96% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an amino acid sequence having at least 97% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an amino acid sequence having at least 98% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an amino acid sequence having at least 99% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47, or an amino acid sequence as in any one of SEQ ID NOs: 1-4, 8-51, or an amino acid sequence as in any one of SEQ ID NOs: 1-4, 8-51.

[0118] In some embodiments, an illustrative example of an mTOT protein for use in the methods of the present invention includes an mTOT protein encoded by a nucleic acid sequence that specifically hybridizes to the complement of an mTOT nucleic acid sequence of SEQ ID NO: 5-7, or 52-53, under stringent hybridization conditions which are: 50% formamide, 5X SCC and 1% SDS, incubating at 42°C and wash in 0.2X SSC and 0.1% SDS at 65°C and wherein said mTOT protein specifically binds to mitoglitazone.

[0119] In some embodiments, the screening method includes the use of BP44 protein as the mTOT protein. In some embodiments, the screening method includes the use of BRP44-Like protein as the mTOT protein. In some embodiments, the screening method includes the use

of BP44-BRP44-Like heterodimer as the mTOT protein, for example as encoded by a ligated nucleotide sequence of SEQ ID Nos: 81 and 86 as described in Example 3.

[0120] In some embodiments, the present invention provides screening assays that are capable of screening candidate compounds that can bind to an mTOT protein. In some embodiments, the screening assays of the present invention also contemplate assays that are capable of screening candidate compounds that can bind to an mTOT protein. Without wishing to be bound by any particular theory, it is believed that compounds that are capable of mediating metabolic protective effects, including, anti-diabetic, anti-obesity, increased insulin sensitivity and reduced inflammation in various cells, including hepatic cells, endothelial cells, epithelial cells, adipocyte cells, muscle cells, neuronal cells and other cells of the brain will also bind to one or more mTOT proteins. The present inventors have surprisingly discovered that mitoglitazone specifically binds to and/or has an affinity to one or more mTOT proteins, for example, BP44 protein and BRP44-Like proteins. Hence, it is believed that candidate compounds that may bind to an mTOT protein or which actively induce the expression of an mTOT protein in a eukaryotic or bacterial cell, for example, a human cell, will also have some physiological effect on other targets of mitoglitazone, by mimicking the activity of mitoglitazone.

[0121] In some embodiments, an optional step in determining whether a candidate compound may exert some physiological effect related to thiazolidinedione activity, and generally involves determining whether the candidate compound which binds to the mTOT protein is able to exert some measureable insulin-sensitizing activity, anti-diabetic activity, anti-obesity activity, metabolic protective activity, neuroprotective activity, either in vitro and/or in vivo. If such a candidate compound binds to an mTOT protein and is subsequently found to exert some measurable insulin-sensitizing activity, anti-diabetic activity, anti-obesity activity, metabolic protective activity, or neuroprotective activity, then the candidate compound is a therapeutic agent.

[0122] The present invention provides for at least three broad types of screening assays that can be performed to identify one or more candidate compounds for further testing on the basis that they selectively bind to one or more mTOT proteins. The first assay type generally determines direct binding of an mTOT protein and a candidate compound and then determining whether the candidate compound is biologically active, i.e. whether the candidate compound exerts some measurable insulin-sensitizing activity, anti-diabetic activity, anti-obesity activity, metabolic protective activity, or neuroprotective activity.

[0123] The second type of screening assays generally involves prokaryotic or eukaryotic cells that have an over-expressed mTOT protein. The over expression of the mTOT protein in the prokaryotic or eukaryotic cell can be intracellular, for example in the cytosol, nucleus or attached to an organelle membrane or may be confined, embedded in or attached to a cell membrane or both. Such over expression of the mTOT protein will result in some inhibition of the activity caused by such over expression. As used herein, the term "over-expression" can include expression of the mTOT protein in the treated cell that is greater than 150% of the expression of the mTOT protein on a percentage weight basis (% wt/wt) of an identical non-treated, normal or wild-type cell.

[0124] The third general class of screening assays involve spectrophotometric, NMR or some other physical analytical method that can measure the spectral nature of the mTOT protein in the presence and absence of a candidate compound. In some embodiments, the screening assay is a fluorescence polarization assay or a FRET assay.

[0125] As used herein, a candidate compound can include, but is not limited to: nucleic acids, peptides, proteins, sugars, polysaccharides, glycoproteins, lipids, and small organic molecules. A "candidate compound" is a compound that can be tested in a screening assay of the present invention. The term "small organic molecules" typically refers to molecules of a size comparable to those organic molecules generally used in pharmaceuticals. Small organic molecules generally exclude biological polymers (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da. more preferably up to 2000 Da. and most preferably up to about 1000 Da. In some embodiments, the candidate compound can be at least one of a metal, a peptide, a protein, a lipid, a polysaccharide, a nucleic acid, a library of small organic molecules, and a drug.

[0126] In some embodiments, determining whether a candidate compound has bound specifically with an mTOT protein requires that at least one of the mTOT protein and candidate compound is labeled with a marker or label. As used herein, a label can include a fluorescent molecule, a radionuclide, a protein tag, or combinations thereof. Fluorescent molecules can include naturally occurring or synthetic organic and organometallic compounds known in the art. In some embodiments, an illustrative fluorescent molecule can include a compound of Formula (I) or Formula (II) as described herein. In some embodiments, an mTOT protein or candidate compound can be labeled with a radionuclide or radioisotope. Illustrative examples of radionuclide agents useful as labels for use in the screening assays herein can include, but not limited to: ^3H , ^{14}C , ^{32}P , or ^{35}S .

[0127] In some embodiments, the mTOT protein or candidate compound can be labeled with a protein tag. Several examples of protein tags can include: glutathione-S-transferase, c-myc, Heme-agglutinin (HA), FLAG, avidin, biotin, streptavidin, or a fluorescent protein, in addition to other protein or amino acid based tags that can be used in fusion constructs comprising an mTOT protein or a protein/peptide candidate protein, or linked chemically to an mTOT or candidate compound. In one embodiment, a protein tag of the present invention can be readily identified by native fluorescence upon proper excitation and emission wavelength application, by reacting in an enzymic reaction operable to release a detectable signal, or can be detected using a conjugate antibody.

[0128] In some embodiments, the protein tag can include a fluorescent protein or a protein that is capable of fluorescing under appropriate wavelength absorption and emission. In some embodiments, the mTOT protein can be recombinantly engineered to be expressed as a fusion construct having a fluorescent protein tag. In some embodiments, illustrative fluorescent tags can include: green fluorescent protein, enhanced green fluorescent protein, AcGFP1 Fluorescent Protein, AmCyan1 Fluorescent Protein, AsRed2 Fluorescent Protein, mBanana Fluorescent Protein, mCherry Fluorescent Protein, Dendra2, Fluorescent Protein, DsRed2 Fluorescent Protein, DsRed-Express Fluorescent Protein, DsRed-Monomer Fluorescent Protein, E2-Crimson Fluorescent Protein, GFPuv Fluorescent Protein, HcRed1 Fluorescent Protein, mOrange Fluorescent Protein, PAmCherry Fluorescent Protein, mPlum Fluorescent Protein, mRaspberry Fluorescent Protein, mStrawberry Fluorescent, tdTomato Fluorescent Protein, Timer Fluorescent Protein, ZsGreen1 Fluorescent Protein, and ZsYellow1 Fluorescent Protein. All of these fluorescent proteins are commercially available as DNA which can be readily coupled to a gene of interest, i.e. an mTOT gene. In some embodiments, the fluorescent proteins can be in monomeric form and can be coupled to either the N-terminus or the C-terminus and are available in vectors suitable for fusion protein construction from Clontech, (Mountainview, CA USA), for example, bacterial expression vectors cat. No. 632412-pDsRed-Express vector, or plasmid or lentiviral vectors operable to clone a fusion construct comprising an mTOT protein fused to a fluorescent protein (See an exemplary commercially available vector from Clontech pEF-1alpha-DsRed Monomer C1 vector Cat. No. 631977). Excitation and emission spectra for all of the exemplified fluorescent proteins are well known and can be found from Clontech product literature or the Clontech website at www.clontech.com/OA_MEDIA/xxclt_media/MainWP063553.html and are incorporated herein in their entirety.

[0129] In some embodiments, the screening assay of the present invention includes the screening of one or more candidate compounds in the form of a library of compounds. The library of compounds comprises a combinatorial chemical library as exemplified and described below. Combinatorial chemical libraries can include a plurality of small organic molecules. Typically, the combinatorial chemical library can contain at least 1000 candidate compounds. In various embodiments, the candidate compound is a small organic molecule.

[0130] Conventionally, new chemical entities with useful properties are generated by identifying a candidate compound with some desirable property or activity, creating variants of the candidate compound, and evaluating the property and activity of those variant compounds. However, the current trend is to shorten the time scale for all aspects of drug discovery. Because of the ability to test large numbers quickly and efficiently, high throughput screening (HTS) methods are replacing conventional lead compound identification methods.

[0131] In some embodiments, high throughput screening methods involve providing a library containing a large number of potential therapeutic compounds (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity, for example, capable of binding to an mTOT protein, or increase the expression of an mTOT protein in a cell, for example an adipocyte cell or hepatic cell or a eukaryotic cell line.

[0132] A combinatorial chemical library, or chemical library, is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide (e.g., mutein) library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Thousands to millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks. For example, systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds.

[0133] Preparation of combinatorial chemical libraries are well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Pat. No. 5,010,175. Peptide synthesis is by no means the only approach envisioned and intended for use with the present invention. Other chemistries for generating

chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (PCT Publication No WO 91/19735, 26 Dec. 1991), encoded peptides (PCT Publication WO 93/20242, 14 Oct. 1993), random bio-oligomers (PCT Publication WO 92/00091, 9 Jan. 1992), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides, vinylogous polypeptides, nonpeptidal peptidomimetics with a Beta-D-Glucose scaffolding, analogous organic syntheses of small compound libraries, oligocarbamates, and/or peptidyl phosphonates. See, generally, nucleic acid libraries (see, e.g., Strategene, Corp.), peptide nucleic acid libraries (see, e.g., U.S. Pat. No. 5,539,083) antibody libraries (see, e.g., PCT/US96/10287), carbohydrate libraries (see, e.g., U.S. Pat. No. 5,593,853), and small organic molecule libraries (see, e.g., benzodiazepines, isoprenoids U.S. Pat. No. 5,569,588, thiazolidinones and metathiazanones U.S. Pat. No. 5,549,974, pyrrolidines U.S. Pat. Nos. 5,525,735 and 5,519,134, morpholino compounds U.S. Pat. Nos. 5,506,337, benzodiazepines 5,288,514, and the like).

[0134] Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville Ky., Symphony, Rainin, Woburn, Mass., 433A Applied Biosystems, Foster City, Calif., 9050 Plus, Millipore, Bedford, Mass.). A number of well-known robotic systems have also been developed for solution phase chemistries. These systems include, but are not limited to, automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist and the Venture™ platform, an ultra-high-throughput synthesizer that can run between 576 and 9,600 simultaneous reactions from start to finish (see Advanced ChemTech, Inc. Louisville, KY, USA). Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, Mo., ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, Pa., Martek Biosciences, Columbia, Md., etc.).

[0135] If the candidate compound binds to an mTOT protein specifically, or causes increased expression or modulates an activity of the mTOT protein as compared to the expression or activity in the absence of the candidate compound, that candidate compound can be said to be a "lead candidate compound". Subsequently, or concurrently, the lead

candidate compound may be validated using an assay capable of demonstrating insulin sensitizing activity, anti-diabetic activity, anti-obesity activity, metabolic protective activity, or neuroprotective activity of the lead candidate compound. If the lead candidate compound demonstrates insulin sensitizing activity, anti-diabetic activity, anti-obesity activity, metabolic protective activity, or neuroprotective activity, the lead candidate compound is a therapeutically active agent. The therapeutically active agent thereby possesses PPAR γ sparring and/or insulin sensitizing activity suitable for the treatment of a metabolic disease, such as metabolic syndrome, diabetes mellitus, cardiovascular disease, gastrointestinal disease and neurodegenerative diseases.

[0136] A. Direct Binding Assays

[0137] In some embodiments, the screening assays described herein are useful for identifying a lead candidate compound from plurality of candidate compounds. Upon confirmation of its binding specificity to an mTOT protein, subsequent activity assays are employed that measure the insulin-sensitizing activity or anti-diabetic activity, or metabolic protective activity, or neuroprotective activity, the lead candidate compound is a therapeutic active agent that putatively modulates a metabolic disease condition in a subject for example a human subject based on its effect on mitochondrial function and/or binding to an mTOT protein. In some embodiments, the direct binding screening assay method comprises: (i) screening one or more candidate compounds in a direct binding assay that identifies candidate compounds which bind to an mTOT protein. In one illustrative screening method, screening one or more candidate compounds in a binding assay, the binding assay comprising the steps: (i) providing an mTOT protein; (ii) contacting the mTOT protein with a candidate compound; and (iii) detecting if the candidate compound specifically binds to the mTOT protein or inhibits the specific binding of a thiazolidinedione compound to the mTOT protein, wherein the candidate compound is identified as a lead candidate compound if the candidate compound specifically binds to the mTOT protein or inhibits the binding of the thiazolidinedione compound to the mTOT protein.

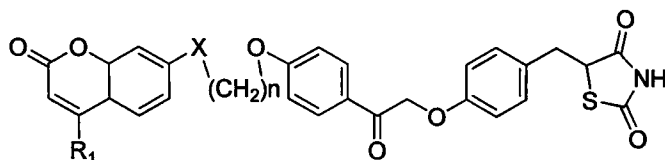
[0138] The mTOT proteins capable of use in the present assays have been described above, and are exemplified in Tables 1, 3, 4 and 5 and shown in Figures 3A, 3B, 4A and 4B. Competitive binding assays can be designed wherein the candidate compound competes with a known thiazolidinedione compound, for example, any one of mitoglitazone, rosiglitazone, pioglitazone or troglitazone. In some embodiments, the competitor compound is mitoglitazone. In one embodiment, the competitor compound is rosiglitazone.

[0139] 1. Fluorescence Based Assays

[0140] In some embodiments, direct binding between a candidate compound and an mTOT protein can be detected and/or measured using fluorescence based assays. Binding of fluorescent candidate compounds can be detected in various ways, including fluorescence energy transfer (FRET), direct spectrophotofluorometric analysis of bound compound, or fluorescence polarization (Rogers, *Drug Discovery Today*, 1997, 2, 156-160; Hill, *Cur. Opinion Drug Disc. Dev.*, 1998, 1, 92-97). In some embodiments, the mTOT protein can be labeled with a fluorescence molecule, for example, a fluorescence protein tag such as GFP, and then incubated in the presence and absence of a candidate compound under conditions which permit binding of the two agents. The non-bound candidate compound(s) can then be removed from the labeled mTOT protein, and the bound candidate compound can be identified using an appropriate method. In an other embodiment, the mTOT protein is unlabeled and the candidate compound is labeled. In this illustrative example, the mTOT protein can be incubated in the presence and absence of a labeled candidate compound and a complex comprising the mTOT protein and labeled candidate compound is subsequently isolated and the candidate compound identified.

[0141] In some embodiments, fluorescence binding assays can involve the use of FRET assays that may be designed for use in screening of compounds with green fluorescent protein (GFP) or some other appropriate fluorescence protein tag. Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. In an illustrative example, candidate compounds can be synthesized which contain a fluorescence probe with the ability to be activated by wavelengths of light that by themselves would not cause a fluorescence fusion tag on an mTOT protein to emit a fluorescence signal. However, once activated the fluorescence probe would then emit a light at a wavelength that activates the fluorescence fusion tag on the mTOT protein to emit a fluorescence signal when the fluorescence probe is bound to, or in close proximity, to the mTOT protein. Hence, when the candidate compound has bound to the mTOT protein, the excitation of the fluorescence probe absorbed by the fluorescence fusion tag attached to the mTOT protein.

[0142] In some examples, a fluorescence probe of the present invention includes a compound of Formula I:



I

wherein

X is -O- or -NR₂;

R₁ is optionally substituted C₁₋₆ straight or branched alkyl or CH₂C(O)OR₃;

R₂ is H, optionally substituted C₁₋₆ straight or branched alkyl, optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl;

R₃ is H, optionally substituted C₁₋₆ straight or branched alkyl, or optionally substituted -CH₂-phenyl; and

n is 2-6.

[0143] In several embodiments, X is -O-. For instance, X is -O- and n is 2. In other instances, X is -O- and n is 3.

[0144] In several embodiments, R₁ is optionally substituted C₁₋₆ straight or branched alkyl. For example, R₁ is optionally substituted C₁₋₆ straight alkyl. In other examples, R₁ is optionally substituted C₃₋₆ branched alkyl. In several alternative examples, R₁ is methyl, ethyl, propyl, *sec*-propyl, butyl, *t*-butyl, pentyl, or 2,2-dimethylbutyl, each of which is optionally substituted.

[0145] In several embodiments, R₂ is optionally substituted C₁₋₆ straight or branched alkyl. For example, R₂ is optionally substituted C₁₋₆ straight alkyl. In other examples, R₂ is optionally substituted C₃₋₆ branched alkyl. In several alternative examples, R₂ is methyl, ethyl, propyl, *sec*-propyl, butyl, *t*-butyl, pentyl, or 2,2-dimethylbutyl, each of which is optionally substituted.

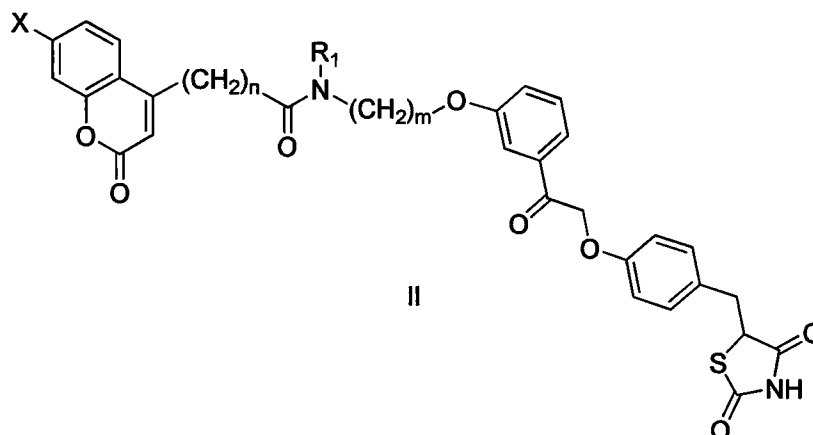
[0146] In several embodiments, R₂ is H.

[0147] In other embodiments, R₂ is optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl.

[0148] In several embodiments, R₃ is H or optionally substituted C₁₋₆ straight or branched alkyl. For example R₃ is H. In other examples, R₃ is optionally substituted C₁₋₆ straight or

branched alkyl. For example, R_3 is optionally substituted C_{1-6} straight alkyl. In other examples, R_3 is optionally substituted C_{3-6} branched alkyl. In several alternative examples, R_3 is methyl, ethyl, propyl, *sec*-propyl, butyl, *t*-butyl, pentyl, or 2,2-dimethylbutyl, each of which is optionally substituted. In several embodiments, R_3 is optionally substituted $-CH_2$ -phenyl.

[0149] In other examples, the probe includes a compound of Formula II:



wherein

X is $-OH$, $-OCH_3$, $-N(R_2)_2$;

R_1 is H, optionally substituted C_{1-6} straight or branched alkyl, optionally substituted phenyl, optionally substituted $-CH_2$ -phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, or optionally substituted 4-pyridyl;

Each R_2 is independently H, optionally substituted C_{1-6} straight or branched alkyl, optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl, or

two R_2 substituents and the nitrogen atom to which they are attached form an optionally substituted 3-7 membered heterocyclic ring;

m is 2-6; and

n is 2-6.

[0150] In several embodiments, X is $-OH$. In other embodiments, X is $-OCH_3$. In other instances, X is $-N(R_2)_2$.

[0151] In several embodiments, R_1 is H.

[0152] In several embodiments, R_1 is optionally substituted C_{1-6} straight or branched alkyl. For example, R_1 is optionally substituted C_{1-6} straight alkyl. In other examples, R_1 is optionally substituted C_{3-6} branched alkyl. In several alternative examples, R_1 is methyl, ethyl, propyl, *sec*-propyl, butyl, *t*-butyl, pentyl, or 2,2-dimethylbutyl, each of which is

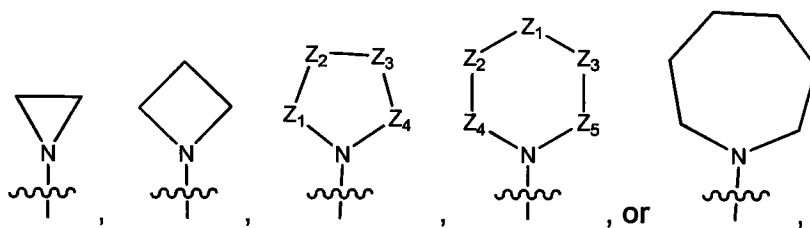
optionally substituted.

[0153] In several embodiments, R_2 is optionally substituted C_{1-6} straight or branched alkyl. For example, R_2 is optionally substituted C_{1-6} straight alkyl. In other examples, R_2 is optionally substituted C_{3-6} branched alkyl. In several alternative examples, R_2 is methyl, ethyl, propyl, *sec*-propyl, butyl, *t*-butyl, pentyl, or 2,2-dimethylbutyl, each of which is optionally substituted.

[0154] In several embodiments, R_2 is H.

[0155] In other embodiments, R_2 is optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl.

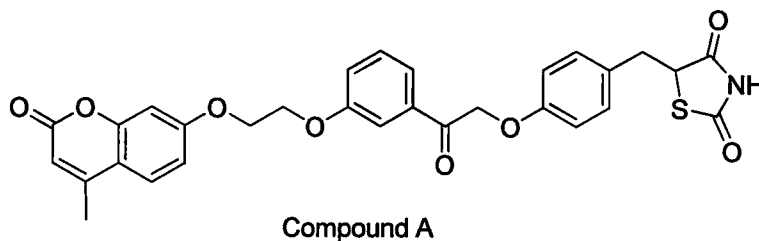
[0156] In several embodiments, two R_2 substituents and the nitrogen atom to which they are attached form an optionally substituted 3-7 membered heterocyclic ring. For example, two R_2 substituents and the nitrogen atom to which they are attached form a ring selected from:



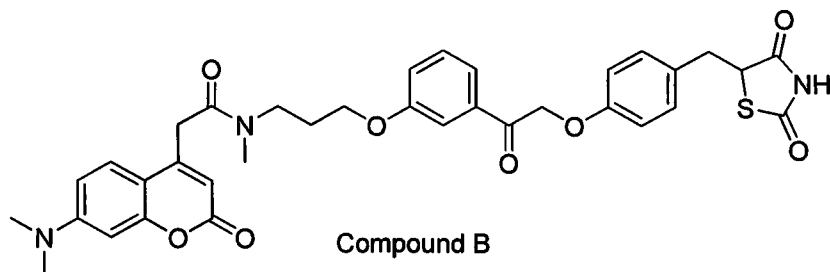
wherein each of Z_1 , Z_2 , Z_3 , Z_4 , and Z_5 are independently selected from -NH-, -O-, -S-, or -CH₂-.

[0157] In other examples, two R_2 substituents and the nitrogen atom to which they are attached form a pyrrolidine-yl, piperidine-yl, piperazine-yl, morpholine-yl, or thiomorpholine-yl, each of which is optionally substituted.

[0158] In some examples, the compound of Formula I is Compound A:

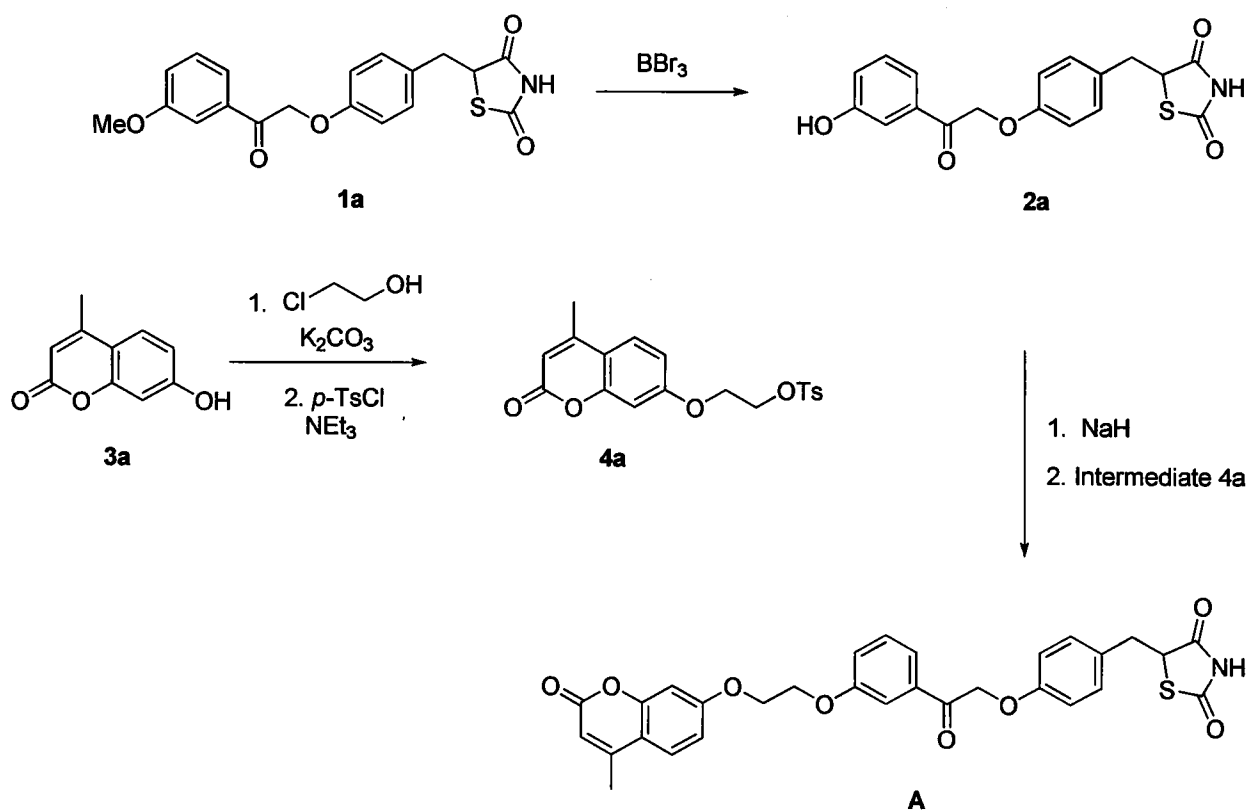


[0159] In other examples, the compound of Formula II is Compound B:



[0160] Exemplary methods for making probes of Formulae I or II include synthetic pathways such as those illustrated in Schemes 1 and 2 below.

[0161] Scheme 1:



[0162] In Scheme 1, commercially available starting material, 5-(4-(2-(3-methoxyphenyl)-2-oxoethoxy)benzyl)thiazolidine-2,4-dione (**1a**), undergoes *O*-dealkylation upon treatment with BBr_3 or other dealkylating reagent (e.g., AlBr_3) to generate intermediate (**2a**). 7-hydroxy-4-methyl-2H-chromen-2-one (**3a**) undergoes *O*-alkylation upon treatment with an appropriate alkylating agent. Preferably, the alkylating agent is functionalized with a hydroxyl group (e.g., 2-chloroethanol). Following alkylation, the alcohol functionality can be converted to a leaving group (e.g., tosyl, mesyl, chloro, bromo, or the like) to generate intermediate (**4a**) using an appropriate reagent (e.g. *p*-toluenesulfonyl chloride, methylsulfonyl chloride, PBr_5 , PCl_5 , or the like). Intermediate (**2a**) is treated with a strong base (e.g., NaH or the like) and reacted with intermediate (**4a**) to generate exemplary

compound A of Formula I.

[0163] One exemplary experimental procedure used to generate compound A of Formula I includes: heating a mixture of 7-hydroxy-4-methylcoumarin (1.76 g, 10 mmol), potassium carbonate (2.07 g, 15 mmol), 2-chloroethanol (1.34 mg, 20.0 mmol), and potassium iodide (80 mg, 0.5 mmol) in N,N-dimethylformamide (50 ml, 600 mmol) at 110 °C for about 5 hours, cool to room temperature, and stir at room temperature.

[0164] The reaction mixture was partitioned between EtOAc and water, and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with brine, dried on Na₂SO₄, filtered, and evaporated in vacuo. The residue was purified by flash chromatography on a small Biotage column eluting with 0-10% acetone/DCM. Fractions containing product were combined and evaporated in vacuo to give 535 mg of the alcohol intermediate 6-(2-hydroxyethoxy)-4-methyl-2H-chromen-2-one.

[0165] Alcohol intermediate 6-(2-hydroxyethoxy)-4-methyl-2H-chromen-2-one (208 mg, 0.000944 mol) was dissolved in 5 ml of DCM at 0°C. To this reaction mixture, triethylamine (0.145 ml, 0.00104 mol) was added followed by p-toluenesulfonylchloride (0.189 g, 0.000992 mmol), and warmed to room temperature. After stirring for 4 hours, the reaction mixture was partitioned between EtOAc and 1M aqueous HCl, and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with brine, dried on Na₂SO₄, filtered, and evaporated in vacuo. The residue was purified by flash chromatography on a small Biotage column eluting with 0-10% acetone/DCM. Fractions containing product were combined and evaporated in vacuo to give 243 mg of intermediate 4a as a colorless, clear oil.

[0166] To a solution of 5-(4-(2-(3-methoxyphenyl)-2-oxoethoxy)benzyl)-1,3-thiazolidine-2,4-dione (164 mg, 0.442 mmol) at 0 °C was added 1.0M of borontribromide in methylenechloride (5.0 ml, 5.0 mmol). The solution was warmed to room temperature and stirred for about 1 hour. The reaction mixture was evaporated in vacuo. The residue was partitioned between EtOAc and water, and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with brine, dried on Na₂SO₄, filtered, and evaporated in vacuo. The residue was dissolved in DCM and a minimal volume of acetone and chromatographed on a small Biotage column eluting with 0-10% acetone/DCM. Fractions containing product were combined and evaporated in vacuo to give 113 mg of intermediate 2a as a white solid.

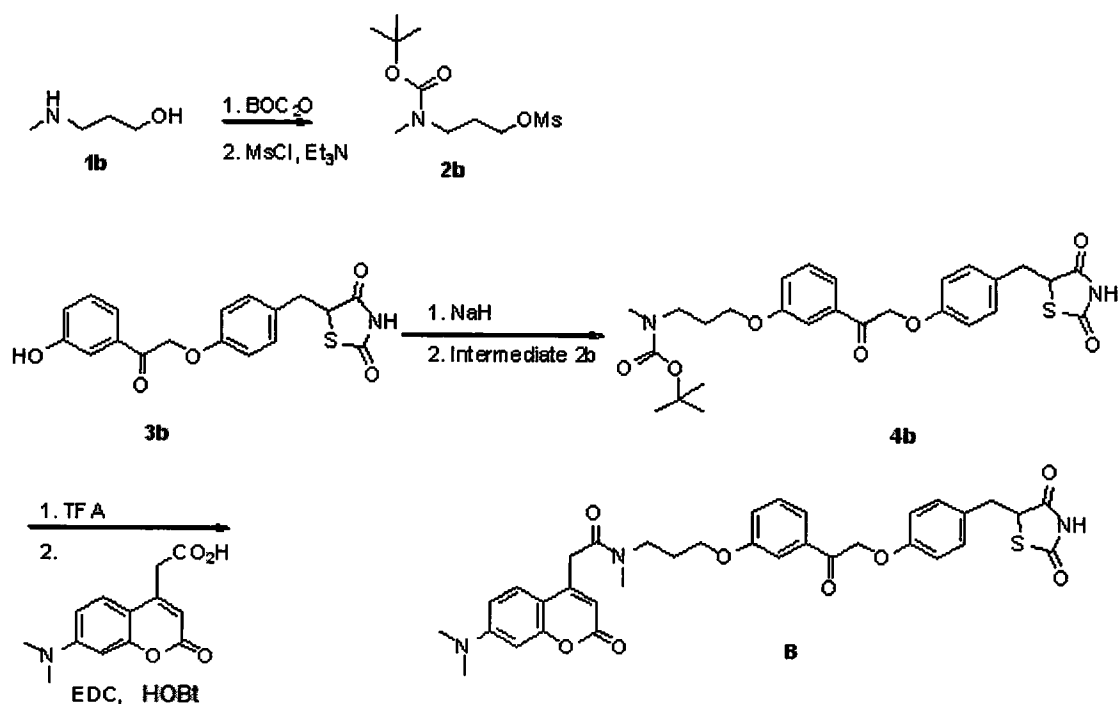
[0167] NaH was washed 3× with hexanes, suspended in DMF (1ml) and cooled to 0 °C. To this NaH was added a solution of 5-(4-(3-(3-hydroxyphenyl)-3-oxopropyl)benzyl)-1,3-

thiazolidine-2,4-dione (100 mg, 0.3 mmol) in DMF (0.75 ml). This mixture was stirred at room temperature for about 90 minutes, and a solution of 2-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)ethyl-4-methylbenzenesulfonate (111 mg, 0.295 mmol) in DMF (1 ml) was added. The reaction mixture was heated to 50 °C and left to stir overnight. The reaction mixture was evaporated in vacuo, and the residue was partitioned between EtOAc and water, and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with brine, dried on Na₂SO₄, filtered, and evaporated in vacuo. The residue was dissolved in DCM and a minimal volume of acetone and chromatographed on a small Biotage column eluting with 0-20% acetone/DCM. Fractions containing product were combined and evaporated in vacuo. The residue was treated with saturated NaHCO₃ and extracted 2× with EtOAc. Combined extracts were dried on Na₂SO₄, filtered and evaporated to give 24 mg of compound A as a white solid.

[0168] Compound A of formula I is characterized by the following ¹H NMR spectrum:

[0169] ¹H NMR (400MHz, DMSO-d₆): δ 12.02(brs, 1H), 7.71(d, J = 8.7Hz, 1H), 7.61(m, 2H), 7.50(t, J = 7.9Hz, 1H), 7.33(dd, J = 8.2, 2.4Hz, 1H), 7.15(d, J = 8.5Hz, 2H), 7.05(m, 2H), 6.91(d, J = 8.5Hz, 2H), 6.23(s, 1H), 5.55(s, 2H), 4.87(dd, J = 9.2, 4.3Hz, 1H), 4.47(m, 4H), 3.30(m, 1H), 3.05(dd, J = 14.2, 9.2Hz, 1H), 2.40(s, 3H). Mass spectrum: 560.0 m/z (M+1), 558.2 m/z (M-1).

[0170] Scheme 2:



[0171] The starting material (**1b**) undergoes protection of its secondary amine group using

any suitable protecting group (e.g., a BOC protecting group) followed by conversion of the primary alcohol functionality to a leaving group (e.g., a mesyl or tosyl group) to generate intermediate (2b). Starting material (3b) is treated with a strong base (e.g., NaH or the like) and reacted with intermediate (2b) to generate the BOC-protected intermediate (4b), which is deprotected with a strong acid (e.g., trifluoroacetic acid (TFA)) and reacted with an optionally substituted 2-(2-oxo-2H-chromen-4-yl)acetic acid in the presence of a coupling reagent(s), (e.g., EDC and HOBt) to generate exemplary compound B of Formula II.

[0172] In some embodiments, candidate compounds that block the interaction of a compound labeled with an exemplified probe of formula I or formula II and which is known to specifically and/or directly bind to a fluorescently tagged mTOT protein will result in a decrease in fluorescence. Such blocking candidate compounds are potentially active agents with respect to metabolic diseases and can be validated using assays described in Examples 1 and 2. In some embodiments, the fluorescence based assays described herein may use intact cells or membranes isolated from cells that express the fluorescence tagged mTOT protein. In one embodiment, the fluorescence tagged mTOT protein can include any one of GFP labeled mTOT protein. The reduction of the fluorescence transfer by binding of the candidate compound to the mTOT protein, competing with the known target binding compound, will help select compounds to pursue for possible anti diabetic, anti-obesity, metabolic protective, or neuroprotective therapeutics that are discovered using a new non-PPAR γ activity pathway.

[0173] Other assays may be used to identify candidate compounds capable of exerting some metabolic disease therapeutic effect, including assays that identify candidate compounds operable to bind to the mTOT protein through measuring direct binding of candidate compounds to the mTOT protein. Other exemplary screening methods employ direct binding between an mTOT protein and a TZD that is known to bind to the mTOT protein of interest, for example, BP44 and mitoglitazone. After the combination of an mTOT protein and a known binding TZD to form a binding complex, candidate compounds are added to the binding complex one at a time and the dissociation of the mTOT protein from the TZD, for example, mitoglitazone can be detected using affinity ultrafiltration and ion spray mass spectroscopy/HPLC or other physical and analytical methods. Candidate compounds that inhibit the specific binding of known compounds that bind to mTOT are then identified as lead candidate compounds.

[0174] 2. Yeast –Two-Hybrid Assays

[0175] In some embodiments, the binding of an mTOT protein with one or more candidate

compounds can be evaluated indirectly using the yeast two hybrid system described in Fields et al., *Nature*, 340:245-246 (1989), and Fields et al., *Trends in Genetics*, 10:286-292 (1994), both of which are incorporated herein by reference in its entirety.

[0176] The two-hybrid system is a genetic assay for detecting interactions between two proteins or polypeptides. It can be used to identify proteins that bind to a known protein of interest, or to delineate domains or residues critical for an interaction. Variations on this methodology have been developed to clone genes that encode DNA binding proteins, to identify peptides that bind to a protein, and to screen for drugs. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA binding domain that binds to an upstream activation sequence (UAS) of a reporter gene, and is generally performed in yeast. In some embodiments, the assay requires the construction of two hybrid genes encoding (1) a DNA-binding domain that is fused to a first protein and (2) an activation domain fused to a second protein. The DNA-binding domain targets the first hybrid protein to the UAS of the reporter gene; however, because most proteins lack an activation domain, this DNA-binding hybrid protein does not activate transcription of the reporter gene. The second hybrid protein, which contains the activation domain, cannot by itself activate expression of the reporter gene because it does not bind the UAS. However, when both hybrid proteins are present, the non-covalent interaction of the first and second proteins tethers the activation domain to the UAS, activating transcription of the reporter gene. For example, when the first protein is an mTOT protein, or fragment thereof, that is known to interact with another protein or nucleic acid, this assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene is monitored as different candidate compounds are added to the system. The presence of an inhibitory agent (for example a competitive binding compound) results in lack of a reporter signal.

[0177] The yeast two-hybrid screening assay of the present invention can also be used to identify proteins that bind to the gene product. In an assay to identify proteins that bind to an mTOT protein, or a functional fragment thereof, a fusion polynucleotide encoding both an mTOT protein (or fragment) and a UAS binding domain (i.e., a first protein) may be used. In addition, a large number of hybrid genes each encoding a different second protein fused to an activation domain are produced and screened in the assay. Typically, the second protein is encoded by one or more members of a total cDNA or genomic DNA fusion library, with each second protein-coding region being fused to the activation domain. This system is applicable to a wide variety of proteins, and it is not even necessary to know the identity or function of

the second binding protein. The system is highly sensitive and can detect interactions not revealed by other methods; even transient interactions may trigger transcription to produce a stable mRNA that can be repeatedly translated to yield the reporter protein.

[0178] 3. Protein Folding Assays

[0179] Other direct binding assays that find utility in the present screening methods rely on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a candidate compound binds to the folded form of an mTOT protein (i.e., when the candidate compound specifically binds to the mTOT protein), the mTOT protein conformation bound by the candidate protein remains in its folded state. Thus, the folded mTOT protein is present in a sample in a greater proportion in the presence of the candidate compound that binds the mTOT protein, than in the absence of the binding candidate compound. The quantitative measurement of bound candidate compound to the mTOT protein can be determined by any method that distinguishes between the folded and unfolded states of the mTOT protein, for example, by adding an antibody that recognizes the folded state but not the unfolded state and vice versa. Then the amount of bound antibody can be measured and quantified using standard immunological procedures, for example Enzyme Linked Immunosorbent Assay (ELISA). Antibodies to the mTOT proteins, for example, BP44 and BRP44-Like are known and commercially available. The function of the mTOT protein need not necessarily be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a candidate compound, including, but not limited to, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

[0180] 4. Competitive Binding Assays

[0181] In some embodiments, the binding assays of the present invention involve measuring the binding of a candidate compound in the presence or absence of a competitive compound that is known to bind to the mTOT protein used in the assay. In some embodiments, the competitive binding assay to identify a lead candidate compound effective against an insulin resistance disease or disorder includes the steps: (a) providing a candidate compound and at least one mTOT protein; (b) incubating the at least one mTOT protein with a competitor compound in the presence of the candidate compound to produce a test combination; (c) incubating the at least one mTOT protein with said competitor compound in the absence of the candidate compound to produce a corresponding control combination; (d) measuring the amount of the competitor compound bound to the mTOT protein in the test combination and in the control combination; and (e) selecting as a lead candidate compound any candidate

compound that causes a measurable decrease in the amount of competitor compound bound to the mTOT protein measured in step (d) in the test combination relative to the control combination.

[0182] The magnitude in reduction of competitor compound bound to the mTOT protein in the test combination relative to the control combination reflects the affinity of the candidate compound for the at least one mTOT protein. In some embodiments, the method further includes repeating steps (b)-(d) in a high throughput screen.

[0183] In some embodiments, providing the candidate compound and at least one mTOT protein can include affixing the mTOT protein to a solid substrate, for example, a glass slide, a silicon chip, or other solid substrates used in high-throughput applications in which 1 to 1,000 reaction spots are printed or spotted in an array format. In some embodiments, providing the candidate compound and at least one mTOT protein is carried out in solution in a reaction vessel or some containment receptacle. In some embodiments, providing the candidate compound and at least one mTOT protein can include admixing the mTOT protein and candidate compound in solution contained within a reaction vessel or receptacle. In some embodiments, a reaction vessel or receptacle can include a well in a microtiter plate, or other multi-well plate, or plastic or other solid polymeric vial or glass vial or tubes, eppendorf tubes or such containers or liquid handling devices conventionally used in chemistry and biological syntheses and reactions.

[0184] In some embodiments, the mTOT protein can include an isolated mTOT protein or a fusion construct thereof. In some embodiments, the mTOT protein is unlabeled. In some embodiments, the mTOT protein is a fusion of an mTOT protein and a tag or label which facilitates specific binding or isolation from a mixture using the tag or label, for example a GST, c-myc, heme-agglutinin (HA), avidin, streptavidin, biotin, FLAG, hexa-Histidine (His6), a fluorophore or fluorescent protein (e.g. GFP, EGFP, DsRed and other fluorescent proteins described herein) and the like. In the competitive assays of the present invention, the mTOT protein can include at least one of: a BP44 protein, a BRP44-Like protein or a heterodimer of BP44-BRP44Like proteins. In some embodiments, the mTOT protein is a human BP44, BRP44-Like or a heterodimer of BP44-BRP44Like proteins. In some embodiments, the mTOT protein is a protein provided in tables 1,3,4 and 5 and Figures 3A-4B.

[0185] In some embodiments, the competitor compound can be a TZD which is known to bind to a target compound as described herein. The competitor compound can be any one of mitoglitazone, rosiglitazone, pioglitazone, MSDC-160, UK5099, or troglitazone. In some embodiments, the competitor compound can be labeled with a radionuclide, protein tag or a

fluorescent molecule or the competitor compound is unlabelled, but competes with a labeled photoaffinity crosslinker, such as ^{125}I MSDC -1101 as shown in FIG. 9B. Competitive assays useful in the present screening methods are described in the examples below, for example, with reference to FIG. 9C. In some embodiments of the present invention, direct binding screening methods can comprise using competitive screening assays in which a neutralizing antibodies capable of binding to an mTOT protein, specifically compete with a candidate compound for binding to the mTOT protein. In some embodiments, the competitor compound is a radiolabelled antibody capable of specifically binding to an mTOT protein. Such antibodies have been described herein. Other labels such as radiolabeled competitive binding studies are described in A. H. Lin et al., *Antimicrobial Agents and Chemotherapy*, 1997, vol. 41, no. 10. pp. 2127-2131, the disclosure of which is incorporated herein by reference in its entirety.

[0186] In various examples of the competitive assays of the present invention, the competitor compound is labeled with, for example, a radioactive isotope, for example, ^3H , ^{32}P , ^{35}S or ^{14}C . In some embodiments, the competitor compound is a TZD compound labeled with a radioisotope. In some embodiments, the competitor compound is radiolabelled mitoglitazone or radiolabelled rosiglitazone. In some embodiments, the competitor compound is a TZD compound labeled with ^3H . In some embodiments, the competitor compound is ^3H mitoglitazone or ^3H rosiglitazone. In some embodiments, the competitor compound is ^3H mitoglitazone. In some embodiments, the competitor compound is ^3H rosiglitazone.

[0187] In various embodiments, measuring the amount of the competitor compound bound to the mTOT protein in the test combination and in the control combination can include determining the amount of competitor compound bound to the mTOT protein. Alternatively, measuring the amount of the competitor compound bound to the mTOT protein in the test combination and in the control combination can include determining the amount of candidate compound bound to the competitor compound and by logical operation, the more candidate compound bound to the mTOT protein indicates that less competitor compound is bound to the mTOT protein. In some embodiments, the competitive assays of the present invention can include the step of measuring the amount of labeled competitor compound bound to an mTOT protein by measuring the amount of labeled competitor compound remaining in solution or attached to a solid substrate after incubation with a non-labeled candidate compound. In one example, measuring the amount of the competitor compound bound to the mTOT protein in the test combination and in the control combination includes measuring the

amount of labeled competitor compound remaining bound to the mTOT protein after removal of unbound candidate compound and unbound labeled competitor compound. If the candidate compound reduces the amount of labeled competitor compound bound to the mTOT protein in the test combination relative to the control combination, then the candidate compound is a lead candidate compound. In the various direct binding assays described herein, an optional step can include a subsequent activity assay to identify whether the bound candidate compound is a therapeutic active agent. Once a candidate compound has been identified as a compound that specifically binds to an mTOT protein it is a lead candidate compound. Lead candidate compounds can be confirmed as being a therapeutic active agent by further evaluating its activity in activity assays used to confirm the insulin sensitizing or anti-diabetic activity of thiazolidinediones, such as mitoglitazone. Representative activity assays include the mitochondrial membrane competitive binding crosslinking assay, the induction of BP44 protein expression in brown adipose tissue assay, and the *Drosophila melanogaster* model of diet-induced insulin resistance assay. Representative activity assays are further described in the examples section.

[0188] 5. High-Throughput Assays

[0189] In some embodiments, an illustrative high-throughput screening method for identifying candidate compounds capable of directly binding to an mTOT protein is described in Wieboldt et al., Anal. Chem., 69:1683-1691 (1997), incorporated herein by reference in its entirety. This assay method is capable of screening in high-throughput fashion combinatorial libraries of 20-30 agents at a time in solution phase for binding to the mTOT protein. In some embodiments, candidate compounds that bind to the mTOT protein can be separated from other library components by simple membrane washing. The bound candidate compound(s) that are retained on the filter can be subsequently liberated from the mTOT protein, and analyzed by HPLC and electrospray (ion spray) ionization mass spectroscopy. This procedure selects candidate compounds originally present in the library with the greatest affinity for the mTOT protein, and is particularly useful for small molecule libraries.

[0190] B. Over-Expression Assays

[0191] In some embodiments, binding between a candidate compound and an mTOT protein can be measured by over expressing the mTOT protein on the surface of a cell or over expressing the mTOT protein within a cell as described above. As a result of such over expression, some measureable effect occurring within the cell as a result can be made. For example, HEK 293 cells, can be cultured in vitro to express the mTOT protein in either a transient or stable fashion using the methods herein for expressing an mTOT protein in

prokaryotic or eukaryotic cells recombinantly. Other human or mammalian cell lines amenable for expression of an mTOT protein either on a membrane surface or expressed intracellularly (nuclear, organelle or cytoplasmic, for example, expressed on the surface of a mitochondrial membrane) may be employed in the present assays. While not wishing to be bound by any particular theory, it is believed that the mTOT protein over expression can result in changes in cell function relative to control cells (non-transfected or cells not over-expressing the mTOT protein). In some embodiments, changes in intracellular calcium will occur due to over expression of the mTOT protein in cell culture, for example, HEK 293 cells. Such calcium levels in the cell can be determined using a calcium-sensitive dye. Candidate compound is added to a tissue culture medium (at concentrations ranging from 0.01-50 micromolar) harboring the cells over expressing the mTOT protein. Specific binding between candidate compound and mTOT protein can be detected as reversal of the calcium fluctuations and return them to levels of intracellular calcium shown in control cells without overexpression of the mTOT protein. An illustrative example of such an assay is as follows. Cells over expressing the mTOT protein fail to grow, but the additions of a positive modulator, for example mitoglitazone, known to have positive metabolic activity return the cells to normal growth. This system can then be used in vitro or in vivo to select other candidate compounds that can bind to the mTOT protein and in a similar fashion to the positive modulator mitoglitazone, return the cells to normal growth. Such candidate compounds can then be evaluated and validated using other activity assays for anti-diabetic, anti-obesity, metabolic protective, or neuroprotective actions as described herein.

[0192] C. Intrinsic Fluorescence Assays

[0193] Determining whether a test compound binds to an mTOT protein can also be accomplished by measuring the intrinsic fluorescence of the mTOT protein and determining whether the intrinsic fluorescence is modulated in the presence of a candidate compound. Preferably, the intrinsic fluorescence of the mTOT protein is measured as a function of the tryptophan residue(s) of the mTOT protein. Preferably, fluorescence of the mTOT protein is measured and compared to the fluorescence intensity of the mTOT protein in the presence of the candidate compound, wherein a decrease in fluorescence intensity indicates binding of the test compound to an mTOT protein. In some embodiments, a method for performing the intrinsic fluorescence measurement is set forth in "Principles of Fluorescence Spectroscopy" by Joseph R. Lakowicz, New York, Plenum Press, 1983 (ISBN 0306412853) and "Spectrophotometry And Spectrofluorometry" by C. L. Bashford and D. A. Harris Oxford, Washington D.C., IRL Press, 1987, each of which is incorporated herein by reference in its

entirety.

[0194] In the various direct binding assays described herein, an optional step can include a subsequent activity assay to identify whether the bound candidate compound is a therapeutic active agent. Once a candidate compound has been identified as a compound that specifically binds to an mTOT protein it is a lead candidate compound. Lead candidate compounds can be confirmed as being a therapeutic active agent by further evaluating its activity in standard known activity assays used to confirm the activity of thiazolidinediones, such as mitoglitazone. Representative activity assays include the mitochondrial membrane competitive binding crosslinking assay, the induction of BP44 protein expression in brown adipose tissue assay, and the *Drosophila melanogaster* model of diet-induced insulin resistance assay.

[0195] III. FUNCTIONAL NUCLEIC ACIDS THAT INHIBIT THE EXPRESSION OF MTOT PROTEIN GENE PRODUCTS

[0196] In further aspects, the present invention is also drawn to the discovery of a previously unrecognized target of the insulin sensitizers TZDs called mTOT protein. As used herein mTOT proteins and mTOT protein are used interchangeably. Because mTOT proteins of the present invention are well conserved phylogenetically, the present inventors evaluated whether not a fly mTOT protein could bind and respond to insulin sensitizers. Indeed, mitochondrial fractions from wild type flies contained a protein of about 18-19 kDa that specifically interacted with the TZD probe. Since informatics indicated that the mTOT protein ortholog, CG9399 (SEQ ID NOs: 31-33) was a member of a small protein family, which also contained another well-conserved orthog in flies, the present inventors obtained several fly lines in which either of the orthologs were knocked down.

[0197] Unexpectedly, binding of the TZD probe was lost with the knockdown of either mTOT proteins BP44 or BRP44-Like gene product, suggesting that both mTOT proteins might be involved in a functional complex in regulating insulin sensitivity. The present inventors made antibodies to both mTOT proteins (BP44 and BRP44-Like) and found that either was able to immunoprecipitate the other mTOT protein to varying degrees, supporting the possibility of an mTOT protein complex (comprising BP44-BRP44-Like protein complex). The present inventors had previously developed a *Drosophila melanogaster* model of diet-induced insulin resistance that bears strong similarity to the pathophysiology of TZD in humans. Animals raised on a high sugar diet have elevated hemolymph glucose and reduced longevity compared to those reared on control diets. Treatment of these flies with

compounds that bound this mTOT protein complex both lowered glucose and extended life span, while a modified analog that did not bind this complex was ineffective.

[0198] In one aspect of the present invention, the inventors have conducted preliminary studies which indicate that the knock down of either the mTOT protein BP44 or BRP44-Like gene products, produces reduced hemolymph glucose levels and extends life span as do the active drugs, suggesting that the TZDs may be attenuating the activity of the recently identified mitochondrial mTOT protein complex.

[0199] The present invention provides compositions and methods for modulating mTOT protein gene product activity involved in insulin sensitivity.

[0200] In another aspect, the present invention is directed to compounds, particularly functional nucleic acids, for example, antisense oligonucleotides, which are targeted to a nucleic acid encoding an mTOT protein as provided herein. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of an mTOT protein in cells, tissues or in an organism. In some embodiments, the method comprises contacting said cells, tissues or organism with one or more functional nucleic acid compounds or compositions of the present invention to inhibit, reduce or prevent the expression and/or activity of an mTOT protein in the cell, tissue or organism. Further provided are methods of treating an animal, particularly a mammal, and more particularly a human, having, or suspected of having, or being prone to a disease or condition associated with expression of an mTOT protein, for example, diabetes mellitus, cardiovascular disease, gastrointestinal disease, or Alzheimer's disease, by administering a therapeutically or prophylactically effective amount of one or more of the functional nucleic acid compounds or compositions of the invention.

[0201] In another embodiment, a functional nucleic acid, for example, an antisense oligonucleotide which modulates the expression of an mTOT protein gene product is administered in a liposome formulation. In some embodiments, the liposome formulation is an amphoteric liposome formulation. In some embodiments, the amphoteric liposome formulation comprises one or more amphoteric lipids. In some embodiments, the amphoteric liposome is formed from a lipid phase comprising a mixture of lipid components with amphoteric properties, which may, for example, be selected from the group consisting of (i) a stable cationic lipid and a chargeable anionic lipid, (ii) a chargeable cationic lipid and chargeable anionic lipid and (iii) a stable anionic lipid and a chargeable cationic lipid. In some embodiments, the liposome may further comprise neutral lipids.

[0202] In some embodiments, the amphoteric liposomes comprise DOPE, POPC, CHEMS and MoChol. In some embodiments, the molar ratio of POPC/DOPE/MoChol/CHEMS is about 6/24/47/23.

[0203] In another aspect, the present invention provides co-therapies comprising a functional nucleic acid compound that hybridizes to at least a portion of oligonucleotide sequence selected from SEQ ID NOs: 5-7, 52-73 or the complement thereof, and another TZD therapy agent, wherein the TZD therapy agent is selected from a therapeutically effective amount of mitoglitazone, pioglitazone, rosiglitazone or combinations thereof.

[0204] In another embodiment, the functional nucleic acid compound includes an additional oligonucleotide or oligonucleoside. The additional functional nucleic acid can include any one of SEQ ID NOs: 54-73 or the complementary nucleotide sequence thereof.

[0205] In yet another embodiment, the oligonucleotides are between 15 and 35 base pairs in length. In still another embodiment, the oligonucleotides have a phosphorothiolate backbone.

[0206] In another embodiment, the method of treating a subject with an insulin insensitivity disorder, for example, diabetes mellitus, metabolic syndrome, cardiovascular disease, gastrointestinal disease or Alzheimer's disease, where the daily dose of functional nucleic acid compound is from 0.1 mg/m² to 300 mg/m² oligomer of body surface area of a patient.

[0207] In other embodiments, the oligonucleotide is administered intravenously to a diabetes type II patient.

[0208] The present invention utilizes the finding that mTOT proteins, for example, BP44 proteins, BRP44 Like proteins, or BP44-BRP44-Like heterodimers among others exemplified herein, are specific targets of thiazolidinedione compounds, for example, rosiglitazone and PPAR γ sparring mitoglitazone. Compounds that bind to mTOT proteins are postulated to play a role in insulin sensitization, particularly at sites involving the mitochondria.

[0209] In one embodiment, an inhibitor of mTOT protein gene expression can be a functional nucleic acid. As used herein, the category of "functional nucleic acids" encompasses siRNA molecules, shRNA molecules, miRNA molecules, and antisense nucleic acid molecules. The term "siRNA" refers to small inhibitory RNA duplexes that induce the RNA interference (RNAi) pathway. These molecules can vary in length (generally 18-30 base pairs) and contain varying degrees of complementarity to their target mRNA in the antisense strand. Some, but not all, siRNA have unpaired overhanging bases on the 5' or 3' end of the sense strand and/or the antisense strand. The term "siRNA" includes duplexes of two separate strands, as well as single strands that can form hairpin structures comprising a duplex region.

[0210] As used herein, the terms "an oligonucleotide having a nucleotide sequence encoding a gene" and "polynucleotide having a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene, or in other words the nucleic acid sequence that encodes a gene product. The coding region may be present in a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide or polynucleotide may be single-stranded (i.e., the sense strand) or double-stranded.

[0211] As used herein, the term "oligonucleotide," refers to a short length of single-stranded polynucleotide chain. Oligonucleotides are typically less than 200 residues long (e.g., between 8 and 100), however, as used herein, the term is also intended to encompass longer polynucleotide chains (e.g., as large as 5000 residues). Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by hybridizing to other polynucleotides. Such structures can include, but are not limited to, duplexes, hairpins, cruciforms, bends, and triplexes.

[0212] As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (i.e., a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T", is complementary to the sequence "T-C-A". Complementarity may be "partial", in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.

[0213] As used herein, the term "completely complementary," for example when used in reference to a functional nucleic acid compound of the present invention refers to an functional nucleic acid, for example an antisense oligonucleotide where all of the nucleotides are complementary to a target sequence (e.g., a gene).

[0214] As used herein, the term "partially complementary," for example when used in reference to a functional nucleic acid compound of the present invention, refers to an functional nucleic acid, for example an antisense oligonucleotide, where at least one nucleotide is not complementary to the target sequence. Exemplary partially complementary oligonucleotides are those that can still hybridize to the target sequence under physiological conditions. The term "partially complementary" refers to oligonucleotides that have regions of one or more non-complementary nucleotides both internal to the oligonucleotide or at

either end. Oligonucleotides with mismatches at the ends may still hybridize to the target sequence.

[0215] The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is a nucleic acid molecule that at least partially inhibits a completely complementary nucleic acid molecule from hybridizing to a target nucleic acid is "substantially homologous". The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous nucleic acid molecule to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target that is substantially non-complementary (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

[0216] When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any functional nucleic acid that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

[0217] When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any functional nucleic acid that can hybridize (i.e., it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

[0218] As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids. A single molecule that contains pairing of complementary nucleic acids within its structure is said to be "self-hybridized".

[0219] As used herein, the term " T_m " is used in reference to the "melting temperature". The melting temperature is the temperature at which a population of double-stranded nucleic

acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (See e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization [1985]). Other references include more sophisticated computations that take structural as well as sequence characteristics into account for the calculation of T_m .

[0220] As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. Under "low stringency conditions" a nucleic acid sequence of interest will hybridize to its exact complement, sequences with single base mismatches, closely related sequences (e.g., sequences with 90% or greater homology), and sequences having only partial homology (e.g., sequences with 50-90% homology). Under "medium stringency conditions," a nucleic acid sequence of interest will hybridize only to its exact complement, sequences with single base mismatches, and closely related sequences (e.g., 90% or greater homology). Under "high stringency conditions," a nucleic acid sequence of interest will hybridize only to its exact complement, and (depending on conditions such a temperature) sequences with single base mismatches. In other words, under conditions of high stringency the temperature can be raised so as to exclude hybridization to sequences with single base mismatches.

[0221] "High stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4 H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

[0222] "Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4 H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

[0223] "Low stringency conditions" comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4

H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

[0224] The present invention is not limited to the hybridization of probes of about 500 nucleotides in length. The present invention contemplates the use of probes between approximately 8 nucleotides up to several thousand (e.g., at least 5,000) nucleotides in length, for example, 8 to 5,000, or 8 to 2,000, or 8 to 1,000, or 8 to 500, or 8 to 250, or 8 to 200, or 8 to 100, or 8 to 75, or 8 to 50, or 8 to 30 nucleotides in length. One skilled in the relevant understands that stringency conditions may be altered for probes of other sizes (See e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization [1985] and Sambrook et al., Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2001, and Current Protocols in Molecular Biology, M. Ausubel et al., eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., and supplements through 2006)).

[0225] It is well known in the art that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions that promote hybridization under conditions of high stringency (e.g., increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.) (See definition above for "stringency").

[0226] As used herein, the term "physiological conditions" refers to specific stringency conditions that approximate or are conditions inside an animal (e.g., a human). Exemplary physiological conditions for use in vitro include, but are not limited to, 37°C, 95% air, 5% CO₂, commercial medium for culture of mammalian cells (e.g., DMEM media available from Gibco, MD), 5-10% serum (e.g., calf serum or horse serum), additional buffers, and optionally hormone (e.g., insulin and epidermal growth factor).

[0227] As used herein, the term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is

identified and separated from at least one component or contaminant with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids as nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding a given protein includes, by way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. In some embodiments, when an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to inhibit the translation of an mTOT protein, the oligonucleotide or polynucleotide will contain at a minimum the non-coding strand or the antisense strand that binds to the sense mRNA encoding an mTOT protein (i.e., the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (i.e., the oligonucleotide or polynucleotide may be double-stranded).

[0228] As used herein, the term "purified" or "to purify" refers to the removal of components (e.g., contaminants) from a sample. For example, recombinant polypeptides are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

[0229] In one example, the functional nucleic acid compounds of the present invention target the mTOT protein gene or a messenger RNA transcript or cDNA thereof. In some embodiments, exemplary functional nucleic acids hybridize to a coding sequence mRNA copy of the cDNA shown in SEQ ID NOs: 5-7 and 52-53 or a complementary nucleotide sequence thereof. In some embodiments, the exemplary functional nucleic acids stringently hybridize to a mRNA copy of the cDNA shown in SEQ ID NOs: 5-7 and 52-53. It is noted that mTOT protein cDNA sequences of SEQ ID NOs: 5-7 and 52-53 and mRNA sequences or complementary sequences thereof are merely exemplary mTOT protein mRNA sequences, other variant sequences (including splicing variants) which encode other isoforms of mTOT protein gene products, for example, human BP44 or BP44-Like polypeptides of Tables 1-5 and shown in Figures 3A, 3B, 4A and 4B are also contemplated herein.

[0230] A. siRNA Inhibitors of mTOT protein Gene Expression

[0231] The rational design process can involve the use of a computer program to evaluate the criteria for every sequence of 18-30 base pairs or only sequences of a fixed length, e.g., 19 base pairs. Preferably the computer program is designed such that it provides a report ranking of all of the potential siRNAs 18-30 base pairs, ranked according to which sequences generate the highest value. A higher value refers to a more efficient siRNA for a particular target gene. The computer program that may be used may be developed in any computer language that is known to be useful for scoring nucleotide sequences. Additionally, rather than run every sequence through one and/or another formula, one may compare a subset of the sequences, which may be desirable if for example only a subset are available. For instance, it may be desirable to first perform a BLAST (Basic Local Alignment Search Tool) search and to identify sequences that have no homology to other targets. Alternatively, it may be desirable to scan the sequence and to identify regions of moderate GC context, then perform relevant calculations using one of the above-described formulas on these regions. These calculations can be done manually or with the aid of a computer.

[0232] In some embodiments, inhibition of mTOT protein genes, includes inhibition of human BP44, and/or human BRP44-Like genes, i.e. genomic DNA, cDNA, mRNA, mutant or alternative splice variants thereof, or complementary nucleotide sequences thereof.

[0233] The term "target" is used in a variety of different forms throughout this disclosure and is defined by the context in which it is used. "Target mRNA" refers to a messenger RNA to which a given siRNA can be directed against. "Target sequence" and "target site" refer to a sequence within the mRNA to which the sense strand of an siRNA shows varying degrees of homology and the antisense strand exhibits varying degrees of complementarity. The phrase "siRNA target" can refer to the gene, mRNA, cDNA or protein against which an siRNA is directed. Similarly, "target silencing" can refer to the state of a gene, or the corresponding mRNA or protein.

[0234] The siRNA can comprise partially purified RNA, substantially pure RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, including modifications that make the siRNA resistant to nuclease digestion. The siRNA can be targeted to any stretch of approximately 18-30 contiguous nucleotides in any of the target mRNA sequences, for example, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30

nucleotides in each strand, wherein one of the strands is substantially complementary to, e.g., at least 80% (or more, e.g., 85%, 90%, 95%, or 100%) complementary to, e.g., having for example 3, 2, 1, or 0 mismatched nucleotide(s), a target mRNA sequence. Techniques for selecting target sequences for siRNA are provided, for example, in Tuschl, T. et al., "The siRNA User Guide", revised Oct. 11, 2002, the entire disclosure of which is herein incorporated by reference. "The siRNA User Guide" is available on the world wide web at a website maintained by Dr. Thomas Tuschl, Department of Cellular Biochemistry, AG 105, Max-Planck-Institute for Biophysical Chemistry, 37077 Gottingen, Germany, and can be found by accessing the website of the Max Planck Institute and searching with the keyword "siRNA". Thus, the sense strand of the present siRNA comprises a nucleotide sequence identical to any contiguous stretch of about 18 to about 30 nucleotides in the mTOT protein mRNA. In some embodiments, specific siRNAs for downregulating the activity and/or expression of an mTOT proteins can include: 5'-GCTGATGCTGCCCCGAGAAATT-3' (SEQ ID NO:54) starting at position 217 of SEQ ID NOs: 5 or 5'-GTGTGCTGGATTGGCTGATAT-3' (SEQ ID NO: 55) starting at position 313 of SEQ ID NO: 5.

[0235] In some embodiments, one or both strands of the siRNA can also comprise a 3' overhang. As used herein, a "3' overhang" refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand. In some embodiments, the siRNA comprises at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxynucleotides) in length, preferably from 1 to about 5 nucleotides in length, more preferably from 1 to about 4 nucleotides in length, and particularly preferably from about 2 to about 4 nucleotides in length. In the embodiment in which both strands of the siRNA molecule comprise a 3' overhang, the length of the overhangs can be the same or different for each strand. In some embodiments, the 3' overhang is present on both strands of the siRNA, and is 2 nucleotides in length. For example, each strand of the siRNA can comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

[0236] In some embodiments, one or both strands of the siRNA can also comprise a hairpin insert. As used herein, a "hairpin insert" refers to at least one nucleotide insert ranging from about 3 to about 10 nucleotides, preferably from 3 to 6 nucleotides in length positioned within the siRNA sequence, for example approximating the center of the sequence. In some embodiments, exemplary siRNA sequences useful in the treatment, inhibition or prevention methods of the present invention can comprise or consist of: 5'-

GCTGATGCTGCCCCGAGAAATT-3' (SEQ ID NO: 56) starting at position 217 of SEQ ID

NO: 5 or 5'-GTGTGCTGGATTGGCTGATAT-3' (SEQ ID NO: 57) starting at position 313 of SEQ ID NO: 5 or 5'-GGCTTATCAAACACGAGATGA-3' (SEQ ID NO: 58) starting at position 412 of SEQ ID NO: 52 or 5'-GCTGCTGAGTCACAGATTTC-3' (SEQ ID NO: 59) starting at position 500 of SEQ ID NO: 52 or 5'-GCTGCCTTACAAGTATTAAAT-3' (SEQ ID NO: 60) starting at position 618 of SEQ ID NO: 52 or 5'-GTATTTCCATGCAGTGTATAT-3' (SEQ ID NO: 61) starting at position 892 of SEQ ID NO: 52 which can be chemically synthesized and annealed. These rationally designed siRNAs can be synthesized and may be commercially available from Dharmacon or InvivoGen.

[0237] In still other embodiments, siRNAs, shRNAs and lentiviral vectors capable of expressing siRNA or shRNA sequences useful in inhibiting mTOT protein expression and/or activity are commercially available under Catalog Nos.: sc-141751, and sc-95332 from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

[0238] In further embodiments, siRNA useful in inhibiting the expression of mTOT protein proteins in vitro and in vivo can be obtained using a number of techniques known to those of skill in the art. For example, the siRNA can be chemically synthesized or recombinantly produced using methods known in the art, such as the *Drosophila* in vitro system described in U.S. Patent Application Publication No. 2002/0086356, filed March 30, 2001 to Tuschl et al., the entire disclosure of which is herein incorporated by reference.

[0239] Preferably, the siRNA are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo. USA), Pierce Chemical (part of Perbio Science, Rockford, Ill. USA), Glen Research (Sterling, Va. USA), ChemGenes (Ashland, Mass. USA) and Cruachem (Glasgow, UK).

[0240] Alternatively, siRNA can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing siRNA from a plasmid include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment.

[0241] The siRNA expressed from recombinant plasmids can either be isolated from cultured cell expression systems by standard techniques, or can be expressed intracellularly at or near the area of neovascularization in vivo. The use of recombinant plasmids to deliver siRNA to cells in vivo is discussed in more detail below.

[0242] siRNA can be expressed from a recombinant plasmid either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

[0243] Selection of plasmids suitable for expressing siRNA, methods for inserting nucleic acid sequences for expressing the siRNA into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the skill in the art. See, for example Tuschl, T. (2002), Nat. Biotechnol, 20: 446-448; Brummelkamp T R et al. (2002), Science 296: 550-553; Miyagishi M et al. (2002), Nat. Biotechnol. 20: 497-500; Paddison P J et al. (2002), Genes Dev. 16: 948-958; Lee N S et al. (2002), Nat. Biotechnol. 20: 500-505; and Paul C P et al. (2002), Nat. Biotechnol. 20: 505-508, the entire disclosures of which are herein incorporated by reference. A plasmid comprising nucleic acid sequences for expressing an siRNA can comprise a sense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter, and an antisense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter. The plasmid is ultimately intended for use in producing a recombinant adeno-associated viral vector or retroviral vector comprising the same nucleic acid sequences for expressing an siRNA in vivo or in vitro upon appropriate transfection. An exemplary selective siRNA with hairpin insert pairs operable to be used in a plasmid for expressing the shRNA can comprise or consist of the pair:

5'-GCTGATGCTGCCCCGAGAAATTCAAGAGAATTCTCGGGCAGCATCAGC-3'
(SEQ ID NO: 62) &

5'-GCTGATGCTGCCCCGAGAAATTCTCTTGAAATTTCTCGGGCAGCATCAGC-3'
(SEQ ID NO: 63).

[0244] In other embodiments, exemplary selective siRNA with hairpin insert oligonucleotide pairs operable to be used in a plasmid for expressing the shRNA to inhibit an mTOT protein mRNA (cDNA of SEQ ID NO: 52) can comprise or consist of the pairs:

5'- TGTGCTGGATTGGCTGATATTCAAGAGATATCAGCCAATCCAGCACAC-3' (SEQ ID NO: 64) &

5'-GTGTGCTGGATTGGCTGATATCTCTTGAATATCAGCCAATCCAGCACAC-3'

(SEQ ID NO: 65); the pair: 5'

GGCTTATCAAACACGAGATGATCAAGAGTCATCTCGTGTTTGATAAGCC 3' (SEQ ID NO: 66) & 5'-

GGCTTATCAAACACGAGATGACTCTTGATCATCTCGTGTTTGATAAGCC-3' (SEQ ID NO: 67); the pair: 5'-

GCTGCTGAGTCACAGATTTTCATCAAGAGTGAAATCTGTGACTCAGCAGC-3' (SEQ ID NO: 68) & 5'-

GCTGCTGAGTCACAGATTTCACTCTTGATGAAATCTGTGACTCAGCAGC-3' (SEQ ID NO: 69); the pair: 5'-

GCTGCCTTACAAGTATTAAATTCAAGAGATTTAATACTTGTAAGGCAGC-3' (SEQ ID NO: 70) & 5'-

GCTGCCTTACAAGTATTAAATCTCTTGAATTTAATACTTGTAAGGCAGC-3' (SEQ ID NO: 71); the pair 5'-

GTATTTCCATGCAGTGTATATTCAAGAGATATACACTGCATGGAAATAC-3' (SEQ ID NO: 72) & 5'-

GTATTTCCATGCAGTGTATATCTCTTGAATATACACTGCATGGAAATAC-3' (SEQ ID NO: 73).

[0245] As used herein, "in operable connection with a polyT termination sequence" means that the nucleic acid sequences encoding the sense or antisense strands are immediately adjacent to the polyT termination signal in the 5' direction. During transcription of the sense or antisense sequences from the plasmid, the polyT termination signals act to terminate transcription.

[0246] As used herein, "under the control" of a promoter means that the nucleic acid sequences encoding the sense or antisense strands are located 3' of the promoter, so that the promoter can initiate transcription of the sense or antisense coding sequences.

[0247] The siRNA can also be expressed from recombinant viral vectors intracellularly at or near the area of the mTOT protein gene expressed in vitro or in vivo. The recombinant viral vectors of the invention comprise sequences encoding the siRNA and any suitable promoter for expressing the siRNA sequences. Suitable promoters include, in addition to others described specifically herein, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment.

[0248] In some embodiments, the siRNA can be expressed from a recombinant viral vector either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

[0249] Any viral vector capable of accepting the coding sequences for the siRNA molecule(s) to be expressed can be used, for example vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (e.g., lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of the viral vectors can also be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses. For example, an AAV vector of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like.

[0250] Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing the siRNA into the vector, and methods of delivering the viral vector to the cells of interest are within the skill in the art. See, for example, Dornburg R (1995), *Gene Therap.* 2: 301-310; Eglitis M A (1988), *Biotechniques* 6: 608-614; Miller A D (1990), *Hum Gene Therap.* 1: 5-14; and Anderson W F (1998), *Nature* 392: 25-30, the entire disclosures of which are herein incorporated by reference. In some cases, a commercially available viral delivery system can be used (see, e.g., vectors for siRNA delivery that are available from Ambion, Austin, Tex.). Other methods for delivery are known to those in the art (e.g., siRNA Delivery Centre, sirna.dk/index.html).

[0251] Preferred viral vectors are those derived from AV and AAV. In a particularly preferred embodiment, the siRNA is expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector comprising, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter.

[0252] A suitable AV vector for expressing the siRNA, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H et al. (2002), *Nat. Biotech.* 20: 1006-1010.

[0253] Suitable AAV vectors for expressing the siRNA, methods for constructing the recombinant AAV vector, and methods for delivering the vectors into target cells are described in Samulski R et al. (1987), *J. Virol.* 61: 3096-3101; Fisher K J et al. (1996), *J. Virol.*, 70: 520-532; Samulski R et al. (1989), *J. Virol.* 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

[0254] The ability of an siRNA containing a given target sequence to cause RNAi-mediated degradation of the mTOT protein encoding mRNA can be evaluated using standard techniques for measuring the levels of RNA or protein in cells. For example, siRNA can be delivered to cultured cells, and the levels of target mRNA can be measured by Northern blot or dot blotting techniques, or by quantitative RT-PCR. Alternatively, the levels of mTOT protein in the infected cells can be measured by ELISA or Western blot.

[0255] B. shRNA Inhibitory Molecules

[0256] In some embodiments, a shRNA nucleic acid molecule refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. One portion or segment of a duplex stem of the shRNA structure is anti-sense strand or complementary, e.g., fully complementary, to a section of about 18 to about 40 or more nucleotides of the mRNA of the target gene, for example, mTOT protein BP44 and/or BRP44-Like. In contrast to siRNAs, shRNAs mimic the natural precursors of micro RNAs (miRNAs). miRNAs are noncoding RNAs of approximately 22 nucleotides which can regulate gene expression at the post transcriptional or translational level during plant and animal development. One common feature of miRNAs is that they are all excised from an approximately 70 nucleotide precursor RNA stem-loop termed pre-miRNA, probably by Dicer, an RNase III-type enzyme, or a homolog thereof. Naturally-occurring miRNA precursors (pre-miRNA) have a single strand that forms a duplex stem including two portions that are generally complementary, and a loop, that connects the two portions of the stem. In typical pre-miRNAs, the stem includes one or more bulges, e.g., extra nucleotides that create a single nucleotide "loop" in one portion of the stem, and/or one or more unpaired nucleotides that create a gap in the hybridization of the two portions of the stem to each other. Short hairpin RNAs, or engineered RNA precursors, of the invention are artificial constructs based on these naturally occurring pre-miRNAs, but which are engineered to deliver desired RNAi agents (e.g., siRNAs of the invention). By substituting the stem sequences of the pre-miRNA with sequence complementary to the target mRNA, a shRNA is formed. The shRNA is processed by the entire gene silencing pathway of the cell, thereby efficiently mediating RNAi.

[0257] In some embodiments, the shRNA molecules of the invention are designed to produce any of the siRNAs described above when processed in a cell e.g., by Dicer present

within the cell. The requisite elements of a shRNA molecule include a first portion and a second portion, having sufficient complementarity to anneal or hybridize to form a duplex or double-stranded stem portion. The two portions need not be fully or perfectly complementary. The first and second "stem" portions are connected by a portion having a sequence that, has insufficient sequence complementarity to anneal or hybridize to other portions of the shRNA. This latter portion is referred to as a "loop" portion in the shRNA molecule. The shRNA molecules are processed to generate siRNAs. shRNAs can also include one or more bulges, i.e., extra nucleotides that create a small nucleotide "loop" in a portion of the stem, for example a one-, two- or three-nucleotide loop. The stem portions can be the same length, or one portion can include an overhang of, for example, 1-5 nucleotides. The overhanging nucleotides can include, for example, uracils (Us), e.g., all Us. Such Us are notably encoded by thymidines (Ts) in the shRNA-encoding DNA which signal the termination of transcription.

[0258] One strand of the stem portion of the shRNA is further sufficiently complementary (e.g., antisense) to a target RNA of mTOT protein (e.g., mRNA of BP44 or BRP44-Like, as provided in SEQ ID NO:5-7 & 52-53, or any mRNA encoding any of the mTOT proteins of Tables 1, 3, 4 and 5) sequence to mediate degradation or cleavage of said target RNA via RNA interference (RNAi). The antisense portion can be on the 5' or 3' end of the stem. The stem portions of a shRNA are preferably about 15 to about 50 nucleotides in length. Preferably the two stem portions are about 18 or 19 to about 25, 30, 35, 37, 38, 39, or 40 or more nucleotides in length. When used in mammalian cells, the length of the stem portions should be less than about 30 nucleotides to avoid provoking non-specific responses like the interferon pathway. In non-mammalian cells, the stem can be longer than 30 nucleotides. In fact, a stem portion can include much larger sections complementary to the target mRNA (up to, and including the entire mRNA). The two portions of the duplex stem must be sufficiently complementary to hybridize to form the duplex stem. Thus, the two portions can be, but need not be, fully or perfectly complementary.

[0259] The loop in the shRNAs or engineered RNA precursors may differ from natural pre-miRNA sequences by modifying the loop sequence to increase or decrease the number of paired nucleotides, or replacing all or part of the loop sequence with a tetraloop or other loop sequences. Thus, the loop portion in the shRNA can be about 2 to about 20 nucleotides in length, i.e., about 2, 3, 4, 5, 6, 7, 8, 9, or more, e.g., 15 or 20, or more nucleotides in length. A preferred loop consists of or comprises a "tetraloop" sequences. Exemplary tetraloop

sequences include, but are not limited to, the sequences GNRA, where N is any nucleotide and R is a purine nucleotide, GGGG, and UUUU.

[0260] In some embodiments, shRNAs of the invention include the sequences of a desired siRNA molecule described above. In other embodiments, the sequence of the antisense portion of a shRNA can be designed essentially as described above or generally by selecting an 18, 19, 20, 21 nucleotide, or longer, sequence from within the target RNA (e.g., mTOT protein mRNA), for example, from a region 100 to 200 or 300 nucleotides upstream or downstream of the start of translation. In general, the sequence can be selected from any portion of the target RNA (e.g., mRNA) including the 5' UTR (untranslated region), coding sequence, or 3' UTR, provided said portion is distant from the site of the gain-of-function mutation. This sequence can optionally follow immediately after a region of the target gene containing two adjacent AA nucleotides. The last two nucleotides of the nucleotide sequence can be selected to be UU. This 21 or so nucleotide sequence is used to create one portion of a duplex stem in the shRNA. This sequence can replace a stem portion of a wild-type pre-miRNA sequence, e.g., enzymatically, or is included in a complete sequence that is synthesized. For example, one can synthesize DNA oligonucleotides that encode the entire stem-loop engineered RNA precursor, or that encode just the portion to be inserted into the duplex stem of the precursor, and using restriction enzymes to build the engineered RNA precursor construct, e.g., from a wild-type pre-miRNA.

[0261] In some embodiments, the efficacy of the functional nucleic acids useful herein can be increased when the functional nucleic acids, for example, siRNAs are mTOT protein targeted, delivered systemically, repeatedly and safely. Low transfection efficiency, nuclease degeneration, poor tissue penetration and non-specific immune degradation can be overcome when the functional nucleic acids are incorporated into protective and functional vehicles, for example, viral vectors, liposomes complexed with polyethyleneimine (PEI), linked with vascular endothelial growth factor (VEGF) receptor- 2 and PEI that was PEGylated with an RGD peptide ligand at the distal end, protamine-antibody fusion protein, and tumor-targeting immunoliposome complexes. Any combination of these strategies can ameliorate and abrogate the above described problems previously seen with first generation delivery methods. In some embodiments, the functional nucleic acid is administered to the subject either as naked siRNA, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the siRNA.

[0262] C. Antisense Oligonucleotide Inhibitors of mTOT protein Proteins

[0263] Isolated functional nucleic acid molecules that are antisense to an mTOT protein nucleotide sequence are useful for reducing activity or expression of the mTOT protein mRNA or polypeptide. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

[0264] As used herein, the terms "target nucleic acid" and "nucleic acid encoding mTOT protein" encompass DNA encoding mTOT protein, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This inhibition of function of a target nucleic acid by compounds that specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. An "antisense" functional nucleic acid (antisense oligonucleotide) can include a nucleotide sequence that is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule, or complementary to an mRNA sequence, for example, as provided in any one or more of SEQ ID NOs: 5-7, 52 or 53. The antisense nucleic acid can be complementary to an entire mTOT protein coding strand, or to only a portion thereof (e.g., coding region of a human mTOT protein nucleotide sequence). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding an mTOT protein protein (e.g., the 5' or 3' untranslated regions).

[0265] An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally

occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used (see, e.g., *Protocols for Oligonucleotide Conjugates*. Totowa, N.J.: Humana Press, 1994; and commercially available services from, for example, Dharmacon, Lafayette, CO, USA. and Ambion, Austin, TX, USA.). The antisense nucleic acid also can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Antisense nucleic acids can also be produced from synthetic methods such as phosphoramidite methods, H-phosphonate methodology, and phosphite trimer methods. Antisense nucleic acids can also be produced by PCR methods. Such methods produce cDNA and cRNA sequences complementary to the mRNA.

[0266] In some embodiments, antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in inhibition of peptide synthesis, for example, inhibition in the expression of one or more mTOT protein proteins. Antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in inhibition of peptide synthesis, for example, inhibition in the expression of mTOT protein proteins. The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules can also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm.

[0267] An antisense nucleic acid can be an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other. The antisense nucleic acid molecule can also comprise a 2'-O-methylribonucleotide or a chimeric RNA-DNA analog, and can have mixed internucleoside linkages (see, e.g., *Protocols for Oligonucleotide Conjugates*. Totowa N.J.: Humana Press, 1994).

[0268] In some embodiments, methods for treating diabetes mellitus, metabolic syndrome, cardiovascular disease, gastrointestinal disease or Alzheimer's disease with one or more functional nucleic acid comprises administering a functional nucleic acid to a subject in need

thereof. In some embodiments, antisense nucleic acid molecules are typically administered to a subject (systemically, e.g., by intravenous administration, or locally, e.g. by direct injection at a tissue site), or are generated in situ such that they hybridize with or bind to cellular RNA (e.g., mRNA) and/or genomic DNA encoding an mTOT protein protein, for example, as provided in Tables 1, 3, 4, 5 and shown in Figures 3A, 3B, 4A and 4B and/or splice variants thereof, to thereby inhibit expression of the mTOT protein protein, e.g., by inhibiting transcription and/or translation. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies that bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter. Methods of administering antisense nucleic acid molecules are also known in the art (e.g., Wacheck et al. *Chemosensitisation of malignant melanoma by BCL2 antisense therapy* (2000) *Lancet* 356:1728-1733; Webb et al. *BCL-2 antisense therapy in patients with non-Hodgkin lymphoma* (1997) *Lancet* 349:1137-1141).

[0269] In some embodiments, mTOT protein gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the mTOT protein (e.g., the mTOT protein promoter and/or enhancers) to form triple helical structures that prevent transcription of an mTOT protein gene in target cells (see generally, Hurst, H.C., *Breast Cancer Res* 2001, 3:395-398; Helene (1991) *Anticancer Drug Des.* 6(6):569-84; Helene et al. (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *Bioassays* 14:807-15), these references are incorporated herein by reference in their entireties. The potential sequences that can be targeted for triple helix formation can be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

[0270] In some embodiments, the present invention employs functional nucleic acids, particularly antisense oligonucleotides, for use in inhibiting, reducing or preventing the expression of one or more mTOT proteins, ultimately inhibiting the amount of mTOT protein produced. Antisense technology is emerging as an effective means for reducing the

expression of specific gene products and is uniquely useful in a number of therapeutic, diagnostic, and research applications for the inhibition of mTOT protein.

[0271] The overall effect of such interference with target nucleic acid function is inhibition of the expression of an mTOT protein as described herein. In the context of the present invention, inhibition is the preferred form of gene expression and mRNA is a preferred target. In one embodiment of the present invention, the functional nucleic acids of the present invention inhibit expression of mTOT protein in vitro or in vivo, by at least 10% as measured in a suitable assay, such as those described in the examples below. In another embodiment, the compounds of the present invention inhibit expression of mTOT protein in vitro or in vivo, by at least 25%. In still another embodiment of the invention, the compounds of the present invention inhibit expression of mTOT protein in vitro or in vivo, by at least 40%. In yet a further embodiment of this invention, the compounds of the present invention inhibit expression of mTOT protein in vitro or in vivo, by at least 50%. In a further embodiment of this invention, the compounds of the present invention inhibit expression of mTOT protein in vitro or in vivo, by at least 60%. In another embodiment of this invention, the compounds of the present invention inhibit expression of mTOT protein in vitro or in vivo, by at least 70% or at least 80% or higher.

[0272] It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense oligonucleotide to a particular nucleic acid, in the context of this invention, is a multistep process. In some embodiments, the process begins with the identification of a nucleic acid sequence encoding an mTOT protein of interest for which the function is to be inhibited. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or inhibition of expression of the protein, results. An antisense nucleic acid can be designed such that it is complementary to the entire coding region of mTOT protein mRNA, but in general, is an oligonucleotide that is antisense to only a portion of the coding or noncoding region of mTOT protein mRNA.

[0273] Within the context of the present invention, one embodiment of an intragenic site for the mTOT protein is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of mTOT protein mRNA, e.g., between the -10 and +10 regions of the target gene nucleotide sequence of interest.

[0274] Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon", the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding mTOT protein, regardless of the sequence(s) of such codons.

[0275] It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. An antisense oligonucleotide can be, e.g., about 5-100 or from about 7, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or more nucleotides in length.

[0276] The open reading frame (ORF) or "coding region", which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is another embodiment of a region of the mTOT protein gene that may be targeted effectively. In another embodiment the 5' untranslated region (5'UTR) of mTOT protein, known in the art to refer to the portion of the mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of the mRNA or corresponding nucleotides on the gene, is the target region. In yet another embodiment, the 3' untranslated region (3'UTR) of mTOT protein, known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus

including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene is the target region. A further target region includes the 5' cap of the mRNA for mTOT protein that comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of the mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. In yet another embodiment, the 5' cap region itself is also a target region according to this invention.

[0277] Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns", which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. In still other embodiments of this invention, mRNA splice sites, i.e., intron-exon junctions, are target regions of the gene encoding mTOT protein, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. In further embodiment, aberrant fusion junctions due to rearrangements or deletions are target regions. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". Introns can be effective target regions for antisense oligonucleotides targeted, for example, to DNA or pre-mRNA of mTOT protein.

[0278] It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and extronic regions.

[0279] Upon excision of one or more exon or intron regions or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

[0280] Variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more than one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that

use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

[0281] Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

[0282] In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense oligonucleotide need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable.

[0283] An antisense oligonucleotide is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of expression and/or activity of an mTOT protein, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense oligonucleotide to non-target sequences under conditions in which specific binding is desired. Such conditions include, e.g., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed. In one embodiment, the antisense oligonucleotides of the present invention comprise at least 80% sequence complementarity to a target region within the target nucleic acid of mTOT protein to which they are targeted. In another embodiment, the antisense

oligonucleotides of the present invention comprise at least 90% sequence complementarity to a target region within the target nucleic acid of mTOT protein to which they are targeted. In still another embodiment of this invention, the antisense oligonucleotides of the present invention comprise at least 95% sequence complementarity to a target region within the target nucleic acid of mTOT protein to which they are targeted. For example, an antisense oligonucleotide in which 18 of 20 nucleobases of the antisense oligonucleotide are complementary, and would therefore specifically hybridize, to a target region would represent 90 percent complementarity. Percent complementarity of an antisense oligonucleotide with a region of a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656).

[0284] Antisense oligonucleotides and other functional nucleic acids of the invention, which hybridize to the target mTOT encoding DNA or RNA and inhibit expression of the target, are identified as taught herein. Representative sequences of these compounds are hereinbelow identified as embodiments of the invention. The sites to which these representative antisense oligonucleotides are specifically hybridizable are hereinbelow referred to as "illustrative target regions" and are therefore sites for targeting. As used herein the term "illustrative target region" is defined as at least an 8-nucleobase portion of a target region of mTOT protein, to which an active antisense oligonucleotide is targeted. In another embodiment an illustrative target region is at least 15 nucleobases. In still another embodiment an illustrative target region is at least 20 nucleobases. In another embodiment an illustrative target region is at 30 nucleobases. In yet another embodiment an illustrative target region is at least 40 nucleobases. In still another embodiment an illustrative target region is at least 50 nucleobases. In another embodiment an illustrative target region is at 60 nucleobases. In still another embodiment an illustrative target region is at least 70 nucleobases. In another embodiment an illustrative target region is at least 80 nucleobases or more. In other embodiments, the illustrative target region consists of consecutive nucleobases. Thus, target regions 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative target regions are considered to be suitable target regions as well. While not wishing to be bound by theory, it is presently believed that these illustrative target regions represent regions of the target nucleic acid that are accessible for hybridization.

[0285] While the specific sequences of particular illustrative target regions are set forth below, one of skill in the art will recognize that these serve to illustrate and describe

particular embodiments within the scope of the present invention. Additional target regions may be identified by one having ordinary skill using the teachings of this invention.

[0286] Exemplary additional target regions include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly additional target regions are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the teachings of the illustrative target regions described herein will be able, without undue experimentation, to identify further target regions. In addition, one having ordinary skill in the art using the teachings contained herein will also be able to identify additional compounds, including oligonucleotide probes and primers, that specifically hybridize to these illustrative target regions using techniques available to the ordinary practitioner in the art.

[0287] Antisense oligonucleotides are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense oligonucleotides are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense inhibition has, therefore, been harnessed for research use.

[0288] For use in kits and diagnostics, the antisense oligonucleotides of the present invention, either alone or in combination with other functional nucleic acids or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of mTOT protein genes expressed within cells and tissues.

[0289] Expression patterns within cells or tissues treated with one or more antisense oligonucleotides are compared to control cells or tissues not treated with antisense oligonucleotides and the patterns produced are analyzed for differential levels of mTOT protein gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the mTOT

protein genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds that affect expression patterns.

[0290] Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, 2000, 480, 17-24; Celis, et al., *FEBS Lett.*, 2000, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, 2000, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, 1999, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Jungblut, et al., *Electrophoresis*, 1999, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Larsson, et al., *J. Biotechnol.*, 2000, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, 2000, 286, 91-98; Larson, et al., *Cytoometry*, 2000, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, 2000, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, 1998, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, 1999, 35, 1895-904) and mass spectrometry methods (reviewed in To, *Comb. Chem. High Throughput Screen*, 2000, 3, 235-41).

[0291] The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals, particularly mammals, and including humans. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans. Specific routes of administration and dosages can be ascertained using routine experimentation, to which several clinical trials and experimental protocols for delivering functional nucleic acids such as siRNA, miRNA and antisense oligonucleotides are well known.

[0292] While antisense oligonucleotides are a particular form of functional nucleic acids, the present invention comprehends other functional nucleic acids with the broad category of antisense oligonucleotides, including but not limited to oligonucleotide mimetics such as are described below. The antisense oligonucleotides in accordance with this invention preferably comprise compounds at least about 8 nucleobases in length (i.e. linked nucleosides). In one

embodiment, antisense oligonucleotides of this invention are antisense oligonucleotides of at least about 12 nucleobases in length. In another embodiment, antisense oligonucleotides of this invention comprise about 20 nucleobases in length. In still another embodiment, antisense oligonucleotides of this invention comprise about 30 nucleobases in length. In yet another embodiment, antisense oligonucleotides of this invention comprise about 40 nucleobases in length. In still another embodiment, antisense oligonucleotides of this invention comprise about 50 nucleobases in length. In another embodiment, antisense oligonucleotides of this invention comprise about 60 nucleobases in length. In still another embodiment, antisense oligonucleotides of this invention comprise about 70 nucleobases in length. In yet another embodiment, antisense oligonucleotides of this invention comprise about 80 nucleobases in length. Antisense oligonucleotides include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides that hybridize to the target nucleic acid encoding mTOT protein and modulate its expression.

[0293] Antisense oligonucleotides spanning from 8 to 80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative antisense oligonucleotides described herein are considered to be suitable antisense oligonucleotides as well.

[0294] In one embodiment, exemplary antisense oligonucleotides include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense oligonucleotides (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the antisense oligonucleotide which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly, in another embodiment, such antisense oligonucleotides include at least 12 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense oligonucleotides. In yet another embodiment, the antisense oligonucleotide includes at least 20 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense oligonucleotides. In a further embodiment, the antisense oligonucleotide includes at least 30 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense oligonucleotides. In yet another embodiment, the antisense oligonucleotide includes at least 50 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense oligonucleotides. In still another embodiment, the antisense oligonucleotide includes at least 60 or more consecutive nucleobases from the 5'-terminus of one of the illustrative antisense oligonucleotides.

[0295] Similarly, in another embodiment antisense oligonucleotides are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense oligonucleotides (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the antisense oligonucleotide which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). In another embodiment, such antisense oligonucleotides include at least 12 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense oligonucleotides. In yet another embodiment, the antisense oligonucleotide includes at least 20 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense oligonucleotides. In a further embodiment, the antisense oligonucleotide includes at least 30 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense oligonucleotides. In yet another embodiment, the antisense oligonucleotide includes at least 50 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense oligonucleotides. In still another embodiment, the antisense oligonucleotide includes at least 60 or more consecutive nucleobases from the 3'-terminus of one of the illustrative antisense oligonucleotides. One having skill in the art, once armed with the antisense oligonucleotides illustrated and other teachings herein will be able, without undue experimentation, to identify further antisense oligonucleotides of this invention.

[0296] As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. In addition, linear structures may also have internal nucleobase complementarity and may therefore fold in a manner as to produce a double stranded structure. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

[0297] Illustrative examples of antisense oligonucleotides useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones 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 this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0298] Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue that may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

[0299] Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside 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; riboacetyl 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.

[0300] In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, 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 aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

[0301] Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones 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.

[0302] Modified oligonucleotides may also contain 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; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, 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, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, poly-alkylamino, 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) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) 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 examples hereinbelow, and 2'-dimethylamino-ethoxyethoxy (also known in the

art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂, also described in examples hereinbelow.

[0303] Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂), 2'-allyl (2'-CH₂-CH=CH₂), 2'-O-allyl (2'-O-CH₂-CH=CH₂) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 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 and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

[0304] A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH₂-)_n group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in International Published Patent Application Nos. WO 98/39352 and WO 99/14226.

[0305] Oligonucleotides may also include 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 (--CC--CH₃) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 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, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido [4,5-b] indol-2-one),

pyridoindole cytidine (H-pyrido[3',2':4,Spyrrolo[2,3-dlpyri- midin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds 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° (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0306] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense oligonucleotides that are chimeric compounds. "Chimeric" antisense oligonucleotides or "chimeras", in the context of this invention, are antisense oligonucleotides, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, increased stability and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as interferon-induced RNaseL which cleaves both cellular and viral RNA. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

[0307] Chimeric antisense oligonucleotides of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described herein. Such compounds have also been referred to in the art as hybrids or gapmers.

[0308] The antisense oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

[0309] IV. FORMULATIONS

[0310] The functional nucleic acid compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S. Pat. Nos.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

[0311] The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in International Published Patent Application No. WO 93/24510 to Gosselin et al., published Dec. 9, 1993 or in International Published Patent Application No. WO 94/26764 and U.S. Pat. No. 5,770,713 to Imbach et al.

[0312] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0313] Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N' dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts", J. of Pharma Sci. , 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well

known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

[0314] The present invention also includes pharmaceutical compositions and formulations that include the antisense oligonucleotides of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

[0315] Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C.sub.1-10 alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

[0316] Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media,

capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydrofusid-ate and sodium glycodihydrofusidate. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Antisense oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcyanoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG).

[0317] Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

[0318] Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

[0319] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[0320] The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[0321] In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

[0322] The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic

systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion.

Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion.

Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

[0323] Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids.

[0324] Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988:, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified

into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

[0325] Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

[0326] A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

[0327] Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

[0328] Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid

and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

[0329] The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

[0330] In one embodiment of the present invention, the compositions of antisense oligonucleotides and functional nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa., 1985, p. 271).

[0331] Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310),

hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

[0332] Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

[0333] Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to

improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories including surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

[0334] A. Liposomes

[0335] In some embodiments, oligonucleotides are sequestered in lipids (e.g., liposomes or micelles) to aid in delivery (See e.g., U.S. Patents 6,458,382, 6,429,200; U.S. Patent Application Publication Numbers: 2003/0099697, 2004/0120997, 2004/0131666, 2005/0164963, and International Publication No. WO 06/048329, each of which is herein incorporated by reference).

[0336] As used herein, “liposome” refers to one or more lipids forming a complex, usually surrounded by an aqueous solution. Liposomes are generally spherical structures comprising lipids fatty acids, lipid bilayer type structures, unilamellar vesicles and amorphous lipid vesicles. Generally, liposomes are completely closed lipid bilayer membranes containing an entrapped aqueous volume. The liposomes may be unilamellar vesicles (possessing a single bilayer membrane) or multilamellar (onion-like structures characterized by multiple membrane bilayers, each separated from the next by an aqueous layer). Liposomes of the present invention may also include a DNAi oligonucleotide as defined below, either bound to the liposomes or sequestered in or on the liposomes. The molecules include, but are not limited to, DNAi oligonucleotides and/or other agents used to treat diseases such as cancer.

[0337] As used herein, “sequestered”, “sequestering”, or “sequester” refers to encapsulation, incorporation, or association of a drug, molecule, compound, including a DNAi oligonucleotide, with the lipids of a liposome. The molecule may be associated with the lipid bilayer or present in the aqueous interior of the liposome or both. “Sequestered” includes encapsulation in the aqueous core of the liposome. It also encompasses situations in which part or all of the molecule is located in the aqueous core of the liposome and part outside of the liposome in the aqueous phase of the liposomal suspension, where part of the molecule is located in the aqueous core of the liposome and part in the lipid portion of the liposome, or part sticking out of the liposomal exterior, where molecules are partially or totally embedded in the lipid portion of the liposome, and includes molecules associated with the liposomes, with all or part of the molecule associated with the exterior of the liposome.

[0338] Particularly, after a systemic application, the oligonucleotide and/or other agents must be stably sequestered in the liposomes until eventual uptake in the target tissue or cells. Accordingly, the guidelines for liposomal formulations of the FDA regulate specific preclinical tests for liposomal drugs (<http://www.fda.gov/cder/guidance/2191dft.pdf>). After injection of liposomes into the blood stream, serum components interact with the liposomes, which can lead to permeabilization of the liposomes. However, release of a drug or molecule that is encapsulated in a liposome depends on molecular dimensions of the drug or molecule. Consequently, a plasmid of thousands of base pairs is released much more slowly than smaller oligonucleotides or other small molecules. For liposomal delivery of drugs or molecules, it is ideal that the release of the drug during circulation of the liposomes in the bloodstream be as low as possible.

[0339] B. Amphoteric liposomes

[0340] In some embodiments, liposomes used for delivery may be amphoteric liposomes, such as those described in US 2009/0220584, incorporated herein by reference. Amphoteric liposomes are a class of liposomes having anionic or neutral charge at about pH 7.5 and cationic charge at pH 4. Lipid components of amphoteric liposomes may be themselves amphoteric, and/or may consist of a mixture of anionic, cationic, and in some cases, neutral species, such that the liposome is amphoteric.

[0341] As used herein, an “amphoteric liposome” is a liposome with an amphoteric character, as defined below.

[0342] As used herein, sequestered, sequestering, or sequester refers to encapsulation, incorporation, or association of a drug, molecule, compound, including a functional nucleic acid compound of the present invention, with the lipids of a liposome. The compound may be associated with the lipid bilayer or present in the aqueous interior of the liposome or both. “Sequestered” includes encapsulation in the aqueous core of the liposome. It also encompasses situations in which part or all of the functional nucleic acid is located in the aqueous core of the liposome and part outside of the liposome in the aqueous phase of the liposomal suspension, where part of the molecule is located in the aqueous core of the liposome and part in the lipid portion of the liposome, or part sticking out of the liposomal exterior, where molecules are partially or totally embedded in the lipid portion of the liposome, and includes molecules associated with the liposomes, with all or part of the molecule associated with the exterior of the liposome.

[0343] As used herein, “polydispersity index” is a measure of the heterogeneity of the particle dispersion (heterogeneity of the diameter of liposomes in a mixture) of the liposomes.

A polydispersity index can range from 0.0 (homogeneous) to 1.0 (heterogeneous) for the size distribution of liposomal formulations.

[0344] The amphoteric liposomes include one or more amphoteric lipids or alternatively a mix of lipid components with amphoteric properties. Suitable amphoteric lipids are disclosed in PCT International Publication Number WO02/066489 as well as in PCT International Publication Number WO03/070735, the contents of both of which are incorporated herein by reference. Alternatively, the lipid phase may be formulated using pH-responsive anionic and/or cationic components, as disclosed in PCT International Publication Number WO02/066012, the contents of which are incorporated by reference herein. Cationic lipids sensitive to pH are disclosed in PCT International Publication Numbers WO02/066489 and WO03/070220, in Budker, et al. 1996, Nat. Biotechnol., 14(6):760-4, and in US Patent Number 6,258,792 the contents of which are incorporated by reference herein, and can be used in combination with constitutively charged anionic lipids or with anionic lipids that are sensitive to pH. Conversely, the cationic charge may also be introduced from constitutively charged lipids that are known to those skilled in the art in combination with a pH sensitive anionic lipid. (See also PCT International Publication Numbers WO05/094783, WO03/070735, WO04/00928, WO06/48329, WO06/053646, WO06/002991 and U.S. Patent publications 2003/0099697, 2005/0164963, 2004/0120997, 2006/159737, 2006/0216343, each of which is also incorporated in its entirety by reference).

[0345] Amphoteric liposomes of the present invention include (1) amphoteric lipids or a mixture of lipid components with amphoteric properties, (2) neutral lipids, (3) one or more functional nucleic acid compounds, (4) a cryoprotectant and/or lyoprotectant, (5) or spray-drying protectant. In addition, the inhibitory mTOT protein functional nucleic acid compounds-liposomes have a defined size distribution and polydispersity index.

[0346] As used herein, “amphoter” or “amphoteric” character refers to a structure, being a single substance (e.g., a compound) or a mixture of substances (e.g., a mixture of two or more compounds) or a supramolecular complex (e.g., a liposome) comprising charged groups of both anionic and cationic character wherein

- (i) at least one of the charged groups has a pK between 4 and 8,
- (ii) the cationic charge prevails at pH 4, and
- (iii) the anionic charge prevails at pH 8,

resulting in an isoelectric point of neutral net charge between pH 4 and pH 8. Amphoteric character by that definition is different from zwitterionic character, as zwitterions do not have a pK in the range mentioned above. Consequently, zwitterions are essentially neutrally

charged over a range of pH values. Phosphatidylcholine or phosphatidylethanolamines are neutral lipids with zwitterionic character.

[0347] As used herein, “Amphoter I Lipid Pairs” refers to lipid pairs containing a stable cation and a chargeable anion. Examples include without limitation DDAB/CHEMS, DOTAP/CHEMS and DOTAP/DOPS. In some aspects, the ratio of the percent of cationic lipids to anionic lipids is lower than 1.

[0348] As used herein, “Amphoter II Lipid Pairs” refers to lipid pairs containing a chargeable cation and a chargeable anion. Examples include without limitation Mo-Chol/CHEMS, DPIM/CHEMS or DPIM/DG-Succ. In some aspects, the ratio of the percent of cationic lipids to anionic lipids is between about 5 and 0.2.

[0349] As used herein, “Amphoter III Lipid Pairs” refers to lipid pairs containing a chargeable cation and stable anion. Examples include without limitation Mo-Chol/DOPG or Mo-Chol/Chol-SO₄. In one embodiment, the ratio of the percent of cationic lipids to anionic lipids is higher than 1.

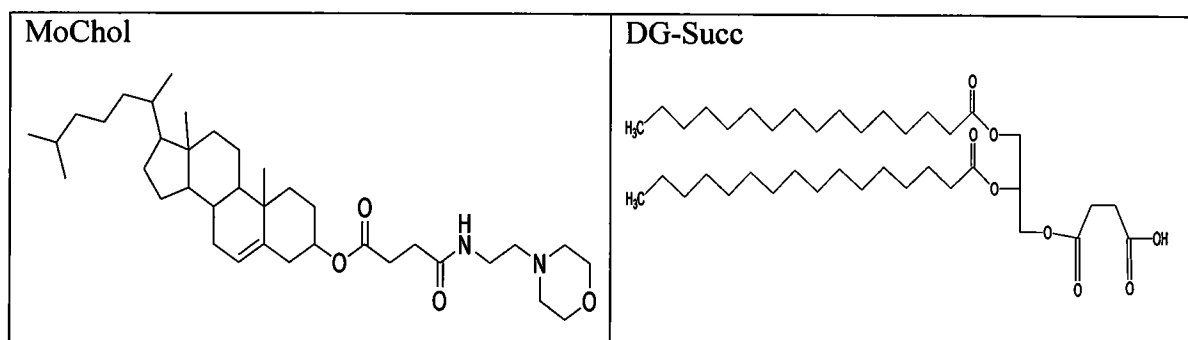
[0350] Abbreviations for lipids refer primarily to standard use in the literature and are included here as a helpful reference:

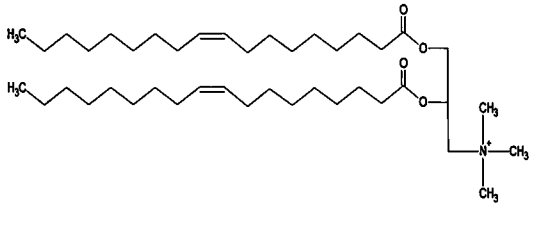
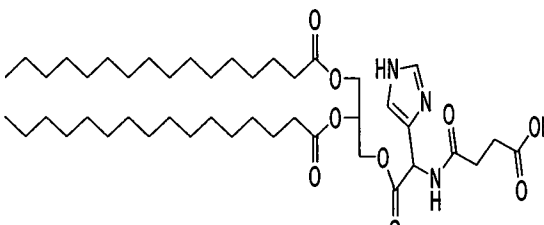
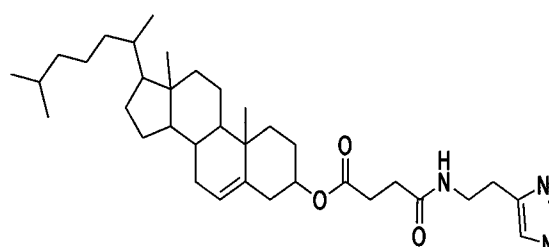
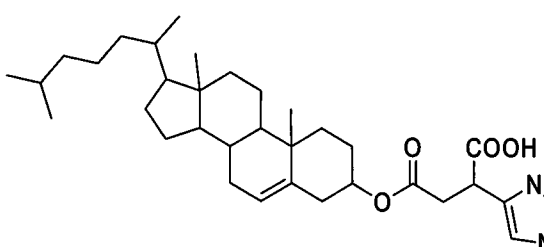
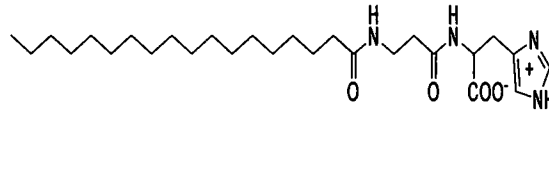
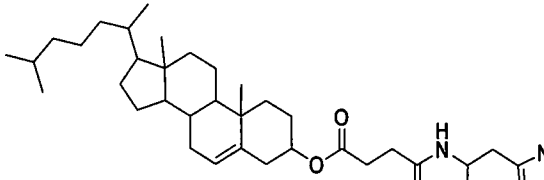
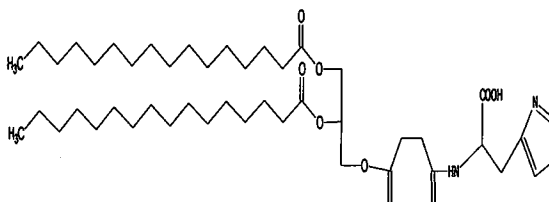
[0351]	DMPC	Dimyristoylphosphatidylcholine
[0352]	DPPC	Dipalmitoylphosphatidylcholine
[0353]	DSPC	Distearoylphosphatidylcholine
[0354]	POPC	Palmitoyl-oleoylphosphatidylcholine
[0355]	OPPC	1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine
[0356]	DOPC	Dioleoylphosphatidylcholine
[0357]	DOPE	Dioleoylphosphatidylethanolamine
[0358]	DMPE	Dimyristoylphosphatidylethanolamine
[0359]	DPPE	Dipalmitoylphosphatidylethanolamine
[0360]	DOPG	Dioleoylphosphatidylglycerol
[0361]	POPG	Palmitoyl-oleoylphosphatidylglycerol
[0362]	DMPG	Dimyristoylphosphatidylglycerol
[0363]	DPPG	Dipalmitoylphosphatidylglycerol
[0364]	DLPG	Dilaurylphosphatidylglycerol
[0365]	DSPG	Distearoylphosphatidylglycerol
[0366]	DMPS	Dimyristoylphosphatidylserine
[0367]	DPPS	Dipalmitoylphosphatidylserine
[0368]	DOPS	Dioleoylphosphatidylserine

[0369]	POPS	Palmitoyl-oleoylphosphatidylserine
[0370]	DMPA	Dimyristoylphosphatidic acid
[0371]	DPPA	Dipalmitoylphosphatidic acid
[0372]	DSPA	Distearoylphosphatidic acid
[0373]	DLPA	Dilaurylphosphatidic acid
[0374]	DOPA	Dioleoylphosphatidic acid
[0375]	POPA	Palmitoyl-oleoylphosphatidic acid
[0376]	CHEMS	Cholesterolhemisuccinate
[0377]	DC-Chol	3- β -[N-(N',N'-dimethylethane) carbamoyl]cholesterol
[0378]	Cet-P	Cetylphosphate
[0379]	DODAP	(1,2)-dioleoyloxypropyl)-N,N-dimethylammonium chloride
[0380]	DOEPC	1,2-dioleoyl-sn-glycero-3-ethylphosphocholine
[0381]	DAC-Chol	3- β -[N-(N,N'-dimethylethane) carbamoyl]cholesterol
[0382]	TC-Chol	3- β -[N-(N',N', N'-trimethylaminoethane) carbamoyl]cholesterol
[0383]	DOTMA (Lipofectin®)	(1,2-dioleoyloxypropyl)-N,N,N-trimethylammoniumchloride)
[0384]	DOGS (Transfectam®)	((C18)2GlySper3+) N,N-dioctadecylamido-glycyl-spermine
[0385]	CTAB	Cetyl-trimethylammoniumbromide
[0386]	CPyC	Cetyl-pyridiniumchloride
[0387]	DOTAP	(1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium salt
[0388]	DMTAP	(1,2-dimyristoyloxypropyl)-N,N,N-trimethylammonium salt
[0389]	DPTAP	(1,2-dipalmitoyloxypropyl)-N,N,N-trimethylammonium salt
[0390]	DOTMA	(1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium chloride)
[0391]	DORIE ammoniumbromide)	1,2-dioleoyloxypropyl)-3 dimethylhydroxyethyl
[0392]	DDAB	Dimethyldioctadecylammonium bromide
[0393]	DPIM	4-(2,3-bis-palmitoyloxy-propyl)-1-methyl-1H-imidazole
[0394]	CHIM	Histaminy-Cholesterolcarbamate
[0395]	MoChol	4-(2-Aminoethyl)-Morpholino-Cholesterolhemisuccinate
[0396]	HisChol	Histaminy-Cholesterolhemisuccinate
[0397]	HCChol	N α -Histidinyl-Cholesterolcarbamate
[0398]	HistChol	N α -Histidinyl-Cholesterol-hemisuccinate

[0399]	AC	Acylcarnosine, Stearyl- & Palmitoylcarnosine
[0400]	HistDG	1,2—Dipalmitoylglycerol-hemisuccinat-N _α -Histidiny-l-hemisuccinate, & Distearoyl, Dimyristoyl, Dioleoyl or palmitoyl-oleoyl derivatives
[0401]	IsoHistSuccDG	1,2-ipalmitoylglycerol-O _α -Histidiny-l-N _α -hemisuccinat, & Distearoyl-, Dimyristoyl, Dioleoyl or palmitoyl-oleoyl derivatives
[0402]	DGSucc	1,2—Dipalmitoylglycerol-3-hemisuccinate & Distearoyl-, dimyristoyl- Dioleoyl or palmitoyl-oleoyl derivatives
[0403]	EDTA-Chol	cholesterol ester of ethylenediaminetetraacetic acid
[0404]	Hist-PS	N _α -histidiny-l-phosphatidylserine
[0405]	BGSC	bisguanidinium-spermidine-cholesterol
[0406]	BGTC	bisguanidinium-tren-cholesterol
[0407]	DOSPER	(1.3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamide
[0408]	DOSC	(1,2-dioleoyl-3-succinyl-sn-glyceryl choline ester)
[0409]	DOGSDO	(1,2-dioleoyl-sn-glycerol-3-succinyl-2-hydroxyethyl disulfide ornithine)
[0410]	DOGSucc	1,2-Dioleoylglycerol-3-hemisuccinate
[0411]	POGSucc	Palmitoyl-oleoylglycerol-oleoyl-3-hemisuccinate
[0412]	DMGSucc	1,2-Dimyristoylglycerol-3-hemisuccinate
[0413]	DPGSucc	1,2-Dipalmitoylglycerol-3-hemisuccinate

[0414] The following table provides non-limiting examples of lipids that are suitable for use in the compositions in accordance with the present invention. The membrane anchors of the lipids are shown exemplarily and serve only to illustrate the lipids of the invention and are not intended to limit the same.



DOTAP 	IsohistsuccDG 
HisChol 	HCChol 
AC 	Hist-Chol 
Hist-DG 	

[0415] Amphoteric lipids are disclosed in PCT International Publication Numbers WO02/066489 and WO03/070735, the contents of both of which are incorporated herein by reference. The overall molecule assumes its pH-dependent charge characteristics by the simultaneous presence of cationic and anionic groups in the "amphoteric substance" molecule portion. More specifically, an amphoteric substance is characterized by the fact that the sum of its charge components will be precisely zero at a particular pH value. This point is referred to as isoelectric point (IP). Above the IP the compound has a negative charge, and below the IP it is to be regarded as a positive cation, the IP of the amphoteric lipids according to the invention ranging between 4.5 and 8.5.

[0416] The overall charge of the molecule at a particular pH value of the medium can be calculated as follows:

$$z = \sum n_i \times ((q_i - 1) + (10^{(pK - pH)}) / (1 + 10^{(pK - pH)}))$$

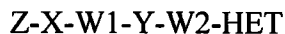
qi: absolute charge of the ionic group below the pK thereof (e.g. carboxyl = 0, single-nitrogen base = 1, di-esterified phosphate group = -1)

ni: number of such groups in the molecule.

[0417] For example, a compound is formed by coupling the amino group of histidine to cholesterol hemisuccinate. At a neutral pH value of 7, the product has a negative charge because the carboxyl function which is present therein is in its fully dissociated form, and the imidazole function only has low charge. At an acid pH value of about 4, the situation is reversed: the carboxyl function now is largely discharged, while the imidazole group is essentially fully protonated, and the overall charge of the molecule therefore is positive.

[0418] In one embodiment, the amphoteric lipid is selected from the group consisting of HistChol, HistDG, isoHistSuccDG, Acylcarnosine and HCChol. In another embodiment, the amphoteric lipid is HistChol.

[0419] Amphoteric lipids can include, without limitation, derivatives of cationic lipids which include an anionic substituent. Amphoteric lipids include, without limitation, the compounds having the structure of the formula:



wherein:

Z is a sterol or an aliphatic;

Sterol is selected from the group consisting of cholesterol, sitosterol, campesterol, desmosterol, fucosterol, 22-ketosterol, 20-hydroxysterol, stigmasterol, 22-hydroxycholesterol, 25 hydroxycholesterol, lanosterol, 7-dehydrocholesterol, dihydrocholesterol, 19-hydroxycholesterol, 5 α cholest-7-en-3 β -ol, 7-hydroxycholesterol, epocholesterol, ergosterol dehydroergosterol, and derivatives thereof;

Each W1 is independently an unsubstituted aliphatic;

Each W2 is independently an aliphatic optionally substituted with HO(O)C-aliphatic-amino or carboxy;

Each X and Y is independently absent, $-(C=O)-O-$, $-(C=O)-NH-$, $-(C=O)-S-$, $-O-$, $-NH-$, $-S-$, $-CH=N-$, $-O-(O=C)-$, $-S-(O=C)-$, $-NH-(O=C)-$, $-N=CH-$, and

HET is an amino, an optionally substituted heterocycloaliphatic or an optionally substituted heteroaryl.

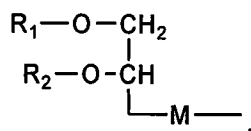
[0420] In some aspects, the HET is an optionally substituted heterocycloaliphatic including at least one nitrogen ring atom, or an optionally substituted heteroaryl including at least one nitrogen ring atom. In other aspects, the HET is morpholinyl, piperidinyl, piperazinyl, pyrimidinyl, or pyridinyl. In another aspect, the cationic lipid has the structure Sterol-X-spacer1-Y-spacer2-morpholinyl or Sterol-X-spacer1-Y-spacer2-imidazolyl. In still further aspects, the sterol is cholesterol.

[0421] In other embodiments, amphoteric lipids include, without limitation, the compounds having the structure of the formula:



wherein:

Z is a structure according to the general formula



wherein R1 and R2 are independently C₈-C₃₀ alkyl or acyl chains with 0, 1 or 2 ethylenically unsaturated bonds and M is selected from the group consisting of -O-(C=O); -NH-(C=O)-; -S-(C=O)-; -O-; -NH-; -S-; -N=CH-; -(O=C)-O-; -S-(O=C)-; -NH-(O=C)-, -N=CH-, -S-S-; and

Sterol is selected from the group consisting of cholesterol, sitosterol, campesterol, desmosterol, fucosterol, 22-ketosterol, 20-hydroxysterol, stigmasterol, 22-hydroxycholesterol, 25 hydroxycholesterol, lanosterol, 7-dehydrocholesterol, dihydrocholesterol, 19-hydroxycholesterol, 5 α cholest-7-en-3 β -ol, 7-hydroxycholesterol, epicholesterol, ergosterol dehydroergosterol, and derivatives thereof;

Each W1 is independently an unsubstituted aliphatic with up to 8 carbon atoms;

Each W2 is independently an aliphatic, carboxylic acid with up to 8 carbon atoms and 0, 1, or 2 ethyleneically unsaturated bonds;

X is absent and Y is -(C=O)-O-; -(C=O)-NH-; -NH-(C=O)-O-; -O-; -NH-; -CH=N-; -O-(O=C)-; -S-; -(O=C)-; -NH-(O=C)-; -O-(O=C)-NH-, -N=CH- and/or -S-S-; and

HET is an amino, an optionally substituted heterocycloaliphatic or an optionally substituted heteroaryl.

[0422] In some aspects, the HET is an optionally substituted heterocycloaliphatic including at least one nitrogen ring atom, or an optionally substituted heteroaryl including at least one nitrogen ring atom. In other aspects, the HET is morpholinyl, piperidinyl, piperazinyl, pyrimidinyl, or pyridinyl. In another aspect, the cationic lipid has the structure

Sterol-X-spacer1-Y-spacer2-morpholinyl or Sterol-X-spacer1-Y-spacer2-imidazolyl. In still further aspects, the sterol is cholesterol.

[0423] Alternatively, the lipid phase can be formulated using pH-responsive anionic and/or cationic components, as disclosed in PCT International Publication Number WO02/066012, the contents of which are incorporated by reference herein. Cationic lipids sensitive to pH are disclosed in PCT International Publication Numbers WO02/066489 and WO03/070220, in Budker, et al. (1996), Nat Biotechnol. 14(6):760-4, and in US Patent Number 6,258,792, the contents of all of which are incorporated by reference herein. Alternatively, the cationic charge may be introduced from constitutively charged lipids known to those skilled in the art in combination with a pH sensitive anionic lipid. Combinations of constitutively (e.g., stable charge over a specific pH range such as a pH between about 4 and 9) charged anionic and cationic lipids, e.g. DOTAP and DPPG are not preferred. Thus, in some embodiments of the invention, the mixture of lipid components may comprise (i) a stable cationic lipid and a chargeable anionic lipid, (ii) a chargeable cationic lipid and chargeable anionic lipid or (iii) a stable anionic lipid and a chargeable cationic lipid.

[0424] The charged groups can be divided into the following 4 groups.

(1) Strongly (e.g., constitutively charged) cationic, $pK_a > 9$, net positive charge: on the basis of their chemical nature, these are, for example, ammonium, amidinium, guanidium or pyridinium groups or primary, secondary or tertiary amino functions.

(2) Weakly cationic, $pK_a < 9$, net positive charge: on the basis of their chemical nature, these are, in particular, nitrogen bases such as piperazines, imidazoles and morpholines, purines or pyrimidines. Such molecular fragments, which occur in biological systems, are, for example, 4-imidazoles (histamine), 2-, 6-, or 9-purines (adenines, guanines, adenosines or guanosines), 1-, 2- or 4-pyrimidines (uracils, thymine, cytosine, uridine, thymidine, cytidine) or also pyridine-3-carboxylic acids (nicotinic esters or amides). Nitrogen bases with preferred pK_a values are also formed by substituting nitrogen atoms one or more times with low molecular weight alkane hydroxyls, such as hydroxymethyl or hydroxyethyl groups. For example, aminodihydroxypropanes, triethanolamines, tris-(hydroxymethyl)methylamines, bis-(hydroxymethyl)methylamines, tris-(hydroxyethyl)methylamines, bis-(hydroxyethyl)methylamines or the corresponding substituted ethylamines.

(3) Weakly anionic, $pK_a > 4$, net negative charge: on the basis of their chemical nature, these are, in particular, the carboxylic acids. These include the aliphatic, linear or branched mono-, di- or tricarboxylic acids with up to 12 carbon atoms and 0, 1 or 2

ethylenically unsaturated bonds. Carboxylic acids of suitable behavior are also found as substitutes of aromatic systems. Other weakly anionic groups are hydroxyls or thiols, which can dissociate and occur in ascorbic acid, N-substituted alloxane, N-substituted barbituric acid, veronal, phenol or as a thiol group.

(4) Strongly (e.g., constitutively charged) anionic, $pK_a < 4$, net negative charge: on the basis of their chemical nature, these are functional groups such as sulfonate or phosphate esters.

[0425] The amphoteric liposomes contain variable amounts of such membrane-forming or membrane-based amphiphilic materials, so that they have an amphoteric character. This means that the liposomes can change the sign of the charge completely. The amount of charge carrier of a liposome, present at a given pH of the medium, can be calculated using the following formula:

$$z = \sum n_i ((q_i - 1) + 10^{(pK - pH)} / (1 + 10^{(pK - pH)}))$$

in which

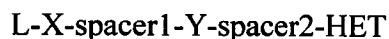
q_i is the absolute charge of the individual ionic groups below their pK (for example, carboxyl = 0, simple nitrogen base = 1, phosphate group of the second dissociation step = -1, etc.)

n_i is the number of these groups in the liposome.

[0426] At the isoelectric point, the net charge of the liposome is 0. Structures with a largely selectable isoelectric point can be produced by mixing anionic and cationic portions.

[0427] In one embodiment, cationic components include DPIM, CHIM, DORIE, DDAB, DAC-Chol, TC-Chol, DOTMA, DOGS, $(C_{18})_2Gly^+ N,N$ -dioctadecylamido-glycine, CTAB, CPyC, DODAP, DMTAP, DPTAP, DOTAP, DC-Chol, MoChol, HisChol and DOEPC. In another embodiment, cationic lipids include DMTAP, DPTAP, DOTAP, DC-Chol, MoChol and HisChol.

[0428] The cationic lipids can be compounds having the structure of the formula



wherein:

L is a sterol or [aliphatic(C(O)O)-]₂-alkyl-;

Sterol is selected from the group consisting of cholesterol, sitosterol, campesterol, desmosterol, fucosterol, 22-ketosterol, 20-hydroxysterol, stigmasterol, 22-hydroxycholesterol, 25 hydroxycholesterol, lanosterol, 7-dehydrocholesterol, dihydrocholesterol, 19-hydroxycholesterol, 5 α cholest-7-en-3 β -ol, 7-hydroxycholesterol, epocholesterol, ergosterol dehydroergosterol, and derivatives thereof;

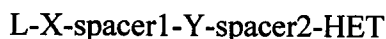
Each spacer 1 and spacer 2 is independently an unsubstituted aliphatic;

Each X and Y is independently absent, $-(C=O)-O-$, $-(C=O)-NH-$, $-(C=O)-S-$, $-O-$, $-NH-$, $-S-$, $-CH=N-$, $-O-(O=C)-$, $-S-(O=C)-$, $-NH-(O=C)-$, $-N=CH-$, and

HET is an amino, an optionally substituted heterocycloaliphatic or an optionally substituted heteroaryl.

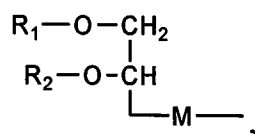
[0429] In some aspects, the HET is an optionally substituted heterocycloaliphatic including at least one nitrogen ring atom, or an optionally substituted heteroaryl including at least one nitrogen ring atom. In other aspects, the HET is morpholinyl, piperidinyl, piperazinyl, pyrimidinyl or pyridinyl. In another aspect, the cationic lipid has the structure Sterol-X-spacer1-Y-spacer2-morpholinyl or Sterol-X-spacer1-Y-spacer2-imidazolyl. In still further aspects, the sterol is cholesterol.

[0430] In another embodiment, pH sensitive cationic lipids can be compounds having the structure of the formula



wherein:

L is a structure according to the general formula



wherein R1 and R2 are independently C₈-C₃₀ alkyl or acyl chains with 0, 1 or 2 ethylenically unsaturated bonds and M is absent, $-O-(C=O)-$; $-NH-(C=O)-$; $-S-(C=O)-$; $-O-$; $-NH-$; $-S-$; $-N=CH-$; $-(O=C)-O-$; $-S-(O=C)-$; $-NH-(O=C)-$; $-N=CH-$, $-S-S-$; and

Sterol is selected from the group consisting of cholesterol, sitosterol, campesterol, desmosterol, fucosterol, 22-ketosterol, 20-hydroxysterol, stigmasterol, 22-hydroxycholesterol, 25 hydroxycholesterol, lanosterol, 7-dehydrocholesterol, dihydrocholesterol, 19-hydroxycholesterol, 5 α -cholest-7-en-3 β -ol, 7-hydroxycholesterol, epicholesterol, ergosterol dehydroergosterol, and derivatives thereof;

Each spacer 1 and spacer 2 is independently an unsubstituted aliphatic with 1-8 carbon atoms;

X is absent and Y is absent, $-(C=O)-O-$; $-(C=O)-NH-$; $-NH-(C=O)-O-$; $-O-$; $-NH-$; $-CH=N-$; $-O-(O=C)-$; $-S-$; $-(O=C)-$; $-NH-(O=C)-$; $-O-(O=C)-NH-$, $-N=CH-$ and/or $-S-S-$; and

HET is an amino, an optionally substituted heterocycloaliphatic or an optionally substituted heteroaryl.

[0431] In some aspects, the HET is an optionally substituted heterocycloaliphatic including at least one nitrogen ring atom, or an optionally substituted heteroaryl including at least one

nitrogen ring atom. In other aspects, the HET is morpholinyl, piperidinyl, piperazinyl, pyrimidinyl or pyridinyl. In another aspect, the cationic lipid has the structure Sterol-X-spacer1-Y-spacer2-morpholinyl or Sterol-X-spacer1-Y-spacer2-imidazolyl. In still further aspects, the sterol is cholesterol.

[0432] The above compounds can be synthesized using syntheses of 1 or more steps, and can be prepared by one skilled in the art.

[0433] The amphoteric mixtures further comprise anionic lipids, either constitutively or conditionally charged in response to pH, and such lipids are also known to those skilled in the art. In one embodiment, lipids for use with the invention include DOGSucc, POGSucc, DMGSucc, DPGSucc, DMPS, DPPS, DOPS, POPS, DMPG, DPPG, DOPG, POPG, DMPA, DPPA, DOPA, POPA, CHEMS and CetylP. In another embodiment, anionic lipids include DOGSucc, DMGSucc, DMPG, DPPG, DOPG, POPG, DMPA, DPPA, DOPA, POPA, CHEMS and CetylP.

[0434] Neutral lipids include any lipid that remains neutrally charged at a pH between about 4 and 9. Neutral lipids include, without limitation, cholesterol, other sterols and derivatives thereof, phospholipids, and combinations thereof. The phospholipids include any one phospholipid or combination of phospholipids capable of forming liposomes. They include phosphatidylcholines, phosphatidylethanolamines, lecithin and fractions thereof, phosphatidic acids, phosphatidylglycerols, phosphatidylinositols, phosphatidylserines, plasmalogens and sphingomyelins. The phosphatidylcholines include, without limitation, those obtained from egg, soy beans or other plant sources or those that are partially or wholly synthetic or of variable lipid chain length and unsaturation, POPC, OPPC, natural or hydrogenated soy bean PC, natural or hydrogenated egg PC, DMPC, DPPC, DSPC, DOPC and derivatives thereof. In one embodiment, phosphatidylcholines are POPC, non-hydrogenated soy bean PC and non-hydrogenated egg PC. Phosphatidylethanolamines include, without limitation, DOPE, DMPE and DPPE and derivatives thereof. Phosphatidylglycerols include, without limitation, DMPG, DLPG, DPPG, and DSPG. Phosphatidic acids include, without limitation, DSPA, DMPA, DLPA and DPPA.

[0435] Sterols include cholesterol derivatives such as 3-hydroxy-5.6-cholestene and related analogs, such as 3-amino-5.6-cholestene and 5,6-cholestene, cholestane, cholestanol and related analogs, such as 3-hydroxy-cholestane; and charged cholesterol derivatives such as cholesteryl-beta-alanine and cholesterol hemisuccinate. In one embodiment neutral lipids include but are not limited to DOPE, POPC, soy bean PC or egg PC and cholesterol.

[0436] In some aspects, the invention provides a mixture comprising amphoteric liposomes and a functional nucleic acid compound. In one embodiment, the amphoteric liposomes have an isoelectric point of between 4 and 8. In a further embodiment, the amphoteric liposomes are negatively charged or neutral at pH 7.4 and positively charged at pH 4.

[0437] In some embodiments, the amphoteric liposomes include amphoteric lipids. In a further embodiment, the amphoteric lipids can be HistChol, HistDG, isoHistSucc DG, Acylcarnosine, HCChol or combinations thereof. In another embodiment, the amphoteric liposomes include a mixture of one or more cationic lipids and one or more anionic lipids. In yet another embodiment, the cationic lipids can be DMTAP, DPTAP, DOTAP, DC-Chol, MoChol or HisChol, or combinations thereof, and the anionic lipids can be CHEMS, DGSucc, Cet-P, DMGSucc, DOGSucc, POGSucc, DPGSucc, DG Succ, DMPS, DPPS, DOPS, POPS, DMPG, DPPG, DOPG, POPG, DMPA, DPPA, DOPA, POPA or combinations thereof.

[0438] In yet another embodiment, the liposomes also include neutral lipids. In a further embodiment, the neutral lipids include sterols and derivatives thereof. In an even further embodiment, the sterols comprise cholesterol and derivatives thereof. The neutral lipids may also include neutral phospholipids. In one embodiment, the phospholipids include phosphatidylcholines or phosphatidylcholines and phosphoethanolamines. In another embodiment, the phosphatidylcholines are POPC, OPPC, natural or hydrogenated soy bean PC, natural or hydrogenated egg PC, DMPC, DPPC or DOPC and derivatives thereof and the phosphatidylethanolamines are DOPE, DMPE, DPPE or derivatives and combinations thereof. In a further embodiment, the phosphatidylcholine is POPC, OPPC, soy bean PC or egg PC and the phosphatidylethanolamines is DOPE.

[0439] In an even further embodiment, the lipids of the amphoteric liposomes include DOPE, POPC, CHEMS and MoChol; POPC, Chol, CHEMS and DOTAP; POPC, Chol, Cet-P and MoChol, or POPC, DOPE, MoChol and DMGSucc.

[0440] In another aspect, the amphoteric liposomes of the mixture of the invention can be formed from a lipid phase comprising a mixture of lipid components with amphoteric properties, wherein the total amount of charged lipids in the liposome can vary from 5 mole% to 70 mole%, the total amount of neutral lipids may vary from 20 mole% to 70 mole%, and a functional nucleic acid compound. In an embodiment of the first aspect, the amphoteric liposomes include 3 to 20 mole% of POPC, 10 to 60 mole% of DOPE, 10 to 60 mole% of MoChol and 10 to 50 mole% of CHEMS. In a further embodiment, the liposomes can include POPC, DOPE, MoChol and CHEMS in the molar ratios of

POPC/DOPE/MoChol/CHEMS of about 6/24/47/23 or 15/45/20/20. In yet another embodiment, the liposomes include 3 to 20 mole% of POPC, 10 to 40 mole% of DOPE, 15 to 60 mole% of MoChol and 15 to 60 mole% of DMGSucc. In a further embodiment, the liposomes include POPC, DOPE, DMGSucc and MoChol in the molar ratios of POPC/DOPE/DMGSucc/MoChol of about 6/24/47/23 or 6/24/23/47. In still another embodiment, the liposomes include 10 to 50 mole% of POPC, 20 to 60 mole% of Chol, 10 to 40 mole% of CHEMS and 5 to 20 mole% of DOTAP. In a further embodiment, the liposomes include POPC, Chol, CHEMS and DOTAP in the molar ratio of POPC/Chol/CHEMS/DOTAP of about 30/40/20/10. In yet another embodiment the liposomes include 10 to 40 mole% of POPC, 20 to 50 mole% of Chol, 5 to 30 mole% of Cet-P and 10 to 40 mole% of MoChol. In a further embodiment, the molar ratio of POPC/Chol/Cet-P/MoChol is about 35/35/10/20.

[0441] In a third aspect, the functional nucleic acid compound contained in the amphoteric liposomal mixture comprises one or more functional nucleic acids that hybridize to SEQ ID NOs: 5-7, 52-53 or to a nucleic acid (DNA or RNA) that encode any one mTOT proteins of Tables 1, 3,4, or 5, and shown in Figures 3A, 3B, 4A and 4B, and complementary nucleotides sequences thereof or portions thereof. In another embodiment, the functional nucleic acid can be one or more of SEQ ID NOs: 54-73 or the complement thereof.

[0442] In another aspect, the functional nucleic acid compounds contained in the liposomal mixture are between 15 and 35 base pairs in length.

[0443] In another aspect, the amphoteric liposome-functional nucleic acid compound mixture includes at least one functional nucleic acid compounds as set forth in SEQ ID NO: 54-73 and amphoteric liposomes comprising POPC, DOPE, MoChol and CHEMS in the molar ratio of POPC/DOPE/MoChol/CHEMS of about 6/24/47/23.

[0444] In another aspect, the amphoteric liposomes of the mixture can include a size between 50 and 500 nm. In one embodiment, the size is between 80 and 300 nm and in another embodiment the size is between 90 and 200 nm.

[0445] In another aspect, the amphoteric liposomes may have an isoelectric point between 4 and 8. In an embodiment of the sixth aspect, the amphoteric liposomes may be negatively charged or neutral at pH 7.4 and positively charged at pH 4.

[0446] In another aspect, the amphoteric liposomes have a functional nucleic acid compound concentration of at least about 2 mg/ml at a lipid concentration of 10 to 100 mM or less.

[0447] In another aspect, the invention provides a method of preparing amphoteric liposomes containing a functional nucleic acid compound. In one embodiment, the method includes using an active loading procedure and in another, a passive loading procedure. In a further embodiment, the method produces liposomes using manual extrusion, machine extrusion, homogenization, microfluidization or ethanol injection. In yet another embodiment, the method has an encapsulation efficiency of at least 35%.

[0448] In some embodiments, amphoteric liposomes formulations may comprise POPC/DOPE/ MoChol/ CHEMS at molar ratios of 6/24/47/23, respectively. Such liposomes are cholesterol-rich and negatively-charged. This is unique among lipid delivery systems and contributes to cellular uptake.

[0449] Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

[0450] If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

[0451] If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

[0452] If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary

ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

[0453] If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

[0454] In some embodiments, formulation and therapeutically effective compositions may optionally contain a penetration enhancer. In some embodiments, a penetration enhancer can include a fatty acid. Various exemplary fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₁₀ alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

[0455] Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier*

Systems, 1990, 7, 1-33; Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita et al., J. Pharm. Sci., 1990, 79, 579-583).

[0456] Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14: 43-51).

[0457] Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7: 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39: 621-626).

[0458] Agents that enhance uptake of functional nucleic acid compounds at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For, example, cationic lipids, such as lipofectin (Junichi et al, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., International Published Patent Application No. WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

[0459] Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

[0460] C. Carriers

[0461] Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

[0462] D. Excipients

[0463] In some embodiments, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

[0464] Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

[0465] Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration that do not deleteriously react with nucleic acids can be used.

[0466] Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

[0467] E. Other Components

[0468] The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

[0469] Aqueous suspensions may contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[0470] In another related embodiment, compositions of the invention may contain one or more antisense oligonucleotides, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense oligonucleotides targeted to a second nucleic acid target. Numerous examples of antisense oligonucleotides are known in the art. Two or more combined compounds may be used together or sequentially.

[0471] V. ADMINISTRATION AND DOSING

[0472] In one embodiment, the invention provides a method of introducing the functional nucleic acid compound-amphoteric liposome mixture to cells or an animal. The functional nucleic acid compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits for a variety of conditions related to the expression of mTOT protein. Among such conditions are inhibition of mTOT protein expression, i.e., the reduction or inhibition of the activity of mTOT protein. Such treatment is useful for the treatment, prophylaxis and management of an insulin resistance disorder. As used herein, an “insulin resistance disorder” as discussed herein, refers to any disease, disorder or condition that is caused by or contributed to by insulin resistance. Examples of insulin resistance disease or disorders include: diabetes mellitus, obesity (obesity can include individuals having a body mass index (BMI) of at least 25 or greater, obesity may or may not be associated with insulin resistance), weight gain, metabolic syndrome, insulin-resistance syndromes, syndrome X, complications associated with insulin resistance, for example: high blood pressure, hypertension, high blood cholesterol, dyslipidemia, hyperlipidemia, dyslipidemia, hyperglycemia, hyperinsulinemia and/or hyperproinsulinemia, impaired glucose tolerance, delayed insulin release, diabetic complications, including cognitive functions in dementia, retinopathy, peripheral neuropathy, nephropathy, glomerulonephritis, glomerulosclerosis, nephrotic syndrome, hypertensive nephrosclerosis, some types of cancer (such as endometrial, breast, prostate, and colon), complications of pregnancy, poor female reproductive health (such as menstrual irregularities, infertility, irregular ovulation, polycystic ovarian syndrome (PCOS)), lipodystrophy, cholesterol related disorders, such as gallstones, cholecystitis and cholelithiasis, gout, obstructive sleep apnea and respiratory problems, osteoarthritis, and bone loss, e.g. osteoporosis.), metabolic or inflammation mediated diseases (e.g., dyslipidemia, central obesity, rheumatoid arthritis, lupus, myasthenia gravis, vasculitis, Chronic Obstructive Pulmonary Disease (COPD), or inflammatory bowel disease), cardiovascular disease (e.g. atherosclerosis, arteriosclerosis, angina pectoris, coronary artery disease, congestive heart failure, stroke, or myocardial infarction) as well as neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder that can be treated by inhibiting the expression of mTOT protein, is treated by administering one or more functional nucleic acid compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense oligonucleotide to a suitable pharmaceutically acceptable diluent or carrier. Administration of the functional nucleic acid compounds and

methods of the invention may also be useful prophylactically, e.g., to prevent or delay insulin insensitivity, enhance glucose utilization and improve mitochondrial function, for example.

[0473] The functional nucleic acid compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding mTOT protein, enabling sandwich and other assays to easily be constructed to exploit this fact.

Hybridization of the functional nucleic acid compounds of the invention with a nucleic acid encoding mTOT protein can be detected by means known in the art. Such means may include conjugation of an enzyme to the functional nucleic acid compounds, radiolabelling of the functional nucleic acid compounds or any other suitable detection means. Kits using such detection means for detecting the level of mTOT protein in a sample may also be prepared.

[0474] The administered mixtures can reduce or stop aberrant glucose metabolism, insulin insensitivity, improved glucose utilization and improved mitochondrial function in mammals. In another embodiment, the introduction of the mixture results in a reduction hyperglycemia. In another embodiment, the mixture is administered to a cell, a non-human animal or a human to treat or prophylactically treat insulin insensitivity disease or disorder. In a further embodiment, the mixture is introduced to an animal at a dosage of between 0.001 μg per kg of body weight to 100 mg per kg of body weight. Precise amounts of the functional nucleic acid to be administered typically will be guided by judgment of a medical practitioner, typically titrating from a high dose to a tolerable lower dose having comparable insulin sensitizing activity. In some embodiments, a unit dose will generally depend on the route of administration and be in the range of 10 ng/kg body weight to 100 mg/kg body weight per day, more typically in the range of 100 ng/kg body weight to about 10 mg/kg body weight per day or most preferably, in the range of about 0.1 mg/kg body weight to about 10 mg/kg body weight. In another embodiment, the method provides administration of a daily dose of one or more functional nucleic acid or antisense oligonucleotides (absent any vehicle) in an amount ranging from about 0.01 mg/m^2 to 300 mg/m^2 functional nucleic acid per body surface area of a patient.

[0475] In other embodiments, the oligonucleotide is administered intravenously to a patient. In still other embodiments, the dose is administered daily for up to 5 days of a three to ten week treatment cycle.

[0476] Alternatively, continuous or intermittent intravenous infusions may be made sufficient to maintain concentrations of at least from about 10 nanomolar to about 100 micromolar of the functional nucleic acid compound in the blood or plasma of the subject.

[0477] In yet another embodiment, the mixture is introduced to the animal one or more times per day or continuously. In still another embodiment, the mixture is introduced to the animal via topical, pulmonary or parenteral administration or via a medical device. In a further embodiment, the mixture administered to the animal or cells further includes a TZD agent, for example, a therapeutically effective dose of mitoglitazone or pioglitazone.

[0478] The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual functional nucleic acid, and can generally be estimated based on EC_{50} found to be effective *in vitro* and *in vivo* animal models. In general, dosage is from 0.001 μ g to 100 mg per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the functional nucleic acid is administered in maintenance doses, ranging from 0.001 μ g to 100 g per kg of body weight, once or more daily, to once every 20 years.

[0479] The term "prodrug" refers to compounds that are transformed (typically rapidly) *in vivo* to yield the parent compound of the above formulae, for example, by hydrolysis in blood. Common examples include, but are not limited to, ester and amide forms of a compound having an active form bearing a carboxylic acid moiety. Examples of pharmaceutically acceptable esters of the compounds of this invention include, but are not limited to, alkyl esters (for example with between about one and about six carbons) the alkyl group is a straight or branched chain. Acceptable esters also include cycloalkyl esters and arylalkyl esters such as, but not limited to benzyl. Examples of pharmaceutically acceptable amides of the compounds of this invention include, but are not limited to, primary amides, and secondary and tertiary alkyl amides (for example with between about one and about six carbons). Amides and esters of the compounds of the present invention may be prepared according to conventional methods. A thorough discussion of prodrugs is provided in T.

Higuchi and V. Stella, "Pro-drugs as Novel Delivery Systems", Vol. 14 of the A.C.S. Symposium Series, and in *Bioreversible Carriers in Drug Design*, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are incorporated herein by reference for all purposes.

[0480] The term "therapeutically effective amount" is an amount of a functional nucleic acid operable to inhibit mTOT protein expression in a cell, tissue or organism, for example, an animal patient, preferably a mammal, such as a human, that when administered to a subject or patient, ameliorates a symptom of the disease. The amount of a functional nucleic acid of the invention which constitutes a "therapeutically effective amount" will vary depending on the functional nucleic acid, the presence of a TZD co-administered agent, the disease state and its severity, the bioavailability characteristics of the compound and/or inhibitor, the age of the patient to be treated, and the like. The therapeutically effective amount can be determined routinely by one of ordinary skill in the art having regard to their knowledge and to this disclosure. The dosage or dosages comprising the therapeutically effective amounts are not toxic and perform to accepted medical practices commensurate with an appropriate risk/benefit ratio.

[0481] "Treating" or "treatment" of a disease, disorder, or syndrome, as used herein, includes any one or more of: (i) preventing the disease, disorder, or syndrome from occurring in a human, i.e. causing the clinical symptoms of the disease, disorder, or syndrome not to develop in an animal that may be exposed to or predisposed to the disease, disorder, or syndrome but does not yet experience or display symptoms of the disease, disorder, or syndrome; (ii) inhibiting the disease, disorder, or syndrome, i.e., arresting its development; and (iii) relieving the disease, disorder, or syndrome, i.e., causing regression of the disease, disorder, or syndrome and (iv) alleviate one or more symptoms of the disease, disorder, or syndrome. As is known in the art, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by one of ordinary skill in the art.

[0482] "Co-administration" or "combined administration" or the like as utilized herein are meant to include modes of administration of the selected active, therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are not necessarily administered by the same route of administration or at the same time. Co-administration can also include delivery of the active ingredients in a "fixed combination", e.g. a functional nucleic acid compound and a TZD are both administered to a patient

simultaneously in the form of a single entity or dosage. The term "non-fixed combination" means that the active ingredients, e.g. a functional nucleic acid compound and a TZD, are both administered to a patient as separate entities, either simultaneously, concurrently or sequentially with no specific time limits, such that the administration provides therapeutically effective levels of the combination of active agents in the body of the patient.

[0483] VI METHODS OF USE

[0484] In another embodiment, the invention is directed to a method of treating a disease or disorder associated, caused or linked with insulin insensitivity, for example, diabetes mellitus, cardiovascular disease and gastrointestinal disease, and Alzheimer's disease, which method comprises administering to a patient a therapeutically effective amount of a functional nucleic acid compound, as defined in the present disclosure, where mitochondrial function or insulin sensitivity is enhanced, at least in part, by the inhibition of mTOT protein; optionally in combination with one or more TZDs. In one aspect, the mTOT protein inhibitor can be a functional nucleic acid compound. In one embodiment, the present invention provides a method for treating a disease or disorder associated with insulin insensitivity in a patient in need thereof, the method comprising administering to said patient a therapeutically effective dose of a functional nucleic acid compound that inhibits the expression of mTOT protein in said patient. In some embodiments, the administration includes administering one or more functional nucleic acid compounds operable to inhibit the expression of an mTOT gene or RNA, for example, the functional nucleic acid can include the nucleotide sequence of any one of SEQ ID NOs: 54-73, or a complement thereof. In a further embodiment, the administration comprises administering a therapeutically effective amount of one or more functional nucleic acids having the nucleotide sequence of any one of SEQ ID NOs: 54-73 or a complement thereof in a pharmaceutical composition. In a further embodiment, the administration comprises administering a therapeutically effective amount of one or more functional nucleic acid compounds having the nucleotide sequence of any one of SEQ ID NOs: 54-73 or a complement thereof in a pharmaceutical composition comprising a liposome. In a further embodiment, the administration comprises administering a therapeutically effective amount of one or more functional nucleic acid compounds having the nucleotide sequence of any one of SEQ ID NOs: 54-73 or a complement thereof in a pharmaceutical composition formulated for systemic administration via intravenous administration. In a further embodiment, the administration comprises administering a combination of a therapeutically effective amount of one or more functional nucleic acid compounds having the nucleotide sequence of any one of SEQ ID NOs: 54-73 or a

complement thereof in a pharmaceutical composition formulated for systemic administration via intravenous administration and a TZD, for example, mitoglitazone or pioglitazone.

[0485] In another aspect, the method of the invention comprises administering to a patient a therapeutically effective amount of a functional nucleic acid compound. In another embodiment, the method of the invention comprises administering to a patient a therapeutically effective amount of a functional nucleic acid compound in combination with a therapeutically effective dose of a TZD, for example, mitoglitazone.

[0486] **VII. EXAMPLES**

[0487] **Example 1. Mitochondrial Membrane Competitive Binding Crosslinking Assay**

[0488] A photoaffinity crosslinker can be synthesized by coupling a carboxylic acid analog of pioglitazone to a p-azido-benzyl group containing ethylamine as described in Amer. J. Physiol 256:E252-E260. (See FIG 9B). The crosslinker can be iodinated carrier free, using a modification of the Iodogen (Pierce) procedure and purified using open column chromatography (PerkinElmer). Specific crosslinking is defined as labeling that is prevented by the presence of competing drug. Competitive binding assays are conducted in 50 mM Tris, pH8.0. All crosslinking reactions are conducted in triplicate using 8 concentrations of competitor ranging from 0-25 μ M. (See Figure 9 C, compounds 2 and 3. Each crosslinking reaction tube contains 20 μ g of crude mitochondrial enriched rat liver membranes, 0.1 μ Ci of 125 I-MSDC-1101, and +/- competitor drug with a final concentration of 1% DMSO. The binding assay reaction is performed at room temperature in the dark for 20 minutes and stopped by exposure to 180,000 μ Joules. Following crosslinking, the membranes are pelleted at 20,000 x g for 5 minutes, the pellet is resuspended in Laemmli sample buffer containing 1% BME and run on 10-20% Tricine gels. Following electrophoresis the gels are dried under vacuum and exposed to Kodak BioMax MS film at -80°C The density of the resulting specifically labeled autoradiography bands are quantitated using ImageJ software (NIH) and IC₅₀ values determined by non-linear analysis using GraphPad Prism™. Selected compounds in this assay demonstrated an IC₅₀ of less than 20 μ M, less than 5 μ M or less than 1 μ M. The crosslinking to this protein band is emblematic of the ability of the ability of the PPAR-sparing compounds to bind to the mitochondria, the key organelle responsible for the effectiveness of these compounds for the therapeutic effects sought to be determined.

[0489] The mitochondrial membrane cross-linking assay can also be performed using transfected mTOT cells. In one example, as shown in FIG. 9A a comparison of HEK293 wild type cells with HEK293 cells transiently transfected with pcDNA 3.1+ (human BP44 c-terminal 6-his-tagged) for 48 hours was made. Following transfection of the HEK293 cells,

the cells were fractionated by Dounce homogenization in 50 mM Tris-HCl (pH 8.0), 250 mM sucrose containing a protease inhibitor cocktail (Roche Complete). The homogenates were subjected to differential centrifugation resulting in fractions P1 (800 x g nuclear pellet), P2 (20,000 x g mitochondrial pellet) and S2 (20,000 x g cytosolic supernatant). Each crosslinking reaction tube contains 20 µg of HEK293 fractions, 0.1 µCi of ¹²⁵I-MSDC-1101, and +/- 25 µM MSDC-0160 in a final concentration of 1% DMSO. The binding assay reaction is performed at room temperature in the dark for 20 minutes and stopped by exposure to UV light (180,000 µJoules). Following crosslinking, the membranes are pelleted at 20,000 x g for 5 minutes, the pellet is resuspended in Laemmli sample buffer containing 1% BME and 10 µg total protein is run on 10-20% Tricine gels. Following electrophoresis the gels are dried under vacuum and exposed to Kodak BioMax MS film at -80°C. Arrows on the autoradiography film indicate the specifically labeled native BP44 (~14 kDa) from the wild type HEK293 P2 fraction. The BP44 (~14 kDa) and BP44 6-His(~15.6 kDa) indicated by the two arrows in the P2 fraction from the transiently transfected HEK 293 cells show the increased size of the expressed his-tagged protein is specifically crosslinked. FIG.9B shows the chemical structure of the photoaffinity crosslinker, ¹²⁵I-MSDC-1101.

[0490] Example 2. Induction Of BP44 In Brown Adipose Tissue (BAT) Assay

[0491] In some embodiments, PPARγ-sparing thiazolidinedione like activity can be measured by assaying the effect of a candidate compound's ability to induce expression of BP44 in an adipocyte precursor cell. Brown adipose precursor cells can be isolated from mouse interscapular brown fat pads and inoculated into 35 mm culture dishes. Cultures can be maintained in Dulbecco's modified-Eagle's medium containing 10% fetal calf serum. The medium is changed the following day and every second day thereafter. Following 7 days in culture, the precursor cells are confluent and the appropriate control vehicle, candidate compounds, or a prototype PPARγ-sparing thiazolidinedione such as MSDC-0160 (a positive control PPARγ-sparing TZD) are added to the medium of each culture dish. The medium containing the candidate compound is replaced every other day. Each treatment group is typically assayed in triplicate. After 5-7 days of treatment with the one or more candidate compounds, the cultures are treated with KHM buffer (20 mM Hepes, pH 7.2, containing 10 mM potassium acetate, 2 mM magnesium acetate) also containing 1% NP-40 detergent plus a protease inhibitor cocktail, transferred to microfuge tubes and pelleted at 8,000 x g for 5 minutes. The post-nuclear lysates contained in the supernatant are collected and stored at -80°C. The lysate protein concentration is equalized to 1 µg/ul in SDS sample buffer and 20 µg/sample is separated by SDS-PAGE under reducing conditions. Following completion

of electrophoresis, Western blot analysis is carried out on the separated proteins. The blot is subjected to incubation with a specific rabbit polyclonal antibody that recognizes only BRP44 and is followed by incubation with a goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase. The ~ 14 kDa BP44 protein is detected using an enhanced chemiluminescence reagent followed by exposure to detection film. Expression levels of the BP44 protein are quantified by densitometry using ImageJ software (U.S. National Institutes of Health). The candidate compound's ability to induce expression of BP44 is compared to the positive and negative control samples. A candidate compound that induces the expression of BP44 in Brown adipose precursor cells relative to negative controls suggests that the candidate compound possesses PPAR γ -sparing thiazolidinedione like activity. Results shown in Figure 2, illustrate the effect of mitoglitazone (a PPAR γ -sparing thiazolidinedione) on the expression of BP44. As can be seen, the induction of expression of BP44 is mitoglitazone dose dependent. Hence, the expression of BP44 in brown adipose cells is proportional to the activity of a PPAR γ -sparing thiazolidinedione mitoglitazone. This validation activity assay can be used to confirm the identity of therapeutic active compounds of those selected lead candidate compounds after screening.

[0492] Example 3. Preparation of HEK293 Cells Expressing mTOT Proteins

[0493] Construction of vectors operable to express human mitochondrial target of TZD (mTOT protein) mTOT proteins. In one experiment, a mammalian cell expression construct expressing mTOT proteins BP44 and BRP44-Like linked via the C-terminus was cloned from a synthesized DNA sequence optimized for human expression. In a first construct, BP44-His₆ nucleotide sequence:

ATGAGTGCTGCCGGGGCTCGGGGGCTGAGGGCTACTTACCACAGACTGCTGGAC
AAGGTCGAACTGATGCTGCCTGAGAACTGAGACCACTGTACAACCACCCCGCA
GGGCCTAGGACCGTGTTCTTTTGGGCCCCCATCATGAAGTGGGGACTGGTCTGCG
CAGGACTGGCAGACATGGCTCGACCTGCAGAGAACTGTCTACCGCCCAGAGTG
CTGTGCTGATGGCCACAGGCTTCATTTGGAGCAGATATTCCCTGGTCATCATTCC
CAAGAACTGGAGCCTGTTTCGCTGTGAATTTCTTTGTCGGCGCCGCTGGGGCCTCT
CAGCTGTTTCGGATTTGGAGATACAACCAGGAACTGAAGGCTAAAGCACATAAG
CACCACCACCACCACCAT – SEQ ID NO: 74 was optimized for human expression and
synthesized using DNA2.0 (Menlo Park, CA USA). The above BP44-His₆ nucleotide
sequence encodes a BP44-His₆ protein with the following amino acid sequence:
MSAAGARGLRATYHRLLDKVELMLPEKLRPLYNHPAGPRTVFFWAPIMKWGLVCA
GLADM

ARPAEKLSTAQSAVLMATGFIWSRYSLVIIPKNWSLFAVNFFVGAAGASQLFRIWRY
NQE

LKAKAHKHHHHH – SEQ ID NO: 75.

[0494] The BP44-His₆ nucleotide sequence cloned into the pcDNA3.1 (Genscript, Piscataway, NJ, USA). The cloned mTOT protein nucleotide insert was confirmed using ACGT (Wheeling IL, USA) using a T7 forward primer:

[0495] T7 Forward Primer

AGCTTGGTACCGAGCTCGGATCCGCCACCATGAGTGCTGCCGGGGCTCGGGGGC
TGAGGGCTACTTACCACAGACTGCTGGACAAGGTCGAACTGATGCTGCCTGAGA
AACTGAGACCACTGTACAACCACCCCGCAGGGCCTAGGACCGTGTTCTTTTGGGC
CCCCATCATGAAGTGGGGACTGGTCTGCGCAGGACTGGCAGACATGGCTCGACC
TGCAGAGAACTGTCTACCGCCAGAGTGCTGTGCTGATGGCCACAGGCTTCATT
TGGAGCAGATATTCCCTGGTCATCATTTCCCAAGAACTGGAGCCTGTTTCGCTGTGA
ATTTCTTTGTGCGCGCCGCTGGGGCCTCTCAGCTGTTTCGGATTTGGAGATACAA
CCAGGAAGTGAAGGCTAAAGCACATAAGCACACCACCACCACCATTGATAAGC
GGCCGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCC
TTCTAGT – SEQ ID NO: 76.

[0496] In a second construct, BRP44-Like-His₆ nucleotide sequence was optimized for human expression and synthesized by DNA2.0 (Menlo Park, CA USA). The BRP44-Like nucleotide sequence was derived from NCBI reference sequence: NM_016098.2, GI: 215983059: SEQ ID NO:52. The second construct encoding the mTOT protein BRP44-Like-His₆ was engineered to express BamH1/NotI restriction sites for cloning into pcDNA3.1 having the 5' extension: GGATCCGCCACC (SEQ ID NO: 77) and a 3' extension: TGATAAGCGGCCGCCCTAGG (SEQ ID NO: 78).

[0497] The final BRP44-Like-His₆ nucleotide sequence insert optimized for human expression included:

GGATCCGCCACCATGGCAGGAGCTCTTGTAAGGAAAGCGGCGGACTATGTCCGG
TCAAAGACTTCAGAGATTACTTGATGTGACGCATTTTTGGGGTCCGGTGGCCA
ACTGGGGGCTGCCCATCGCCGCGATCAACGATATGAAGAAGTCCCCCGAGATCA
TTTCGGGGAGGATGACATTTGCGCTCTGTTGCTACTCACTCACGTTTCATGCGATTT
GCGTATAAAGTGCAGCCTCGCAATTGGTTGCTGTTCGCCTGCCATGCGACCAATG
AGGTCGCACAACCTTATCCAGGGCGGACGGCTCATTAAGCACGAAATGACTAAGA
CAGCAAGCGCCCATCATCACCACCACCACTGATAAGCGGCCGCCCTAGG – SEQ
ID NO:79, which encodes an BRP44-Like-His₆ mTOT protein:

MAGALVRKAADYVRSKDFRDYLMSTHFWGPVANWGLPIAAINDMKKSPEIISGRMT
FALCCYSLTFMRFA YKVQPRNWLLFACHATNEVAQLIQGGRLIKHEMTKTASAHHH
HHH – SEQ ID NO: 99.

[0498] The optimized BRP44-Like-His₆ gene was excised from the DNA2.0 cloning vector pJ221 with BamHI and NotI and ligated into the BamHI/NotI sites of the expression vector pcDNA3.1. Correct clones were selected by restriction analysis and the sequence was confirmed by DNA sequence analysis by ACGT, Inc. (Wheeling, IL USA).

[0499] The resulting BRP44-Like-His₆ nucleotide sequence cloned into the pcDNA3.1 (Genscript, Piscataway, NJ, USA), was sequenced using a T7 forward primer:

[0500] T7 Forward Primer

CTTGGTACCGAGCTCGGATCCGCCACCATGGCAGGAGCTCTTGTAAGGAAAGCG
GCGGACTATGTCCGGTCAAAAGACTTCAGAGATTACTTGATGTGCGACGCATTTTT
GGGGTCCGGTGGCCAACTGGGGGCTGCCCATCGCCGCGATCAACGATATGAAGA
AGTCCCCCGAGATCATTTTCGGGGAGGATGACATTTGCGCTCTGTTGCTACTCACT
CACGTTTCATGCGATTTGCGTATAAAGTGCAGCCTCGCAATTGGTTGCTGTTTCGCC
TGCCATGCGACCAATGAGGTCGCACAACCTTATCCAGGGCGGACGGCTCATTAAG
CACGAAATGACTAAGACAGCAAGCGCCCATCATCACCACCACCACTGATAAGCG
GCCGCTCGAGTCTAGAGGGCCCGTTTAAACCC – SEQ ID NO: 80.

[0501] A dual expression construct comprising BP44 and BRP-44 like target sequences was created. In the first multiple cloning site (MCS1), the human optimized BRP44-Like-His₆ gene was excised from the DNA2.0 cloning vector pJ221 with BamHI and AvrII and ligated into the BamHI/AvrII sites of the multiple cloning site 1 (MCS1) of the expression vector pVITRO1-neo (Catalog No. pvitro1-nmcs, InvivoGen, San Diego, CA USA). Correct clones were selected by restriction analysis and the sequence was confirmed by DNA sequence analysis by ACGT, Inc. (Wheeling, IL USA).

[0502] The BRP44-Like-His₆ nucleotide sequence excised from the DNA2.0 cloning vector had the nucleotide sequence:

GGATCCGCCACCATGGCAGGAGCTCTTGTAAGGAAAGCGGCGGACTATGTCCGG
TCAAAAGACTTCAGAGATTACTTGATGTGCGACGCATTTTTGGGGTCCGGTGGCCA
ACTGGGGGCTGCCCATCGCCGCGATCAACGATATGAAGAAGTCCCCCGAGATCA
TTTCGGGGAGGATGACATTTGCGCTCTGTTGCTACTCACTCACGTTTCATGCGATTT
GCGTATAAAGTGCAGCCTCGCAATTGGTTGCTGTTTCGCCTGCCATGCGACCAATG
AGGTCGCACAACCTTATCCAGGGCGGACGGCTCATTAAGCACGAAATGACTAAGA
CAGCAAGCGCCCATCATCACCACCACCACTGATAAGCGGCCCGCCCTAGG- SEQ ID

NO: 81. The sequence of the BRP44-Like-His₆ insert within the pVITRO1 expression vector was determined using sequencing primers which hybridized to the MSC1 site of pVITRO1:
Sequencing Primers for MCS1:

PVMCS1 Forward: 5'-GGCTAATTCTCAAGCCTCTTAGCG-3' SEQ ID NO:82.

PVMCS1 Reverse: 5'-GCGAGTGTTAGTAACAGCACTG-3' SEQ ID NO:83.

3'-CGCTCACAATCATTGTCGTGAC-5' SEQ ID NO: 84.

5'-CAGTGCTGTTACTAACACTCGC-3' SEQ ID NO:85.

[0503] The human optimized target BP44-NoHis₆ gene was excised from the pcDNA3.1/MTOT PROTEIN-NoH construct using BamHI and the XbaI site downstream of the stop codon derived from the multiple cloning region of pcDNA3.1. This fragment was ligated into the compatible ends of BglII and NheI in MCS2 of pVITRO1. Correct clones were selected by restriction analysis and the sequence was confirmed by DNA sequence analysis by ACGT, Inc. (Wheeling, IL USA).

[0504] The BP44- NoHis₆ nucleotide sequence excised from the DNA2.0 cloning vector had the nucleotide sequence:

GGATCCGCCACCATGAGTGCTGCCGGGGCTCGGGGGCTGAGGGCTACTTACCAC
AGACTGCTGGACAAGGTCGAACTGATGCTGCCTGAGAACTGAGACCACTGTAC
AACCACCCCGCAGGGCCTAGGACCGTGTTCTTTTGGGCCCCCATCATGAAGTGGG
GACTGGTCTGCGCAGGACTGGCAGACATGGCTCGACCTGCAGAGAACTGTCTA
CCGCCAGAGTGCTGTGCTGATGGCCACAGGCTTCATTTGGAGCAGATATTCCCT
GGTCATCATTTCCCAAGAACTGGAGCCTGTTCGCTGTGAATTTCTTTGTGCGCGCC
GCTGGGGCCTCTCAGCTGTTTCGGATTTGGAGATACAACCAGGAAGTGAAGGCTA
AAGCACATAAGTGATAAGCGGCCGCTCGAGTCTAGA- SEQ ID NO: 86.

The sequence of the BP44-His₆ insert within the pVITRO1 expression vector was determined using sequencing primers that hybridized to the MSC2 site of pVITRO1:

Sequencing Primers for MCS1:

PVMCS2 Reverse: 5'-CCTCTACAAATGTGGTATGG-3'(SEQ ID NO:87).

3'-GGAGATGTTTACACCATAACC-5'(SEQ ID NO:88).

5'-CCATACCACATTTGTAGAGG-3'(SEQ ID NO:89).

[0505] PVMCS2 Reverse Translated

cttttttaggtgtgtgaaaaccaccgctaattcaaagcaaccggtgatatcaaagatcc

L F - V L - K P P L I Q S N R - Y Q R S

gccaccatgagtgtgcccggggctcgggggctgagggtactaccacagactgtggac

A T M S A A G A R G L R A T Y H R L L D

aaggctgaactgatgctgcctgagaaactgagaccactgtacaaccacccgcagggcct

K V E L M L P E K L R P L Y N H P A G P

aggaccgtgttcttttgggcccccatcatgaagtggggactggctgcgcaggactggca

R T V F F W A P I M K W G L V C A G L A

gacatggctcgacctgcagagaaactgtctaccgcccagagtgtgtgctgatggccaca

D M A R P A E K L S T A Q S A V L M A T

ggcttcatttggagcagatattccctgggtcatcattcccaagaactggagcctgttcgct

G F I W S R Y S L V I I P K N W S L F A

gtgaatttcttctgctggcgccgctggggcctctcagctgtttcggatttggagatacaac

V N F F V G A A G A S Q L F R I W R Y N

caggaactgaaggctaaagcacataagcaccaccaccaccattgataagcggccgct

Q E L K A K A H K H H H H H - - A A A

Cgagtctagctggccagacatgataagatacattgatgagtttgacaaaccacaactag SEQ ID NO: 90.

R V - L A R H D K I H - - V W T N H N - SEQ ID NO: 91.

Amino acid sequence of pVITRO MCS2 Flanking vector region:

Met S A A G A R G L R A T Y H R L L D K V E L Met L P E K L R P L Y N H P A G P R
T V F F W A P I Met K W G L V C A G L A D Met A R P A E K L S T A Q S A V L Met A
T G F I W S R Y S L V I I P K N W S L F A V N F F V G A A G A S Q L F R I W R Y N Q
E L K A K A H K H H H H H H StopStop. SEQ ID NO: 92.

Comparison of the nucleotide sequence of the BP44-His₆ insert within the pVITRO1 to the original BP44-His₆ insert sequence from the pcDNA3.1 is provided in FIG.5.

[0506] In another cloning strategy, the human optimized BRP44-Like gene was derived by PCR using Accuprime Pfx DNA Polymerase (Catalog No. 12344024, Invitrogen, Carlsbad, CA USA) from the BRP44-Like-His₆ gene synthesized by DNA2.0. The untagged insert was designed with a BamHI site at the N terminus and an AvrII site at the C terminus. The resulting PCR product was digested with BamHI and AvrII and ligated into the BamHI/AvrII sites of MCS1 of the expression vector pVITRO1. Correct clones were selected by restriction analysis and the sequence was confirmed by DNA sequence analysis by ACGT, Inc. (Wheeling IL, USA).

[0507] PCR Primers used in the isolation of BRP44-Like gene include:

BRP44-Like-5' (BamHI site):

5'-GCATATGGATCCGCCACCATGGCAGGAGCTCTTGTAAG-3' – SEQ ID NO: 93.

BRP44-Like NoH-3' (AvrII and XgaI sites)

5'-CTAAGACAGCAAGCGCCTAGTGACCTAGGTCTAGACATACG-3' – SEQ ID NO: 94.

3'-GATTCTGTCGTTTCGCGGATCACTGGATCCAGATCTGTATGC-5' – SEQ ID NO: 95.

5'-CGTATGTCTAGACCTAGGTCAGGCGCTTGCTGTCTTAG-3' – SEQ ID NO: 96.

DNA Sequence Analysis ACGT pVITROMCS1/BRP44-Like-NoH

PVMCS1 Forward

AAAGCAATCCGGAGTATACGGATCCGCCACCATGGCAGGAGCTCTTGTAAGGAA
 AGCGGCGGACTATGTCCGGTCAAAAGACTTCAGAGATTACTTGATGTCGACGCAT
 TTTTGGGGTCCGGTGGCCAACTGGGGGCTGCCCATCGCCGCGATCAACGATATGA
 AGAAGTCCCCCGAGATCATTTCTGGGGAGGATGACATTTGCGCTCTGTTGCTACTC
 ACTCACGTTTCATGCGATTTGCGTATAAAGTGCAGCCTCGCAATTGGTTGCTGTTC
 GCCTGCCATGCGACCAATGAGGTCGCACAACCTTATCCAGGGCGGACGGCTCATT
 AAGCACGAAATGACTAAGACAGCAAGCGCCTAGTGACCTAGGAGCAGGTTTCCC
 CAA- SEQ ID NO: 97.

Amino Acid: Met A G A L V R K A A D Y V R S K D F R D Y L Met S T H F W G P V A
 N W G L P I A A I N D Met K K S P E I I S G R Met T F A L C C Y S L T F Met R F A Y
 K V Q P R N W L L F A C H A T N E V A Q L I Q G G R L I K H E Met T K T A S A
 StopStop– SEQ ID NO: 98.

Comparison of the PCR cloned BRP44-Like NoH protein versus the
 pVITRO1MCS1/BRP44-Like NoH-1 expressed BRP44-Like protein is shown in FIG. 6.

[0508] HEK293 Transfection Protocol

[0509] HEK293 cells were plated twenty-four hours prior to transfection in 6-well plates at
 6 x 10⁵ cells/well in 2mls/well antibiotic free DMEM (Gibco 11965) supplemented with 10%
 Fetal Bovine Serum (Gibco 16000), 1:100 MEM Non-Essential Amino Acids (Gibco
 11140), 1:100 Sodium Pyruvate (Gibco 11360) and 1:100 GlutaMAX (Gibco 35050) or in
 100mm dishes at 3.5 x 10⁶ cells each in 10mls.

[0510] Plasmid DNA for transfection was purified using the Clontech Nucleobond PC 500
 kit (740574.25). Cells were transfected using Lipofectamine 2000 Transfection Reagent
 (Invitrogen 11668) at a 1:1 ratio of DNA (μg) to Lipofectamine (μl) following Invitrogen's
 suggested transfection protocol. Complexes were formed in Opti-MEM I Reduced Serum
 Medium (Gibco 11058-021) by diluting the appropriate amounts of DNA and Lipofectamine
 separately into Opti-MEM I, incubating at room temperature for 5 minutes, combining DNA
 dilutions with Opti-MEM I dilutions and incubating another 20 minutes at room temperature
 before adding transfection complexes dropwise to wells and plates. Plating medium was not

removed or exchanged prior to transfection. The 6-well dishes were transfected using 5µg of DNA and 5µl Lipofectamine in 500µl Opti-MEM I per well. The 100mm plates were transfected using 30µg DNA and 30µl Lipofectamine in 3mls Optim-MEM I per plate. The medium was not replaced after transfection. Cells were harvested 24 hours following transfection.

[0511] Example 4. Preparation of mitochondrial membranes from HEK293 cells expressing His₆ tagged Human mTOT Proteins

[0512] In an experimental example, membrane fractions of human HEK293 cells expressing His₆-tagged BP44 and His₆-tagged BRP44-Like proteins on the surface of mitochondrial membranes were as described in Example 3, were prepared for use in a 96 well competitive binding assays using 3H-Rosiglitazone and unlabeled analogs (See FIG. 9). Affinity of the unlabeled analogs to compete with 3H-Rosiglitazone, (a TZD known to bind to BP44 and BRP44-Like mTOT proteins) for binding to the mTOT proteins will be accomplished by a scintillation proximity assay (SPA) using copper SPA beads to capture the His₆ tagged mTOT proteins and then measure the relative DPMs by scintillation counting. This assay is a representative competitive binding assay of the present invention.

[0513] Methods and Reagents:

1. Fractionation Buffer (FB) 50 mLs:
 - a. 250 mM sucrose, 50 mM Tris-HCl pH 8
 - b. 4.28 g Sucrose (Sigma, S0389), 250 mM
 - c. 2.5 ml 1M Tris-HCl, pH 8 (Sigma, T3038), 50 mM; and
 - d. q.s. to 50 ml w/ molecular grade water (Sigma, W4502)

The prepared FB can be placed on ice until use.

2. Fractionation Buffer w/ Protease Inhibitors (Roche mini-Complete, 11 836 170 001) (PI) and Phosphatase Inhibitors (Sigma, Phosphatase inhibitor cocktail) (PPI): Contains:
 - a. 10 ml FB + 1 Roche PI tablet; and
 - b. 100 up PPI (100x); and
 - c. Keep on ice. Use fresh FB w/ PI + PPI within 4 hrs.
3. Preparation of HEK293 Cells:
 - a. Harvest HEK cells from 100 mm dishes;
 - b. Aspirate the media from dishes;
 - c. Rinse/aspirate plates with 2 X 4 mL of cold Phosphate Buffered Saline (PBS);
 - d. Add 1ml of cold PBS, scrape cells with rubber policeman and transfer to tube;
 - e. Pellet at 200 x g for 5 min at 4°C; and

- f. Remove supernatant.
4. Homogenization of HEK293 Cells:
 - a. Resuspend cell pellet in FB + PI +PPI;
 - b. Transfer to Potter-Elvehjem Teflon homogenizer tube on ice and homogenize with Eurostar power control-visc IKA Labortechnik with 9 x 15 strokes at 800 rpm on ice;
 - c. Transfer homogenate to microfuge tubes (high G force VWR, 20170-038) and centrifuge for 5 min at 1000 x g at 4°C;
 - d. Save supernatant (S1) on ice and keep pellet (P1)
 - e. Re-suspend the low speed pellet (P1) in 1 ml of fresh FB w/ PI + PPI and repeat homogenization 6 x 15 strokes on ice.
 - f. Centrifuge re-suspended pellet sample at 1000 x g for 5 min at 4°C and then add supernatant to first (S1) fraction;
 - g. Re-suspend the (P1) pellet in 0.5 ml of cold 50 mM Tris-HCl pH 8;
 - h. Freeze in 50 µL aliquots at -80°C;
 - h. Spin the supernatant sample (S1) at 20,000 x g for 20 min at 4°C in TOMY to yield the pellet sample (P2);
 - i. Freeze supernatant (S2) in microfuge tubes at -80°C;
 - j. Re-suspend the pellet (P2 containing the mitochondrial fraction) in cold 50 mM Tris-HCl pH 8 and store at -80°C in 50 µL aliquots; and
 - k. Optionally, perform BCA protein assay on (P2) mitochondrial fractions to determine appropriate concentration for SPA assay. Mitochondrial membrane extracts from the HEK293 transfected cells were run on polyacrylamide gels and then subject to Western blotting against BP44, BRP44-Like and Hex-His antibodies and are shown in FIG. 7.

[0514] Example 5. Candidate Compound Screening Using A Scintillation Proximity Assay (SPA) Using Copper SPA Beads

[0515] Use CHAPS solubilized mitochondrial membranes containing His6 tagged Human BP44 and BRP44-Like mTOT proteins for use in a 96 well competitive binding assay using 3H-Rosiglitazone or MRL24 (non-TZD) and unlabeled analogs. Affinity of the unlabeled analogs to compete with either 3H-Rosiglitazone or MRL24 for binding to the mitochondrial mTOT proteins will be accomplished by a scintillation proximity assay (SPA) using copper SPA beads to capture the His6 tagged mTOT proteins and measure the relative DPMs by scintillation counting.

[0516] Methods and Reagents:

1. Labeling mTOT proteins located on the mitochondrial membrane
 - a. Incubate His-tagged P2 extract +/- unlabeled test material (unknown compounds);
 - b. Add 10 μ L containing either 30 μ g of P2 membranes containing the hexahistidine-BP44 or hexahistidine-BRP44 constructs;
 - c. Add 20 μ L of 0.3% BSA as a carrier protein;
 - d. Add 10 μ L of 4%DMSO/50 mM Tris pH 8 with or without 0.4-300 μ M unknown candidate compounds (final concentration= 0.1-75 μ M); Total volume = 40 μ L;
 - e. Shake plate (60 RPM) @ room temperature for 10 minutes;
 - f. Label mitochondrial membranes with 3 H-Rosiglitazone (60 Ci/mmol; #ART-1231; American Radiolabelled Chemicals, Inc; St. Louis, MO USA) or 3 H-MRL-24 (30-40 Ci/mmol) (Quotient Radiosynthesis, Cardiff, UK);
 - g. Add 20 μ L (0.05 uCi) of 3 H-Rosiglitazone or 3 H-MRL-24 in 5mM CHAPS/1% DMSO to each well. Total volume/well = 60 μ L in 50 mM Tris pH 8;
 - h. Shake plate (60 RPM) @ room temperature for 60 minutes;
 - i. Incubate PVT Copper His-Tag SPA beads (Perkin-Elmer #RPNQ0095, Perkin-Elmer, San Jose CA USA) with 0.1% BSA;
 - j. In a 5 mL vial, add 1 mL Cu Beads in water;
 - k. Add 1 mL of 2X Assay Buffer 2% DMSO/10 mM CHAPS/100 mM Tris pH 8;
 - l. Add 400 μ L of 0.6% BSA in 50 mM Tris pH 8/1% DMSO/5 mM CHAPS;
 - m. Shake tube (60 RPM) @ RT for 20 minutes;
 - n. To each well add 60 μ L of Cu Beads with or without BSA from Stepsj-m to a final volume of 120 μ L;
 - o. Shake microtiter plate (60 RPM) @ room temperature for 30 minutes; and
 - p. Count radioactivity on Beckman Top Count, 20 min per well.
2. Optionally, the mitochondrial membranes are pre-bound to the beads before the addition of competitor compounds and the tritiated ligands.

[0517] Example 6. Anti-Diabetic Activity Validation Assay

[0518] In one experimental validation assay, or an assay to measure the anti-diabetic activity of a lead candidate compound shown to bind to an mTOT protein of the present invention, the lead candidate compound is added to a high-sugar growth medium, and its anti-diabetic activity is measured by determining the modulation of growth-rate, and developmental arrest in the absence and presence of the lead candidate compound. Methods

for screening such candidate compounds using a *Drosophila melanogaster* fly model is provided in U.S. Patent Application Publication No. 2005/0260135, Serial No. 11/036897 to Baranski, T.J. et al, filed on 01/14/2005, said application is herein incorporated by reference in its entirety. Similarly, the method is described in Musselmann, L.P. et al., "*A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila*", Disease Models And Mechanisms (2011), 4:842-849, also incorporated by reference in its entirety herein. In brief, the anti-diabetic validation assay includes the following assay details.

[0519] A 96-well microtiter plates is used, such as those commonly commercially available and typically used for various laboratory assay techniques, including other high throughput drug assay techniques. A suitable example is a Falcon #3912 flexible 96-well plate.

However, other multiple-well microtiter plates can be used. Into each well of the 96 well microtiter plate is pipetted 50-400 μ L of either standard (control) or high glucose (experimental) *Drosophila* growth medium. Any one of several standard *Drosophila* growth medium recipes as known in the art of breeding *Drosophila* for research can be used. A known recipe for a standard growth medium includes, for example, water: 1 liter, cornmeal: 61 grams, yeast: 32 grams, agar: 9.3 grams, glucose: 129 grams (or molasses: 67 grams), methyl p-hydrobenzoate: 2.7 grams, and propionic acid: 0.05 liters.

[0520] In the validations assays to measure anti-diabetic effect of a lead candidate compound, a high glucose growth medium is, for example, such a standard *Drosophila* growth medium supplemented with an additional 1 mole of glucose. For example, to prepare a high glucose growth medium, an additional 180 grams of glucose is added to the recipe for standard growth medium as just described. Another embodiment, for example, of a standard growth medium is that prepared as follows: 1 gram agar, 8 grams brewers yeast, 2 grams yeast extract, 2 grams peptone, 3 grams sucrose, 6 grams glucose, 0.05 grams $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$, 0.05 grams $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 600 μ L propionic acid, 1 milliliter 10% p-Hydroxy-benzoic acid methyl ester in 95% ethanol, brought to 1 liter with water. A high glucose version of this medium is made, for example, by combining 75 milliliters of this medium with 25 milliliters of a 4 molar glucose solution in water.

[0521] To prepare a test well with a known concentration of the lead candidate compound (250 μ M stock concentration- final concentration 25 μ M) is added to each well containing a high-glucose growth medium or a control glucose growth medium and allowed to diffuse through the growth medium for an initial period of about 16-24 hours. Wild type *Drosophila* embryos are collected en masse and, after the initial period of diffusion of the lead candidate compound through the growth medium, sorted several to a well. In one

experiment, ten embryos are sorted to each well. Wild type *Drosophila* embryos are also added to wells with a high-glucose growth medium in the absence of the lead candidate compound.

[0522] Once the *Drosophila* embryos are placed into each well on the growth medium, they hatch out in the form of larvae, and begin feeding after a second period of about 24 hours, bringing the final amount of diffusion time for the subject lead candidate compound to about 40-48 hours. A period of about 24-48 hours is sufficient for full diffusion of most compounds. In some cases where adequate diffusion of the lead candidate compound does not occur within a period of about 48 hours, the growth medium in the plate can be warmed and then sonicated to facilitate mixing of the lead candidate compound with the growth medium. Finally, each well is sealed with a sealing film such as "Aeraseal Sealing Film" (available from Sigma-Aldrich, St. Louis, Mo.; Sigma #A-9224).

[0523] After the addition of the *Drosophila* embryos to both the lead candidate compound containing high-glucose growth medium and the control high-glucose growth medium without the lead candidate compound, developmental arrest can be screened for example, measuring observable delays in pupariation as determined by the timing of significant eclosion, and peak eclosion. For example, control *Drosophila* embryos in the absence of a high-glucose growth medium, show significant eclosion beginning at day 8, while *Drosophila* embryos exposed to high glucose growth medium show significant eclosion beginning at day 11 (1 molar glucose). Similarly, peak eclosion is delayed, for example, from day 10 in *Drosophila* embryos in the absence of a high-glucose growth medium, to day 12 in *Drosophila* embryos exposed to high glucose growth medium (1 molar glucose). Thus, the lead candidate compound is validated to possess anti-diabetic activity by demonstrating reversion or reversal in changes to the delay in significant eclosion and peak eclosion, as compared to the developmental delay induced by high glucose growth medium in the absence of the lead candidate compound.

[0524] In another validation parameter, *Drosophila* embryos grown in high-glucose growth medium in the presence and absence of a lead candidate compound, are studied with respect to the levels of glucose present in their hemolymph. Hemolymph is pooled from five to ten larvae to obtain 1 μ L for a subsequent glucose assay. Glucose is measured by adding to 99 μ L of Thermo Infinity Glucose Reagent (TR15321) commercially available from Thermo Scientific, (Waltham, MA USA) frozen in a 96-well plate, then thawed to allow the detection reactions to occur simultaneously for all wells, and processed as per the manufacturer's instructions. The level of trehalose in the *Drosophila* larvae is measured using the same

reagent after digestion with trehalase, with a ten-fold dilution because trehalose levels are higher than those of glucose. 1 μ L of hemolymph is incubated in 25 μ L 0.25 M sodium carbonate at 95°C for 2 hours in a thermal cycler, cooled to room temperature, and 8 μ L of 1 M acetic acid and 66 μ L of 0.25 M sodium acetate (pH 5.2) are added to make a digestion buffer. 1 μ L of porcine trehalase (Sigma T8778) is added to 40 μ L of this mixture and incubated at 37°C overnight. The resulting glucose is analyzed using 10 μ L reaction + 90 μ L Infinity reagent as above. Glucose and trehalose standards were treated simultaneously and used to quantify the sugar levels in hemolymph.

[0525] Using the above protocol to determine the levels of glucose in the hemolymph of *Drosophila* larvae grown in high-glucose growth medium in the presence and absence of a lead candidate compound, the ability of the lead candidate compound to reduce the level of glucose present in the hemolymph is indicative that the lead candidate compound is a therapeutic active agent which may be suitable to treat or prevent metabolic and insulin resistance diseases or disorders as described herein.

OTHER EMBODIMENTS

[0526] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

CLAIMS

What is claimed is:

1. A method for identifying a lead candidate compound that is effective in treating or preventing an insulin-resistance disease or disorder in a subject, the method comprising:
screening one or more candidate compounds in a binding assay, the binding assay comprising the steps:
 - (i) providing an mTOT protein;
 - (ii) contacting the mTOT protein with a candidate compound; and
 - (iii) detecting if the candidate compound specifically binds to the mTOT protein or inhibits the specific binding of a thiazolidinedione compound to the mTOT protein,wherein the candidate compound is identified as a lead candidate compound if the candidate compound specifically binds to the mTOT protein or inhibits the binding of the thiazolidinedione compound to the mTOT protein.
2. The method according to claim 1, wherein the mTOT protein comprises an amino acid sequence as provided in any one of SEQ ID NOs: 1-4, 8-51, 75, or 99.
3. The method according to claim 2, wherein the mTOT protein comprises an amino acid sequence as provided in any one of SEQ ID NOs: 1-4, 42, and 47.
4. The method according to claim 3, wherein the mTOT protein has an amino acid sequence having at least 85% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
5. The method according to claim 3, wherein the mTOT protein has an amino acid sequence having at least 90% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
6. The method according to claim 3, wherein the mTOT protein has an amino acid sequence having at least 95% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
7. The method according to claim 3, wherein the mTOT protein has an amino acid sequence having at least 96% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
8. The method according to claim 3, wherein the mTOT protein has an amino acid

sequence having at least 97% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.

9. The method according to claim 3, wherein the mTOT protein has an amino acid sequence having at least 98% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.

10. The method according to claim 3, wherein the mTOT protein has an amino acid sequence having at least 99% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.

11. The method according to claim 1, wherein the mTOT protein comprises:

(a) an mTOT protein having an amino acid sequence as in any one of SEQ ID NOs: 1-4, 8-51, 75;

(b) an mTOT protein that possesses at least 90% sequence identity to a polypeptide having an amino acid sequence as in any one of SEQ ID NOs: 1-4, 8-51, 75, or 99;

(c) an mTOT protein as shown in Figures 3A, 3B, 4A, 4B or a having a consensus sequence of FWxPx₃WGLx₂Ax₃DMx₄(E/D)x₂Sx₆Lx₈Rx₆P(K/R)Nx₂LxAx₇Ax₃Qx₂Rx₉Rx₍₉₋₂₁₎AxA; or

(d) an mTOT protein encoded by a nucleic acid sequence that specifically hybridizes to the complement of an mTOT nucleic acid sequence of SEQ ID NO: 5-7, 52-53, 74, or 79 under stringent hybridization conditions which are: 50% formamide, 5X SSC and 1% SDS, incubating at 42°C and wash in 0.2X SSC and 0.1% SDS at 65°C and wherein said mTOT protein specifically binds to mitoglitazone.

12. The method according to claim 1, wherein the thiazolidinedione compound comprises: mitoglitazone, rosiglitazone, pioglitazone, troglitazone, or any combination thereof.

13. The method according to claim 12, wherein the thiazolidinedione compound is mitoglitazone.

14. The method according to claim 12, wherein the thiazolidinedione compound is rosiglitazone.

15. The method according to claim 12, wherein the thiazolidinedione compound is

pioglitazone.

16. The method according to claim 12, wherein the thiazolidinedione compound is troglitazone.
17. The method according to claim 1, wherein the binding assay is a high-throughput binding assay.
18. The method according to claim 1, wherein the candidate compound is at least one of a metal, a peptide, a protein, a lipid, a polysaccharide, a nucleic acid, a library of small organic molecules, and a drug.
19. The method according to claim 1, wherein screening one or more candidate compounds in a binding assay comprises screening a library of compounds.
20. The method according to claim 19, wherein the library of compounds comprises a combinatorial chemical library.
21. The method according to claim 20, wherein said combinatorial chemical library comprises a plurality of small organic molecules.
22. The method according to claim 20, wherein said combinatorial chemical library comprises at least 1000 candidate compounds.
23. The method according to any one of claims 18-22, wherein the candidate compound is a small organic molecule.
24. The method according to any one of claims 1-23, wherein at least one of the mTOT protein and candidate compound is labeled with a fluorescent molecule, a radionuclide, a protein tag, or combinations thereof.
25. The method according to claim 24, wherein the binding assay is a FRET assay or a fluorescence polarization assay.

26. The method according to claim 24, wherein at least one of the mTOT protein and candidate compound is labeled with a radionuclide selected from the group consisting of: ^3H , ^{14}C , ^{32}P , or ^{35}S .

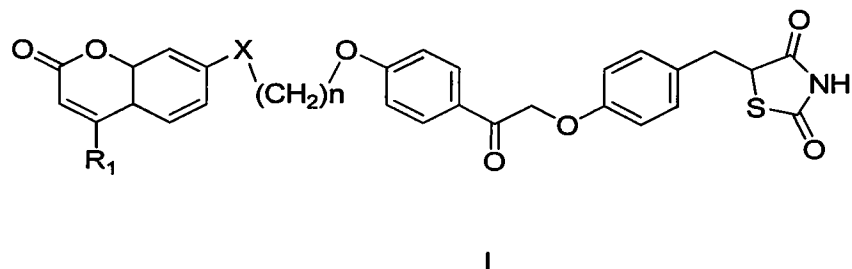
27. The method according to claim 24, wherein the at least one of the mTOT protein and candidate compound is labeled with a protein tag comprising glutathione-S-transferase, c-myc, FLAG, avidin, biotin, streptavidin, or a fluorescent protein.

28. The method according to claim 24, wherein the at least one of the mTOT protein and candidate compound is labeled with a fluorescent protein selected from the group consisting of: green fluorescent protein, enhanced green fluorescent protein, AcGFP1 Fluorescent Protein, AmCyan1 Fluorescent Protein, AsRed2 Fluorescent Protein, mBanana Fluorescent Protein, mCherry Fluorescent Protein, Dendra2, Fluorescent Protein, DsRed2 Fluorescent Protein, DsRed-Express Fluorescent Protein, DsRed-Monomer Fluorescent Protein, E2-Crimson Fluorescent Protein, GFPuv Fluorescent Protein, HcRed1 Fluorescent Protein, mOrange Fluorescent Protein, PAmCherry Fluorescent Protein, mPlum Fluorescent Protein, mRaspberry Fluorescent Protein, mStrawberry Fluorescent, tdTomato Fluorescent Protein, Timer Fluorescent Protein, ZsGreen1 Fluorescent Protein, and ZsYellow1 Fluorescent Protein.

29. The method according to claim 24 wherein the candidate compound is labeled with a fluorescent molecule.

30. The method according to claim 24, wherein the fluorescent molecule is a fluorescent probe.

31. The method according to claim 30, wherein the fluorescent probe is: a compound of Formula I



wherein: X is -O- or -NR₂;

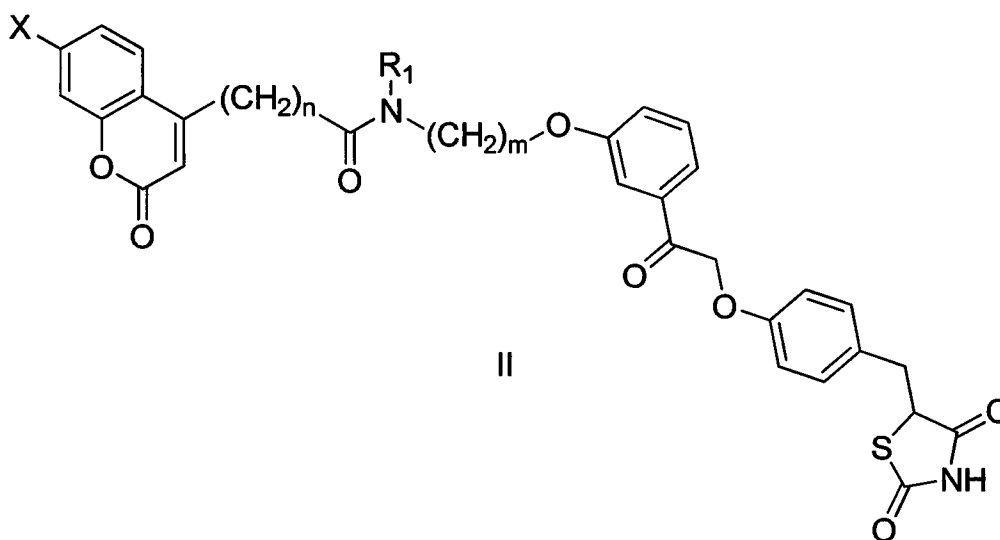
R_1 is optionally substituted C_{1-6} straight or branched alkyl or $CH_2C(O)OR_3$;

R_2 is H, optionally substituted C_{1-6} straight or branched alkyl, optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl;

R_3 is H, optionally substituted C_{1-6} straight or branched alkyl, or optionally substituted $-CH_2$ -phenyl; and

n is 2-6.

32. The method according to claim 30, wherein the fluorescent probe is:
a compound of Formula II:



wherein

X is $-OH$, $-OCH_3$, $-N(R_2)_2$;

R_1 is H, optionally substituted C_{1-6} straight or branched alkyl, optionally substituted phenyl, optionally substituted $-CH_2$ -phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, or optionally substituted 4-pyridyl;

Each R_2 is independently H, optionally substituted C_{1-6} straight or branched alkyl, optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl, or

two R_2 substituents and the nitrogen atom to which they are attached form an optionally substituted 3-7 membered heterocyclic ring;

m is 2-6; and

n is 2-6.

33. The method according to claim 1, wherein the binding assay detects the binding of a candidate compound to an mTOT protein that is over expressed in a cell, and measuring a physiological effect within the cell in the presence and absence of the candidate compound, wherein if the physiological effect in the cell is inhibited, then the candidate compound is determined to have specifically bound specifically to the mTOT protein.
34. The method according to claim 33, wherein measuring a physiological effect within the cell comprises measuring a change in mitochondrial function within the cell.
35. The method according to claim 33, wherein measuring a physiological effect within the cell comprises measuring a change in calcium release, calcium utilization or calcium mobilization.
36. The method according to claim 35, wherein measuring a change in calcium release, calcium utilization or calcium mobilization comprises measuring the activity of a calcium-sensitive dye.
37. The method according to claim 1, wherein detecting if the candidate compound specifically binds to the mTOT protein or inhibits the specific binding of a thiazolidinedione compound to the mTOT protein, comprises detecting the binding of said candidate compound and a fluorescently labeled mTOT protein.
38. The method according to claim 1, wherein detecting if the candidate compound inhibits the specific binding of a thiazolidinedione compound to the mTOT protein comprises detecting the dissociation of the mTOT protein from mitoglitazone.
39. The method according to claim 38, wherein detecting if the candidate compound specifically binds to the mTOT protein comprises detecting the dissociation of the mTOT protein from mitoglitazone using affinity ultrafiltration and ion spray mass spectroscopy/HPLC.
40. The method according to claim 1, wherein detecting if the candidate compound

specifically binds to the mTOT protein comprises detecting binding of an mTOT protein to said candidate compound in a yeast-two hybrid analysis.

41. The method according to claim 1, wherein the lead candidate compound is validated using an activity assay, wherein if the lead candidate compound has activity in the activity assay, then the lead candidate compound is a therapeutic active agent useful in the treatment of an insulin resistance disease or disorder.

42. A method for screening a plurality of candidate compounds to identify a lead candidate compound effective against an insulin resistance disease or disorder, the method comprising:

- (a) providing a candidate compound and at least one mTOT protein;
- (b) incubating the at least one mTOT protein with a competitor compound in the presence of the candidate compound to produce a test combination;
- (c) incubating the at least one mTOT protein with said competitor compound in the absence of the candidate compound to produce a corresponding control combination;
- (d) measuring the amount of the competitor compound bound to the mTOT protein in the test combination and in the control combination; and
- (e) selecting as a lead candidate compound any candidate compound that causes a measurable decrease in the amount of competitor compound bound to the mTOT protein measured in step (d) in the test combination relative to the control combination.

43. The method according to claim 42, further comprising repeating steps (a) – (d) in a high-throughput format.

44. The method according to claim 42, wherein the mTOT protein comprises an amino acid sequence as provided in any one of SEQ ID NOs: 1-4, 8-51, 75 or 99.

45. The method according to claim 42, wherein the mTOT protein comprises an amino acid sequence as provided in any one of SEQ ID NOs: 1-4, 42, and 47.

46. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 85% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.

47. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 90% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
48. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 95% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
49. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 96% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
50. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 97% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
51. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 98% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
52. The method according to claim 45, wherein the mTOT protein has an amino acid sequence having at least 99% sequence identity to any one of SEQ ID NOs: 1-4, 42 or 47.
53. The method according to claim 42, wherein the mTOT protein comprises:
- (a) an mTOT protein having an amino acid sequence as in any one of SEQ ID NOs: 1-4, 8-51;
 - (b) an mTOT protein that possesses at least 90% sequence identity to a polypeptide having an amino acid sequence as in any one of SEQ ID NOs: 1-4, 8-51, 75 or 99;
 - (c) an mTOT protein as shown in Figures 3A, 3B, 4A, 4B or a having a consensus sequence of FWxPx₃WGLx₂Ax₃DMx₄(E/D)x₂Sx₆Lx₈Rx₆P(K/R)Nx₂Lx₁Ax₇Ax₃Qx₂Rx₉Rx₍₉₋₂₁₎Ax₁A; or
 - (d) an mTOT protein encoded by a nucleic acid sequence that specifically hybridizes to the complement of an mTOT nucleic acid sequence of SEQ ID NO: 5-7, 52-53, 74, or 79 under stringent hybridization conditions which are: 50% formamide, 5X SSC and 1% SDS, incubating at 42°C and wash in 0.2X SSC and 0.1% SDS at 65°C and wherein said mTOT protein specifically binds to mitoglitazone.
54. The method according to claim 42, wherein the competitor compound comprises:

mitoglitazone, rosiglitazone, pioglitazone, troglitazone, MRL-24, or UK5099.

55. The method according to claim 54, wherein the competitor compound is mitoglitazone.
56. The method according to claim 54, wherein the competitor compound is rosiglitazone.
57. The method according to claim 54, wherein the competitor compound is pioglitazone.
58. The method according to claim 54, wherein the competitor compound is troglitazone.
59. The method according to any one of claims 42-58, wherein the competitor compound is labeled with at least one of: a fluorescent molecule, a radionuclide, a protein tag, or combinations thereof.
60. The method according to any one of claims 54 - 59, wherein the competitor compound is labeled with a radionuclide selected from the group consisting of: ^3H , ^{14}C , ^{32}P , or ^{35}S .
61. The method according to claim 42, wherein the at least one of the mTOT protein and candidate compound is labeled with a protein tag comprising: glutathione-S-transferase, c-myc, FLAG, avidin, biotin, streptavidin, or a fluorescent protein.
62. The method according to claim 42, wherein the mTOT protein comprises an isolated cell expressing an mTOT protein.
63. The method according to claim 62, wherein the mTOT protein is expressed on the surface of the cell.
64. The method according to claim 62, wherein the mTOT protein is expressed in a cellular organelle.
65. The method according to claim 64, wherein the mTOT protein is expressed in the mitochondria of a cell.

66. The method according to claim 62, wherein the cell is a prokaryotic cell.
67. The method according to claim 62, wherein the cell is a eukaryotic cell.
68. The method according to claim 62, wherein the cell is a yeast cell, an insect cell, an amphibian cell or a mammalian cell.
69. The method according to claim 68, wherein the cell is derived from a tissue culture cell line.
70. The method according to claim 69, wherein the tissue culture cell line is selected from the group consisting of HEK293, Molt4, U937, HeLa, CHO, COS, stem cells, hepatocytes, 2.2.15 cells, Jurkat, cancer cells and fibroblasts.
71. The method according to any one of claims 62 -70, wherein the cell is an isolated cell expressing a recombinant mTOT protein.
72. The method according to claim 71, wherein the mTOT protein comprises a membrane having a recombinant mTOT protein embedded in the membrane.
73. The method according to claim 42, wherein the lead candidate compound is validated as a therapeutic active agent using an activity assay, wherein if the lead candidate compound has activity in the activity assay, then the lead candidate compound is a therapeutic active agent useful in the treatment of an insulin resistance disease or disorder.
74. The method according to claim 73, wherein the activity assay comprises determining the IC₅₀ of said identified candidate compound in a mitochondrial membrane competitive binding crosslinking assay.
75. The method according to claim 74, wherein said mitochondrial membrane competitive binding crosslinking assay is used to determine the therapeutic activity of said identified candidate compound against a metabolic disease or disorder.

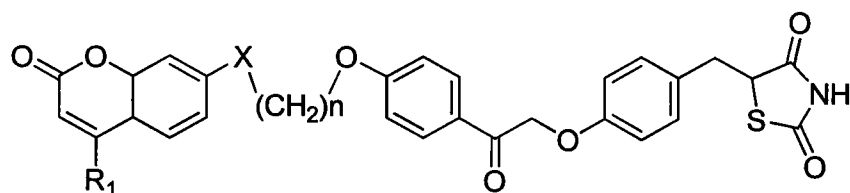
76. The method according to claim 73, wherein the activity assay comprises measuring the lead candidate compound's ability to reduce the glucose level in a *Drosophila melanogaster* high-glucose assay.

77. The method according to claim 73, wherein the activity assay comprises:

- (a) providing a plurality of brown adipose tissue cells;
- (b) contacting said plurality of brown adipose tissue cells with a lead candidate compound; and
- (c) detecting a change in the level of expression of an mTOT protein in said plurality of brown adipose tissue cells in the presence and absence of said lead candidate compound,

wherein an increase in the expression of said mTOT protein in said plurality of brown adipose tissue cells in the presence of said lead candidate compound in comparison to the absence of said candidate compound is indicative of said lead candidate compound being a therapeutic active agent that mimics the activity of a PPAR γ -sparring thiazolidinedione.

78. A compound of Formula I



I

wherein

X is -O- or -NR₂;

R₁ is optionally substituted C₁₋₆ straight or branched alkyl or CH₂C(O)OR₃;

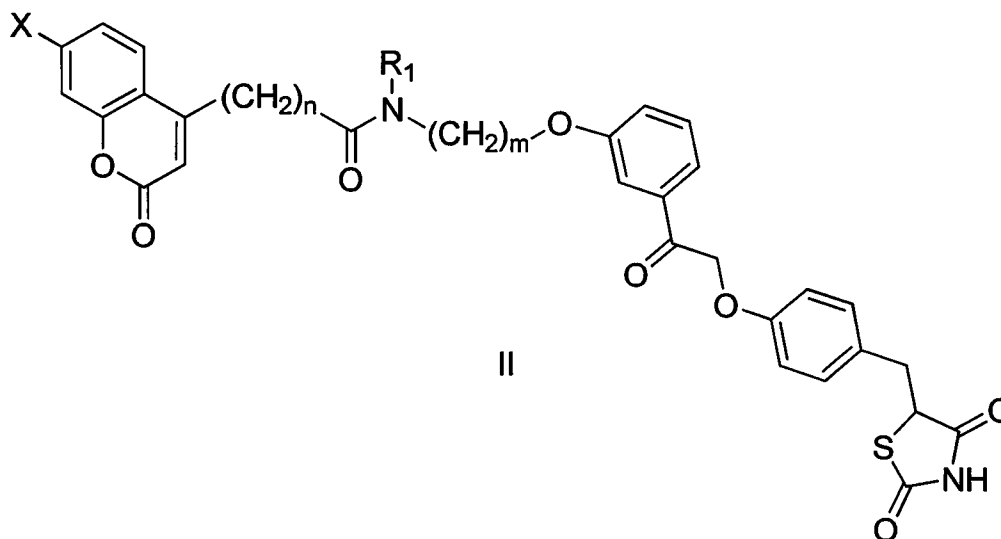
R₂ is H, optionally substituted C₁₋₆ straight or branched alkyl, optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl;

R₃ is H, optionally substituted C₁₋₆ straight or branched alkyl, or optionally substituted

-CH₂-phenyl; and

n is 2-6.

79. A compound of Formula II:



wherein

X is -OH, -OCH₃, -N(R₂)₂;

R₁ is H, optionally substituted C₁₋₆ straight or branched alkyl, optionally substituted phenyl, optionally substituted -CH₂-phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, or optionally substituted 4-pyridyl;

Each R₂ is independently H, optionally substituted C₁₋₆ straight or branched alkyl, optionally substituted phenyl, optionally substituted 2-pyridyl, optionally substituted 3-pyridyl, optionally substituted 4-pyridyl, optionally substituted 2-pyrimidinyl, optionally substituted 3-pyrimidinyl, or optionally substituted 4-pyrimidinyl, or

two R₂ substituents and the nitrogen atom to which they are attached form an optionally substituted 3-7 membered heterocyclic ring;

m is 2-6; and

n is 2-6.

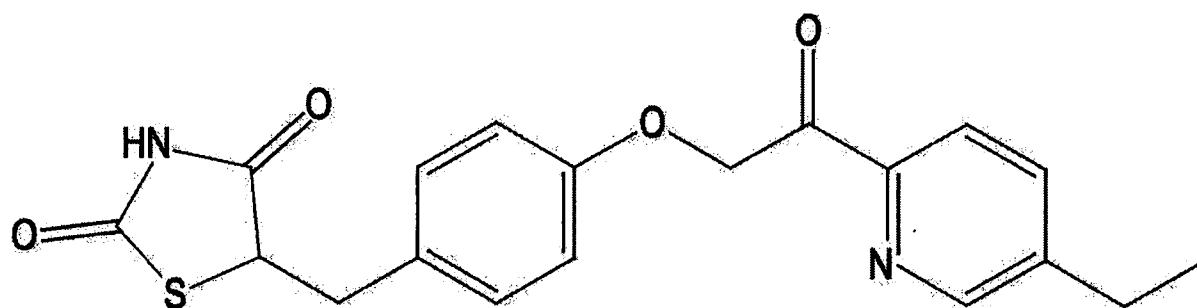
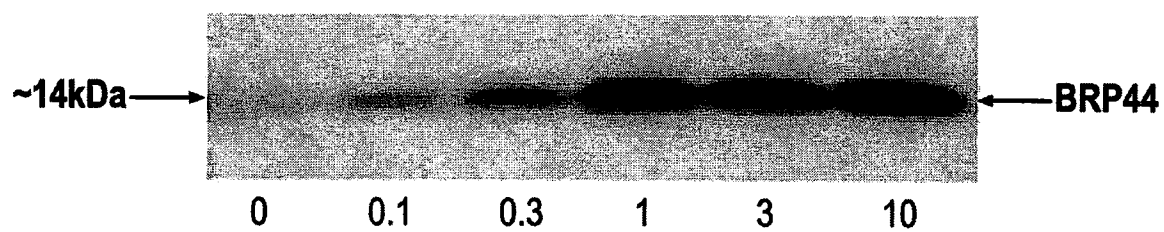
**FIG. 1****FIG. 2**

FIG. 3A

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80	F	I	W	S	R	Y	S	L	V	I	I	P	K	N	W	S	L	F	A	V	N	F	F	V	G	A	A	G	A	S	Q	L	F	R	I	W	R	Y	N	Q	E	L	K	-	-	-	-	122					
80	F	I	W	S	R	Y	S	L	V	I	I	P	K	N	W	S	L	F	A	V	N	F	F	V	G	A	A	G	A	S	Q	L	F	R	I	W	R	Y	N	Q	E	L	K	-	-	-	-	122					
80	F	I	W	S	R	Y	S	L	V	I	I	P	K	N	W	S	L	F	A	V	N	F	F	V	G	A	A	G	A	S	Q	L	F	R	I	W	R	Y	N	Q	E	L	K	-	-	-	-	122					
80	F	I	W	S	R	Y	S	L	V	I	I	P	K	N	W	S	L	F	A	V	N	F	F	V	G	S	A	G	A	S	Q	L	F	R	I	W	K	Y	N	Q	E	L	K	-	-	-	-	122					
80	F	I	W	S	R	Y	S	L	V	I	I	P	K	N	W	S	L	F	A	V	N	F	F	V	G	S	A	G	A	S	Q	L	F	R	I	W	R	Y	N	Q	E	L	K	-	-	-	-	122					
77	L	I	W	S	R	Y	S	L	V	I	I	P	K	N	W	N	L	F	A	V	N	F	F	V	G	S	P	G	G	S	Q	L	Y	R	I	W	M	H	N	R	E	Q	K	-	-	-	-	119					
93	L	I	W	T	R	Y	S	L	V	I	I	P	K	N	Y	S	L	F	A	V	N	L	F	V	S	L	T	Q	L	F	Q	L	G	R	Y	Y	N	Y	Q	W	E	Q	S	R	-	-	-	-	136				
79	A	I	W	T	R	Y	C	L	V	I	I	P	I	N	Y	L	S	S	V	N	F	F	V	M	C	T	G	L	A	Q	L	C	R	I	A	H	Y	R	Y	Q	N	P	-	-	-	-	121						
72	L	I	W	A	R	Y	S	M	V	I	I	P	K	N	W	L	L	F	S	V	N	I	G	L	G	I	T	G	I	N	Q	L	A	R	I	A	N	F	R	I	R	N	G	D	S	F	I	K	I	Q	-	-	107
62	L	I	W	T	R	W	S	F	V	I	I	K	P	K	N	Y	L	A	S	V	N	F	F	L	G	C	T	A	G	Y	H	L	T	R	I	A	N	F	R	I	R	N	G	D	S	F	I	K	I	Q	-	-	109
58	V	I	W	S	R	Y	S	M	V	I	I	N	P	K	N	W	N	L	F	S	V	N	V	A	M	A	G	T	G	I	Y	Q	L	A	R	K	I	K	H	D	Y	A	T	E	A	-	-	-	-	101			

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FIG. 3B

Name Chr	
BRP44	1q24
BRP44L	6q27
Hypo prot	

1q24

6q27

[illegible]

FIG. 4A

HUMAN 044 45 A P I M K W G L V C A G L A D M A R P A E K I S T A Q S A V L M A T G F I W S R Y S L V I I P K 92
BRP44L 29 G P V A N W G L P I A A I N D M K K S P E I I S G R M T F A L C C Y S L T F M R F A Y K V Q P R 76
HYPOTH PRO UPF0041fam 193 G P A F S W G L P L A A F K D M K A S P E I I S G R M T T A L I L Y S A I F M R F A Y R V Q P R 240
347411b 158 G P A F S W G L P L A A F K D M K A S P E I I S G R M T T A L I L Y S A I F M R F A Y R V Q P R 205

HUMAN 044 93 N I W S L F A V N F F V G A A G A S Q L F R I W R Y N Q E - - - - - L K A K A H K 127
BRP44L 77 N I W L L F A C H A T N E V A Q L I Q G R L I K H E M T - - - - - K T A S A 109
HYPOTH PRO UPF0041fam 241 N L L L M A C H C T N V M A Q S V Q A S R Y L L Y Y Y G G G G A E A K A R D P P A T A A A T S 288
347411b 206 N L L L M A C H C T N V M A Q S V Q A S R Y L L Y Y Y G G G G A E A K A R D P P A T A A A T S 253

HUMAN 044 128 127
BRP44L 110 109
HYPOTH PRO UPF0041fam 289 P G S Q P P K Q A S 298
347411b 254 P G S Q P P K Q A S 263

Consensus: FWxPx₃WGLx₂Ax₃DMx₄(E/D)x₂Sx₆Lx₈Rx₆P(K/R)Nx₂LxAx₇Qx₂Rx₉Rx₍₉₋₂₁₎AxA

FIG. 4B

Identities = 109/109 (100%), Positives = 109/109 (100%), Gaps = 0/109 (0%)

Query

1MAGALVRKAADYVRSKDFRDYLMSTHFWGPVANWGLPIAAINDMKKSPEIISGRM
TFALC 60

MAGALVRKAADYVRSKDFRDYLMSTHFWGPVANWGLPIAAINDMKKSPEIISGRMT
FALC 60

Subject

1MAGALVRKAADYVRSKDFRDYLMSTHFWGPVANWGLPIAAINDMKKSPEIISGRM
TFALC 60

Query 61

CYSLTFMRFAYKVQPRNWLLFACHATNEVAQLIQGGRLIKHEMTKTASA 109

CYSLTFMRFAYKVQPRNWLLFACHATNEVAQLIQGGRLIKHEMTKTASA

Subject 61

CYSLTFMRFAYKVQPRNWLLFACHATNEVAQLIQGGRLIKHEMTKTASA 109

FIG. 5

Identities = 133/133 (100%), Positives = 133/133 (100%), Gaps = 0/133 (0%)

Query

1MSAAGARGLRATYHRLLDKVELMLPEKLRPLYNHPAGPRTVFFWAPIMKWGLVC
AGLADM 60

MSAAGARGLRATYHRLLDKVELMLPEKLRPLYNHPAGPRTVFFWAPIMKWGLVCA
GLADM

Subject

1MSAAGARGLRATYHRLLDKVELMLPEKLRPLYNHPAGPRTVFFWAPIMKWGLVC
AGLADM 60

Query

61ARPAEKLSTAQSAVLMATGFIWSRYSLVIIPKNWSLFAVNFFVGAAGASQLFRIWR
YNQE

120ARPAEKLSTAQSAVLMATGFIWSRYSLVIIPKNWSLFAVNFFVGAAGASQLFRIW
RYNQE

Subject

61ARPAEKLSTAQSAVLMATGFIWSRYSLVIIPKNWSLFAVNFFVGAAGASQLFRIWR
YNQE 120

Query 121

LKAKAHKHHHHHH 133

LKAKAHKHHHHHH

Sbjct 121

LKAKAHKHHHHHH 133

FIG. 6

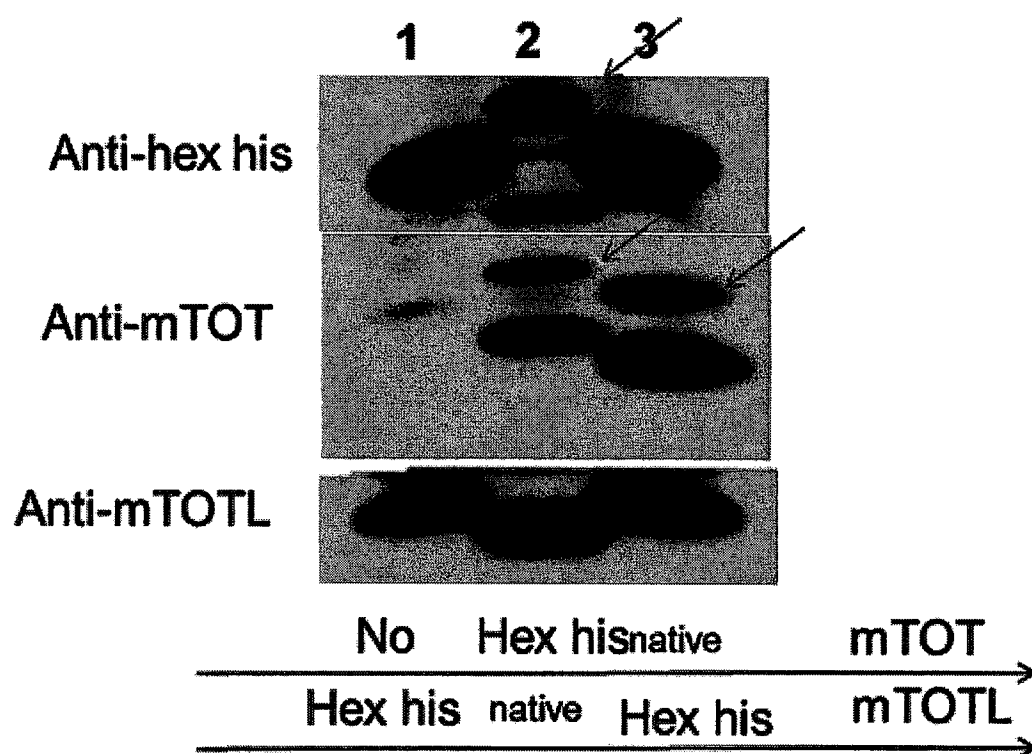


FIG. 7

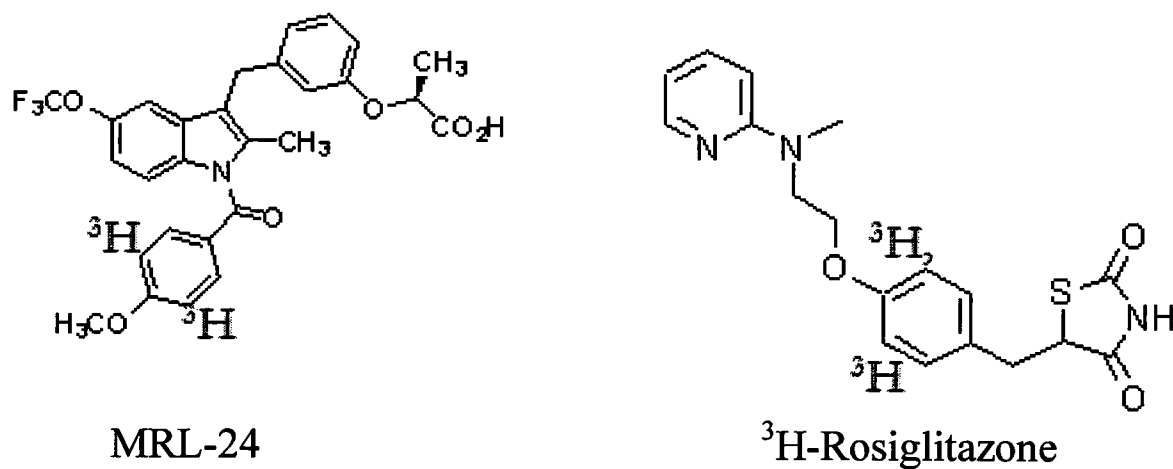
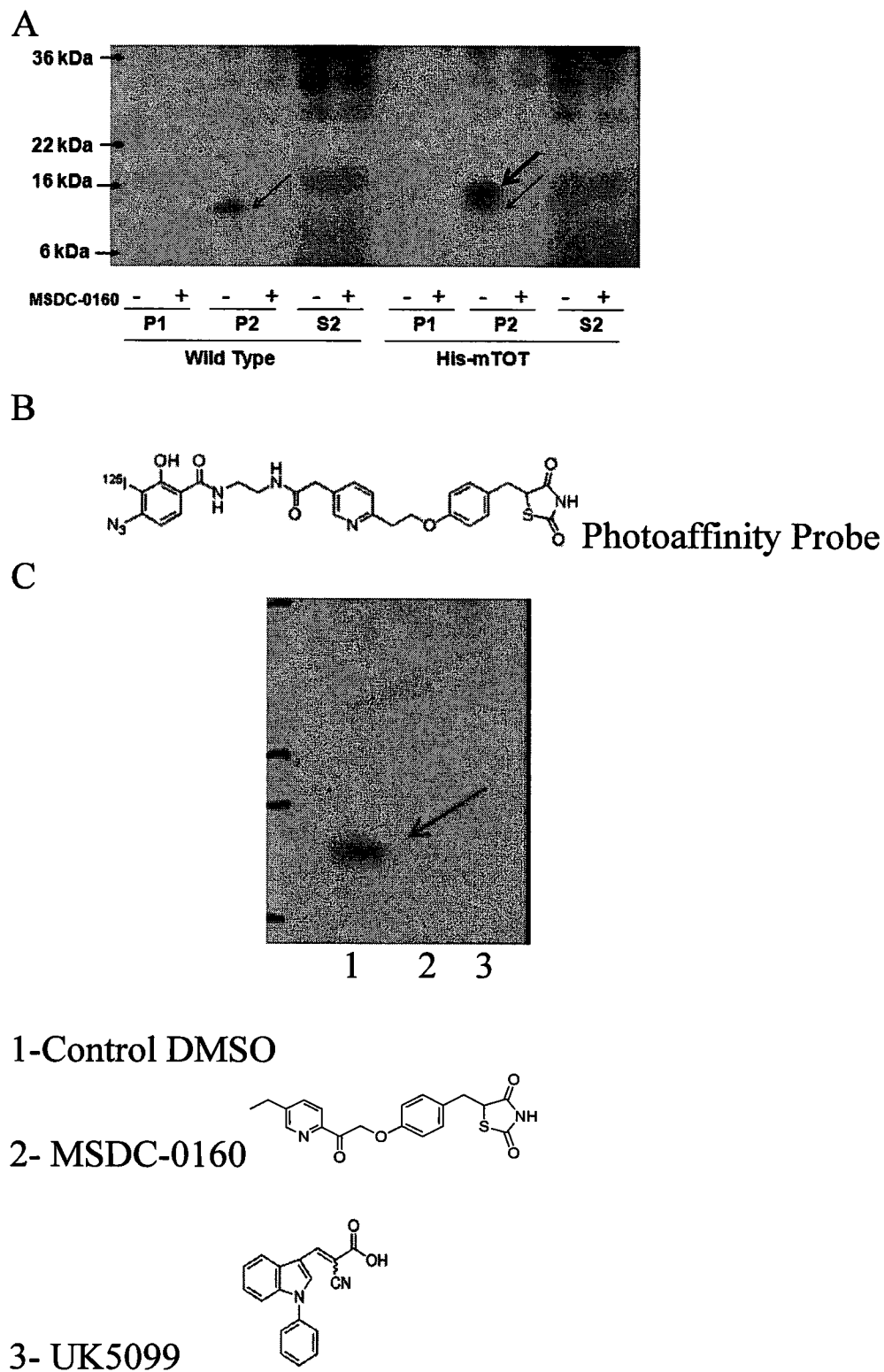


FIG. 8

**FIG. 9**

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/024561

A. CLASSIFICATION OF SUBJECT MATTER INV. G01N33/52 C07D417/10 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) G01N C07D		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BEILSTEIN Data, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010/105048 A1 (METABOLIC SOLUTIONS DEV COMPAN [US]; COLCA GERARD R [US]; LARSEN SCOTT) 16 September 2010 (2010-09-16) claims	1-77
X	WO 2009/038681 A1 (METABOLIC SOLUTIONS DEV COMPAN [US]; COLCA GERARD R [US]; GADWOOD ROBE) 26 March 2009 (2009-03-26) claims	78,79
----- -/--		
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
3 July 2012	02/08/2012	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Vogt, Titus	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/024561

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-77(partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/024561

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLCA J R ET AL: "Identification of a novel mitochondrial protein (mitoNEET)", AMERICAN JOURNAL OF PHYSIOLOGY: ENDOCRINOLOGY AND METABOLISM, AMERICAN PHYSIOLOGICAL SOCIETY, BETHESDA, MD, US, vol. 286, 21 October 2003 (2003-10-21), pages 252-260, XP002997343, ISSN: 0193-1849, DOI: 10.1152/AJPENDO.00424.2003 the whole document -----	1-77
Y	FEINSTEIN D L ET AL: "Receptor-independent actions of PPAR thiazolidinedione agonists: Is mitochondrial function the key?", BIOCHEMICAL PHARMACOLOGY, PERGAMON, OXFORD, GB, vol. 70, no. 2, 15 July 2005 (2005-07-15), pages 177-188, XP027715525, ISSN: 0006-2952 [retrieved on 2005-07-15] the whole document -----	1-77
A	COLCA ET AL: "Insulin sensitizers may prevent metabolic inflammation", BIOCHEMICAL PHARMACOLOGY, PERGAMON, OXFORD, GB, vol. 72, no. 2, 14 July 2006 (2006-07-14), pages 125-131, XP027905479, ISSN: 0006-2952 [retrieved on 2006-07-14] the whole document -----	1-77
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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/024561

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MAORONG JIANG ET AL: "The molecular characterization of the brain protein 44-like (Brp441) gene of Gekko japonicus and its expression changes in spinal cord after tail amputation", MOLECULAR BIOLOGY REPORTS ; AN INTERNATIONAL JOURNAL ON MOLECULAR AND CELLULAR BIOLOGY, KLUWER ACADEMIC PUBLISHERS, DO, vol. 36, no. 2, 18 November 2007 (2007-11-18), pages 215-220, XP019647202, ISSN: 1573-4978 the whole document	1-77
A	----- WO 2007/008501 A1 (MERCK & CO INC [US]; DROPINSKI JAMES F [US]; MEINKE PETER T [US]; SHI) 18 January 2007 (2007-01-18) claims	78,79
X	----- WO 2004/053059 A2 (PHARMACIA CORP [US]; COLCA JERRY R [US]; MCDONALD WILLIAM G [US]) 24 June 2004 (2004-06-24) paragraph [0147]; claims -----	1,12-30, 33-36, 40,42, 43,59-72

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-77

methods of screening for compounds binding to an mTOR protein.

2. claims: 78, 79

thiazolidinedione compounds of formula I and II

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-77(partially)

Independent claims 1 and 42 specify an mTOT protein. However the class of mTOT proteins is not generally recognized, and moreover, the applicant only appears to have provided one example of an mTOT protein, namely: brain protein 44 and like proteins. Since the determination of the scope of the feature mTOT protein requires extensive testing due to its functional result to be obtained description, and the applicant has only identified one type of protein that was identified by him as belonging to the group of mTOT, the search for the methods of independent claims 1 and 42 was restricted to proteins with SEQ ID NOs: 1-4, 5-51, 75, 99 or having the consensus sequence:

FW.P.{3}WGL.{2}A.{3}DM.{4}[ED].{2}S.{6}L.{8}R.{6}P[KR]N.{2}L.A.{7}A.{3}Q.{2}R.{9}R.{9-21}A.A (cf. [0053-0088]; claims 2-11).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2012/024561

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