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Methods for treatment of metabolic disorders using epimetabolic shifters, multidimensional intracellular molecules, or environmental influencers

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(54) **Title**: METHODS FOR TREATMENT OF METABOLIC DISORDERS USING EPIMETABOLIC SHIFTERS, MULTIDIMENSIONAL INTRACELLULAR MOLECULES, OR ENVIRONMENTAL INFLUENCERS

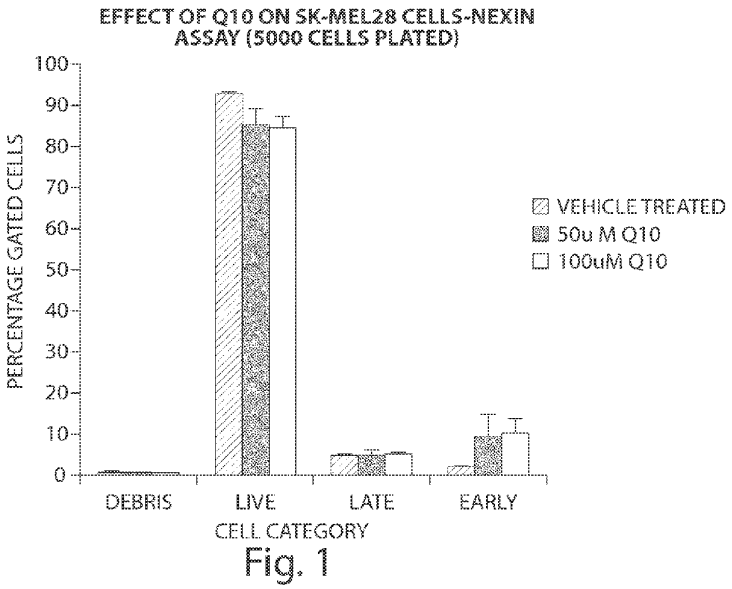

Fig. 1

(57) **Abstract**: Methods and formulations for treating metabolic disorders in humans using epimetabolic shifters, multidimensional intracellular molecules or environmental influencers are described.

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METHODS FOR TREATMENT OF METABOLIC DISORDERS USING EPIMETABOLIC SHIFTERS, MULTIDIMENSIONAL INTRACELLULAR MOLECULES, OR ENVIRONMENTAL INFLUENCERS

Related Applications:

This application claims priority to U.S. Provisional Application No. 61/177,241, filed May 11, 2009, entitled "Methods for Treatment of Oncological Disorders Using an Epimetabolic Shifter (Coenzyme Q10)" (Attorney Docket No.: 117732-00601), U.S. Provisional Application No. 61/177,243, filed May 11, 2009, entitled "Methods for Treatment of Oncological Disorders Using Epimetabolic Shifters, Multidimensional Intracellular Molecules or Environmental Influencers" (Attorney Docket No.: 117732-00701), U.S. Provisional Application No. 61/177,244, filed May 11, 2009, entitled "Methods for the Diagnosis of Oncological Disorders Using Epimetabolic Shifters, Multidimensional Intracellular Molecules or Environmental Influencers" (Attorney Docket No.: 117732-00801), U.S. Provisional Application No. 61/177,245, filed May 11, 2009, entitled "Methods for Treatment of Metabolic Disorders Using Epimetabolic Shifters, Multidimensional Intracellular Molecules or Environmental Influencers" (Attorney Docket No.: 117732-00901), and U.S. Provisional Application No. 61/177,246, filed May 11, 2009, entitled "Methods for the Diagnosis of Metabolic Disorders Using Epimetabolic Shifters, Multidimensional Intracellular Molecules or Environmental Influencers" (Attorney Docket No.: 117732-01001), the entire contents of each of the aforementioned applications are hereby incorporated herein by reference.

Background of the Invention:

The invention relates to the treatment, prevention, and reduction of metabolic disorders, such as diabetes and obesity.

As the levels of blood glucose rise postprandially, insulin is secreted and stimulates cells of the peripheral tissues (skeletal muscles and fat) to actively take up glucose from the blood as a source of energy. Loss of glucose homeostasis as a result of dysregulated insulin secretion or action typically results in metabolic disorders such as diabetes, which may be co-triggered or further exacerbated by obesity. Because these

conditions are often fatal, strategies to restore adequate glucose clearance from the bloodstream are required.

Although diabetes may arise secondary to any condition that causes extensive damage to the pancreas (e.g., pancreatitis, tumors, administration of certain drugs such as corticosteroids or pentamidine, iron overload (e.g., hemochromatosis), acquired or genetic endocrinopathies, and surgical excision), the most common forms of diabetes typically arise from primary disorders of the insulin signaling system. There are two major types of diabetes, namely type 1 diabetes (also known as insulin dependent diabetes (IDDM)) and type 2 diabetes (also known as insulin independent or non-insulin dependent diabetes (NIDDM)), which share common long-term complications in spite of their different pathogenic mechanisms.

Type 1 diabetes, which accounts for approximately 10% of all cases of primary diabetes, is an organ-specific autoimmune disease characterized by the extensive destruction of the insulin-producing beta cells of the pancreas. The consequent reduction in insulin production inevitably leads to the deregulation of glucose metabolism. While the administration of insulin provides significant benefits to patients suffering from this condition, the short serum half-life of insulin is a major impediment to the maintenance of normoglycemia. An alternative treatment is islet transplantation, but this strategy has been associated with limited success.

Type 2 diabetes, which affects a larger proportion of the population, is characterized by a deregulation in the secretion of insulin and/or a decreased response of peripheral tissues to insulin, i.e., insulin resistance. While the pathogenesis of type 2 diabetes remains unclear, epidemiologic studies suggest that this form of diabetes results from a collection of multiple genetic defects or polymorphisms, each contributing its own predisposing risks and modified by environmental factors, including excess weight, diet, inactivity, drugs, and excess alcohol consumption. Although various therapeutic treatments are available for the management of type 2 diabetes, they are associated with various debilitating side effects. Accordingly, patients diagnosed with or at risk of having type 2 diabetes are often advised to adopt a healthier lifestyle, including loss of weight, change in diet, exercise, and moderate alcohol intake. Such lifestyle changes, however, are not sufficient to reverse the vascular and organ damages caused by diabetes.

Coenzyme Q10, also referred to herein as CoQ10, Q10, ubiquinone, or ubidecarenone, is a popular nutritional supplement and can be found in capsule form in nutritional stores, health food stores, pharmacies, and the like, as a vitamin-like supplement to help protect the immune system through the antioxidant properties of ubiquinol, the reduced form of CoQ10. CoQ10 is art-recognized and further described in International Publication No. WO 2005/069916, the entire disclosure of which is incorporated by reference herein.

CoQ10 is found throughout most tissues of the human body and the tissues of other mammals. The tissue distribution and redox state of CoQ10 in humans has been reviewed in a review article by Bhagavan and Chopra (2006 Free Radical Research 40(5):445-453). The authors report that "as a general rule, tissues with high-energy requirements or metabolic activity such as the heart, kidney, liver and muscle contain relatively high concentrations of CoQ10." The authors further report that "[a] major portion of CoQ10 in tissues is in the reduced form as the hydroquinone or uniquinol, with the exception of brain and lungs," which "appears to be a reflection of increased oxidative stress in these two tissues." In particular, Bhagavan report that in heart, kidney, liver, muscle, intenstine and blood (plasma), about 61%, 75%, 95%, 65%, 95% and 96%, respectively, of CoQ10 is in the reduced form. Similarly, Ruiz-Jiminez *et al.* (2007 J. Chroma A, 1175, 242-248) report that when human plasma was evaluated for Q10 and the reduced form of Q10 (Q10H2), the majority (90%) of the molecule was found in the reduced form.

CoQ10 is very lipophilic and, for the most part, insoluble in water. CoQ10 is very lipophilic and, for the most part, insoluble in water. Due to its insolubility in water, limited solubility in lipids, and relatively large moleculer weight, the efficiency of absorption of orally administered CoQ10 is poor. Bhagavan and Chopra report that "in one study with rats it was reported that only about 2-3% of orally-administered CoQ10 was absorbed." Bhagavan and Chopra further report that "[d]ata from rat studies indicate that CoQ10 is reduced to ubiquinol either during or following absorption in the intestine."

Given that the strategies currently available for the management of diabetes are suboptimal, there is a compelling need for treatments that are more effective and are not associated with such debilitating side-effects.

Summary of the Invention:

The present invention is partly based on the finding that mitochondrial dysfunction is associated with a wide range of diseases, including metabolic diseases (such as diabetes and obesity), and that certain endogenous molecules, such as CoQ10, hold the key to the successful diagnosis, treatment, and prevention of such metabolic diseases. The invention is also partly based on the finding that these key endogenous molecules play important roles in maintaining normal mitochondrial function by directly influencing oxidative phosphorylation, and that restoring or promoting more normalized mitochondrial osidative phosphorylation can effectively treat or prevent the progression of metabolic diseases. The invention is further based on the discovery that a class of environmental enfluencers (e.g., CoQ10) can selectively elicit, in disease cells of the metabolic diseases, a cellular metabolic energy shift towards more normalized mitochondrial oxidative phosphorylation. These environmental influencers are capable of modulating intracellular targets that serve as key indices of metabolic disorders (such as diabetes), in a manner representative of therapeutic endpoints.

The present invention is further based, at least in part, on the discovery that application of endogenous Coenzyme Q10 (also referred to as CoQ10 or Q10 herein) to cells results in an apoptotic response. The apoptotic response is preferentially induced in cancer cells. A time and dose response of mitochondrial Q10 levels was observed, wherein after 48 hours, the level of Q10 in cell mitochondria was increased by six fold. The invention is further based on the surprising and unexpected discovery that the Q10 is maintained in the supplied oxidized form (pro-oxidant) and not converted to the reduced (anti-oxidant) form of Q10H2 in any significant amounts. The invention is based on the further discovery that a significant number of proteins and mRNA levels are modulated in cells treated with Q10. These modulated proteins were found to be clustered into several cellular pathways, including apoptosis, cancer biology and cell growth, glycolysis and metabolism, molecular transport, and cellular signaling.

Applicants' data described herein has provided insight into the mechanism of action of Q10. In particular, while not wishing to be bound by theory, Applicants' discoveries indicate that Q10 induces a metabolic shift to the cell microenvironment. Many diseases are known to be associeated with an altered metabolic state. For

example, differential metabolism is known to occur in cancer cells (the Warurg effect), whereby most cancer cells predominantly produce energy by glycolysis followed by lactic acid fermentation in the cytosol, rathe than by oxidative phosphorylation (oxidation of pyruvate) in the mitochondria. In another example, metabolic disorders, such as diabetes and obesity, are associated with an altered glucose metabolism.

Accordingly, the invention provides, in a first aspect, a method for treating, alleviating symptoms of, inhibiting progression of, or preventing a CoQ10 responsive disorder in a mammal, the method comprising: administering to the mammal in need thereof a therapeutically effective amount of pharmaceutical composition comprising at least one environmental influencer (env-influencer), wherein the environmental influencer selectively elicits, in a disease cell of the mammal, a cellular metabolic energy shift towards levels of glycolysis and mitochondrial oxidative phosphorylation observed in a normal cell of the mammal under normal physiological conditions.

In one embodiment, the CoQ10 responsive disorder is a metabolic disorder.

The invention provides, in a related aspect, a method for for treating, alleviating symptoms of, or inhibiting progression of obesity in a mammal, the method comprising: administering to the mammal a therapeutically effective amount of a pharmaceutical composition comprising a sole active ingredient consisting of Coenzyme Q10 (CoQ10) in the amount of about 1% to about 25% w/w of the composition, or a building block of CoQ10.

The invention provides, in a further related aspect, a method for selectively augmenting mitochondrial oxidative phosphorylation, in a disease cell of a mammal in need of treatment for obesity, the method comprising: administering to said mammal a therapeutically effective amount of a pharmaceutical composition comprising a sole active ingredient consisting of Coenzyme Q10 (CoQ10) in an amount of about 1% to about 25% w/w of the composition, or a building block of CoQ10, thereby selectively augmenting mitochondrial oxidative phosphorylation in said disease cell of the mammal.

The invention provides, in yet another aspect, a method of reducing lipid levels in a mammal suffering from obesity comprising administering to the mammal an effective amount of a pharmaceutical composition comprising a sole active ingredient consisting of Coenzyme Q10 in an amount of about 1% to about 25% w/w of the composition, or a building block of CoQ10, thereby reducing lipid levels in the mammal.

The invention provides, in yet another aspect, the use of Coenzyme Q10 (CoQ10) in the manufacture of a medicament for treating, alleviating symptoms of, or inhibiting progression of obesity in a mammal, wherein CoQ10, or a building block

thereof, is the sole active ingredient, and is in the amount of 1% to about 25% w/w of the composition.

The invention provides, in yet another aspect, the use of Coenzyme Q10 (CoQ10) in the manufacture of a medicament for selectively augmenting mitochondrial oxidative phosphorylation, in a disease cell of a mammal in need of treatment for obesity, wherein CoQ10, or a building block thereof, is the sole active ingredient, and is in the amount of 1% to about 25% w/w of the composition.

The invention provides, in yet another aspect, the use of Coenzyme Q10 (CoQ10) in the manufacture of a medicament for reducing lipid levels in a mammal suffering from obesity, wherein CoQ10, or a building block thereof, is the sole active ingredient, and is in the amount of 1% to about 25% w/w of the composition.

The invention provides, in another aspect, a method for treating, alleviating symptoms of, inhibiting progression of, or preventing a metabolic disorder in a mammal, the method comprising administering to the mammal in need thereof a therapeutically effective amount of a pharmaceutical composition comprising at least one environmental influencer (env-influencer), wherein the environmental influencer selectively elicits, in a disease cell of the mammal, a cellular metabolic energy shift towards normalized mitochondrial oxidative phosphorylation.

In one embodiment, the environmental influencer does not substantially elicit, in normal cells of the mammal, the cellular metabolic energy shift towards mitochondrial oxidative phosphorylation.

In one embodiment, the mammal is human (or a non-human mammal).

In one embodiment, the metabolic disorder is responsive or sensitive to treatment by Coenzyme Q10 or its metabolites or analogs thereof.

In one embodiment, the metabolic disorder is characterized by a dysregulated mitochondrial oxidative phosphorylation function that leads to altered gene regulation and/or protein-protein interactions which contribute to or causally lead to the metabolic disease.

In one embodiment, the environmental influencer comprises (a) benzoquinone or at least one molecule that facilitates the biosynthesis of the benzoquinone ring, and (b) at least one molecule that facilitates the synthesis of and/or attachment of isoprenoid units to the benzoquinone ring.

In one embodiment, said at least one molecule that facilitates the biosynthesis of the benzoquinone ring comprises: L-Phenylalanine, DL-Phenylalanine, D-Phenylalanine, L-Tyrosine, DL-Tyrosine, D-Tyrosine, 4-hydroxy-phenylpyruvate, 3-methoxy-4-hydroxymandelate (vanillylmandelate or VMA), vanillic acid, pyridoxine, or panthenol.

In one embodiment, said at least one molecule that facilitates the synthesis of and/or attachment of isoprenoid units to the benzoquinone ring comprises: phenylacetate, 4-hydroxy-benzoate, mevalonic acid, acetylglycine, acetyl-CoA, or farnesyl.

In one embodiment, the environmental influencer comprises (a) one or more of L-Phenylalanine, L-Tyrosine, and 4-hydroxyphenylpyruvate; and (b) one or more of 4-hydroxy benzoate, phenylacetate, and benzoquinone.

In one embodiment, the environmental influencer: (a) inhibits Bcl-2 expression and/or promotes Caspase-3 expression; and/or (b) inhibits cell proliferation.

In one embodiment, the environmental influencer is a multidimensional intracellular molecule (MIM). In one embodiment, the MIM is selected from: alpha ketoglutarate / alpha ketoglutaric acid, Malate / Malic acid, Succinate / Succinic acid, Glucosamine, Adenosine, Adenosine Diphosphate, Glucuronide / Glucuronic acid, Nicotinic Acid, Nicotinic Acid Dinucleotide, Alanine / Phenylalanine, Pyridoxine, Thiamine, or Flavin Adenine Dinucleotide. In one embodiment, the multidimensional intracellular molecule is selected from the group consisting of acetyl Co-A, palmityl Co-A, L-carnitine, and amino acids, e.g., tyrosine, phenylalanine, and cysteine.

In one embodiment, the environmental influencer is an epimetabolic shifter (epi-shifter). In one embodiment, the epimetabolic shifter is selected from Transaldolase, Transketolase, Succinyl CoA synthase, Pyruvate Carboxylase, or Riboflavin. In one embodiment, the epimetabolic shifter is selected from the group consisting of coenzyme Q10, vitamin D3 and extracellular matrix components. In one embodiment, the

epimetabolic shifter is coenzyme Q10. In one embodiment, the extracellular matrix components are selected from the group consisting of fibronectin, immunomodulators (e.g., TNFα or an interleukin), angiogenic factors, and apoptotic factors.

In one embodiment, a population of humans are treated and at least 25% of the population had a systemic environmental influencer level that was therapeutic for the disorder being treated. In other embodiments, a population of humans are treated and at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the population had a systemic Coenzyme Q10 level that was therapeutic for the disorder being treated. It should be understood that ranges having any one of these values as the upper or lower limits are also intended to be part of this invention, *e.g.*, 10% to 25%, 15% to 35%, 25% to 50%, 35% to 60%, 40% to 70%, 50% to 75%, 60% to 85% or 70% to 90%.

In one embodiment, the metabolic disorder being treated is not a disorder typically treated via topical administration with the expectation of systemic delivery of an active agent at therapeutically effective levels.

In one embodiment, the concentration of the environmental influencer in the tissues of the human being treated is different than that of a control standard of human tissue representative of a healthy or normal state.

In one embodiment, the form of the environmental influencer administered to the human is different than the predominant form found in systemic circulation in the human. In one embodiment, the environmental influencer is administered to the human in oxidized form.

In one embodiment, the amount sufficient to treat the metabolic disorder in the human up-regulates or down-regulates mitochondrial oxidative phosphorylation.

In one embodiment, the amount sufficient to treat the metabolic disorder in the human modulates anaerobic use of glucose and/or lactate biosynthesis.

The invention provides, in another aspect, a method for treating or preventing a metabolic disorder in a human, comprising administering an environmental influencer to the human in an amount sufficient to treat or prevent the metabolic disorder, wherein the environmental influencer is administered such that it is maintained in its oxidized form during treatment, thereby treating or preventing the metabolic disorder.

In one embodiment, the form of the environmental influencer administered to the human is different than the predominant form found in systemic circulation in the human.

The invention provides, in still another aspect, a method for treating or preventing a metabolic disorder in a human comprising selecting a human subject suffering from a metabolic disorder; and administering to said human a therapeutically effective amount of an env-influencer capabable of augmenting mitochondrial oxidative phosphorylation and, optionally, blocking anaerobic use of glucose, thereby treating or preventing the metabolic disorder.

The invention provides, in another aspect, a method for selectively augmenting mitochondrial oxidative phosphorylation, in a disease cell of a mammal in need of treatment for a metabolic disorder, the method comprising: administering to said mammal a therapeutically effective amount of a pharmaceutical composition comprising at least one env-influencer, thereby selectively augmenting mitochondrial oxidative phosphorylation in said disease cell of the mammal.

In one embodiment of the methods of the invention, the method further comprises upregulating the expression of one or more genes selected from the group consisting of the molecules listed in Tables 2-4 & Tables 6-28 & Tables 63-68 with a positive fold change; and/or downregulating the expression of one or more genes selected from the group consisting of the molecules listed in Tables 2-4 & Tables 6-28 & Tables 63-68 with a negative fold change, thereby treating or preventing the metabolic disorder. In one embodiment, the method further comprises modulating the expression of one or more genes selected from the group consisting of HNF4-alpha, Bcl-xl, Bcl-xS, BNIP-2, Bcl-2, Birc6, Bcl-2-L11, XIAP, BRAF, Bax, c-Jun, Bmf, PUMA, cMyc, transaldolase 1, COQ1, COQ3, COQ6, prenyltransferase, 4-hydrobenzoate, neutrophil cytosolic factor 2, nitric oxide synthase 2A, superoxide dismutase 2, VDAC, Bax channel, ANT, Cytochrome c, complex 1, complex II, complex III, complex IV, Foxo 3a, DJ-1, IDH-1, Cpt1C and Cam Kinase II.

In one embodiment of the methods of the invention, the treatment occurs via an interaction of the environmental influencer with a molecule selected from the group consisting of the molecules listed in Tables 2-4 & 6-28 & 63-68 . In one embodiment, the treatment occurs via an interaction of the environmental influencer with a protein

selected from the group consisting of HNF4-alpha, Bcl-xl, Bcl-xS, BNIP-2, Bcl-2, Birc6, Bcl-2-L11 (Bim), XIAP, BRAF, Bax, c-Jun, Bmf, PUMA, cMyc, transaldolase 1, COQ1, COQ3, COQ6, prenyltransferase, 4-hydrobenzoate, neutrophil cytosolic factor 2, nitric oxide synthase 2A, superoxide dismutase 2, VDAC, Bax channel, ANT, Cytochrome c, complex 1, complex II, complex III, complex IV, Foxo 3a, DJ-1, IDH-1, Cpt1C and Cam Kinase II. In one embodiment, the treatment occurs via an interaction of the env-influencer with HNF4alpha. In one embodiment, the treatment occurs via an interaction of the env-influencer with transaldolase.

In one embodiment of the methods of the invention, the metabolic disorder is selected from the group consisting of diabetes, obesity, pre-diabetes, Metabolic Syndrome and any key elements of a metabolic disorder.

In one embodiment, the metabolic disorder is diabetes, and the env-influencer affects beta cell function, insulin metabolism, and/or glucagon deposition.

In one embodiment, the metabolic disorder is obesity, and the env-influencer affects beta cell oxidation in the mitochondria, decrease in adipocyte size, and/or control of cortisol levels.

In one embodiment, the metabolic disorder is a cardiovascular disease, and the env-influencer affects decrease in smooth muscle cell proliferation in the tunica media, lipid peroxidation, thromboxane - ax2 synthesis, TNFα, IL-1B, platelet aggregation, decrease in nitric oxide (NO) production, plaque deposition and/or normalized glycemic control.

In one embodiment, key elements of a metabolic disorder include impaired fasting glucose, impaired glucose tolerance, increased waist circumference, increased visceral fat content, increased fasting plasma glucose, increased fasting plasma triglycerides, decreased fasting high density lipoprotein level, increased blood pressure, insulin resistance, hyperinsulinemia, cardiovascular disease, arteriosclerosis, coronary artery disease, peripheral vascular disease, cerebrovascular disease, congestive heart failure, elevated plasma norepinephrine, elevated cardiovascular-related inflammatory factors, elevated plasma factors potentiating vascular endothelial dysfunction, hyperlipoproteinemia, arteriosclerosis or atherosclerosis, hyperphagia, hyperglycemia, hyperlipidemia, and hypertension or high blood pressure, increased plasma postprandial triglyceride or free fatty acid levels, increased cellular oxidative stress or plasma

indicators thereof, increased circulating hypercoagulative state, hepatic steatosis, hetaptic steatosis, renal disease including renal failure and renal insufficiency.

In one embodiment of the methods of the invention, the method further comprises administering an additional therapeutic agent, *e.g*, diabetes mellitus-treating agents, diabetic complication-treating agents, antihyperlipemic agents, hypotensive or antihypertensive agents, anti-obesity agents, diuretics, chemotherapeutic agents, immunotherapeutic agents and immunosuppressive agents.In one embodiment, the metabolic disorder is selected from the group consisting of diabetes, obesity, pre-diabetes, Metabolic Syndrome and any key elements of a metabolic disorder. In one embodiment, a key element of a metabolic disorder is selected from the group consisting of impaired fasting glucose, impaired glucose tolerance, increased waist circumference, increased visceral fat content, increased fasting plasma glucose, increased fasting plasma triglycerides, decreased fasting high density lipoprotein level, increased blood pressure, insulin resistance, hyperinsulinemia, cardiovascular disease, arteriosclerosis, coronary artery disease, peripheral vascular disease, cerebrovascular disease, congestive heart failure, elevated plasma norepinephrine, elevated cardiovascular-related inflammatory factors, elevated plasma factors potentiating vascular endothelial dysfunction, hyperlipoproteinemia, arteriosclerosis or atherosclerosis, hyperphagia, hyperglycemia, hyperlipidemia, and hypertension or high blood pressure, increased plasma postprandial triglyceride or free fatty acid levels, increased cellular oxidative stress or plasma indicators thereof, increased circulating hypercoagulative state, hepatic steatosis, hetaptic steatosis, renal disease including renal failure and renal insufficiency.

In one embodiment of the methods of the invention, the method further comprises administering an additional therapeutic agent, *e.g*., diabetes mellitus-treating agents, diabetic complication-treating agents, antihyperlipemic agents, hypotensive or antihypertensive agents, anti-obesity agents, diuretics, chemotherapeutic agents, immunotherapeutic agents and immunosuppressive agents.

The invention provides, in another aspect, a method of identifying an agent that is effective in treating a metabolic disorder, the method comprising selecting an environmental influencer; identifying an environmental influencer capable of shifting the metabolic state of a cell; and determining whether the environmental influencer is

effective in treating the metabolic disorder; thereby identifying an agent that is effective in treating a metabolic disorder.

In one embodiment, an environmental influencer is identified as capable of shifting the metabolic state of a cell by measuring changes in any one or more of mRNA expression, protein expression, lipid or metabolite concentration, levels of bioenergetic molecules, cellular energetics, mitochondrial function and mitochondrial number.

In one embodiment, the environmental influencer effective in treating a metabolic disorder is capable of reducing glucose levels or lipid levels in a patient.

The invention provides, in still another aspect, a method of identifying a Multidimensional Intracellular Molecule, comprising contacting a cell with an endogenous molecule; monitoring the effect of the endogenous molecule on a cellular microenvironment profile; and identifying an endogenous molecule that induces a change to the cellular microenvironment profile; thereby identifying a Multidimensional Intracellular Molecule.

In one embodiment, the method further comprises comparing the effects of the endogenous molecule on the cellular microenvironment profile of a diseased cell and a normal control cell; and identifying an endogenous molecule that differentially induces a change to the cellular microenvironment profile of the diseased cell as compared to the normal control cell; thereby identifying a MIM.

In one embodiment, the effect on the cellular microenvironment profile is monitored by measuring a change in the level or activity of a cellular molecule selected from the group consisting of mRNA, protein, lipid and metabolite.

The invention provides, in still another aspect, a method of identifying an Epimetabolic shifter, comprising comparing molecular profiles for two or more cells or tissues, wherein the two or more cells or tissues display differential disease states; identifying a molecule from the moleculer profiles for which a change in level correlates to the disease state; introducing the molecule to a cell; and evaluating the ability of the molecule to shift the metabolic state of a cell, wherein a molecule capable of shifting the metabolic state of a cell is identified as an Epimetabolic shifter.

In one embodiment, the molecular profile is selected from the group consisting of a metabolite profile, lipid profile, protein profile or RNA profile.

In one embodiment, the molecule does not negatively effect the health or growth of a normal cell.

The invention provides, in another aspect, a composition comprising an agent identified according to any of the methods of the invention. The invention further provides, in a related aspect, a kit comprising a composition of the invention.

The invention provides, in another aspect, a method of reducing glucose levels in a patient comprising administering to the patient an effective amount of a composition of the invention. The invention provides, in a related aspect, a method of reducing lipid levels in a patient comprising administering to the patient an effective amount of a composition of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Brief Description of the Drawings:

Figure 1: Sensitivity of SK-MEL-28 to 24 hours of Q10 treatment measured by the amount of early and late apoptotic cells.

Figure 2: Sensitivity of SKBR3 to 24 hours of Q10 treatment measured by the amount of early and late apoptotic cells.

Figure 3: Sensitivity of PaCa2 to 24 hours of Q10 treatment measured by the amount of early and late apoptotic cells.

Figure 4: Sensitivity of PC-3 to 24 hours of Q10 treatment measured by the amount of early and late apoptotic cells.

Figure 5: Sensitivity of HepG2 to 24 hours of Q10 treatment measured by the amount of early and late apoptotic cells.

Figure 6: Sensitivity of MCF-7 to 24 hours of Q10 treatment measured by the amount of early and late apoptotic cells.

Figure 7: Measurement of apoptotic cells upon 24 hour treatment with Q10, as measured by Apostrand ELISA method.

Figure 8: Example gel analysis of 2-D gel electrophoresis. Spots excised for identification are marked.

Figure 9: Network of interaction between proteins identified by 2-D gel electrophoresis as being modulated by Q10 in SK-MEL-28 cells.

Figure 10: The pentose phosphate pathway adapted from Verhoeven *et al.* (Am. J. Hum. Genet. 2001 68(5):1086-1092).

Figure 11: 2-D gel of the mitochondrial enriched material of SK-MEL-28 cells. Spots excised and identified by mass spcectrometry characterization are marked.

Figure 12: Comparative plot of the relative amounts of Q10 present in SK-MEL-28 mitochondria following the exogenous addition of 100 μM Q10 into the culture medium.

Figure 13: Apoptosis pathway mapping known processes.

Figure 14: Western blot analysis of Bcl-xl.

Figure 15: Western blot analysis of SK-MEL-28 sample set proved with a Vimentin antibody.

Figure 16: Western blot analysis of cell lysis from a number of cell lines, evaluated with five antibodies targeting oxidative phosphorylation complexes (MitoSciences #MS601).

Figure 17: Western blot comparison of F1-alpha levels.

Figure 18: Western blot comparison of Q10 response with C-III-Core 2.

Figure 19: Western blot comparison of Q10 response with C-II-30.

Figure 20: Western blot comparison of Q10 response with C-IV-COX II.

Figure 21: Western blot comparison of Q10 response with C-I-20 (ND6).

Figure 22: Western blot analysis of a variety of cell types against five mitochondrial protein.

Figure 23: Western blot comparison of Q10 response with Complex V protein C-V-α.

Figure 24: Western blot comparison of Q10 response with C-III-Core 1.

Figure 25: Western blot comparison of Q10 response with Porin (VDAC1).

Figure 26: Western blot comparison of Q10 response with Cyclophilin D.

Figure 27: Western blot comparison of Q10 response with Cytochrome C.

Figure 28: Theoretical model of Q10 (spheres) inserted into the lipid binding channel of HNF4alpha (1M7W.pdb) in the Helix 10 open conformation.

Figure 29: OCR in HDFa cells in various glucose conditions in normoxic and hypoxic conditions.

Figure 30: OCR in HASMC cells in various glucose conditions in normoxic and hypoxic conditions.

Figure 31: OCR values in MCF-7 breast cancer cells in the absence and presence of 31510 and stressors.

Figure 32: OCR values in PaCa-2 pancreatic cancer cells in the absence and presence of 31510 and stressors.

Detailed Description of the Invention:

I. Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited to".

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

The term "such as" is used herein to mean, and is used interchangeably, with the phrase "such as but not limited to".

A "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal, preferably a mammal.

"Therapeutically effective amount" means the amount of a compound that, when administered to a patient for treating a disease, is sufficient to effect such treatment for the disease. When administered for preventing a disease, the amount is sufficient to avoid or delay onset of the disease. The "therapeutically effective amount" will vary depending on the compound, the disease and its severity and the age, weight, etc., of the patient to be treated.

"Preventing" or "prevention" refers to a reduction in risk of acquiring a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop in a patient that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease).

The term "prophylactic" or "therapeutic" treatment refers to administration to the subject of one or more of the subject compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, i.e., it protects the host against developing the unwanted condition, whereas if administered after manifestation of the unwanted condition, the treatment is therapeutic (i.e., it is intended to diminish, ameliorate or maintain the existing unwanted condition or side effects therefrom).

The term "therapeutic effect" refers to a local or systemic effect in animals, particularly mammals, and more particularly humans caused by a pharmacologically active substance. The term thus means any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease or in the enhancement of desirable physical or mental development and conditions in an animal or human. The phrase "therapeutically-effective amount" means that amount of such a substance that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. In certain embodiments, a therapeutically-effective amount of a compound will depend on its therapeutic index, solubility, and the like. For example, certain compounds discovered by the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

By "patient" is meant any animal (e.g., a human), including horses, dogs, cats, pigs, goats, rabbits, hamsters, monkeys, guinea pigs, rats, mice, lizards, snakes, sheep, cattle, fish, and birds.

"Metabolic pathway" refers to a sequence of enzyme-mediated reactions that transform one compound to another and provide intermediates and energy for cellular functions. The metabolic pathway can be linear or cyclic.

"Metabolic state" refers to the molecular content of a particular cellular, multicellular or tissue environment at a given point in time as measured by various chemical and biological indicators as they relate to a state of health or disease.

The term "microarray" refers to an array of distinct polynucleotides, oligonucleotides, polypeptides (e.g., antibodies) or peptides synthesized on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support.

The terms "disorders" and "diseases" are used inclusively and refer to any

deviation from the normal structure or function of any part, organ or system of the body (or any combination thereof). A specific disease is manifested by characteristic symptoms and signs, including biological, chemical and physical changes, and is often associated with a variety of other factors including, but not limited to, demographic, environmental, employment, genetic and medically historical factors. Certain characteristic signs, symptoms, and related factors can be quantitated through a variety of methods to yield important diagnostic information.

The term "expression" is used herein to mean the process by which a polypeptide is produced from DNA. The process involves the transcription of the gene into mRNA and the translation of this mRNA into a polypeptide. Depending on the context in which used, "expression" may refer to the production of RNA, protein or both.

The terms "level of expression of a gene" or "gene expression level" refer to the level of mRNA, as well as pre-mRNA nascent transcript(s), transcript processing intermediates, mature mRNA(s) and degradation products, or the level of protein, encoded by the gene in the cell.

The term "modulation" refers to upregulation (i.e., activation or stimulation), downregulation (i.e., inhibition or suppression) of a response, or the two in combination or apart. A "modulator" is a compound or molecule that modulates, and may be, e.g., an agonist, antagonist, activator, stimulator, suppressor, or inhibitor.

The term "intermediate of the coenzyme biosynthesis pathway" as used herein, characterizes those compounds that are formed between the chemical/biological conversion of tyrosine and Acetyl-CoA to uqiquinone. Intermediates of the coenzyme biosynthesis pathway include 3-hexaprenyl-4-hydroxybenzoate, 3-hexaprenyl-4,5-dihydroxybenzoate, 3-hexaprenyl-4-hydroxy-5-methoxybenzoate, 2-hexaprenyl-6-methoxy-1,4-benzoquinone, 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, 2-hexaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, 3-Octaprenyl-4-hydroxybenzoate, 2-octaprenylphenol, 2-octaprenyl-6-metholxyphenol, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, 2-decaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, 2-decaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, 2-decaprenyl-6-methoxy-1,4-benzoquinone, 2-decaprenyl-6-methoxyphenol, 3-decaprenyl-4-hydroxy-5-methoxybenzoate, 3-decaprenyl-4,5-dihydroxybenzoate, 3-decaprenyl-4-

hydroxybenzoate, 4-hydroxy phenylpyruvate, 4-hydroxyphenyllactate, 4-hydroxy-benzoate, 4-hydroxycinnamate and hexaprenydiphosphate.

As used herein, the phrase "anaerobic use of glucose" or "anaerobic glycolysis" refers to cellular production of energy by glycolysis followed by lactic acid fermentation in the cytosol. For example, many cancer cells produce energy by anaerobic glycolysis.

As used herein, the phrase "aerobic glycolysis" or "mitochondrial oxidative phosphorylation" refers to cellular production of energy by glycolysis followed by oxidation of pyruvate in mitochondria.

As used herein, the phrase "capable of blocking anaerobic use of glucose and augmenting mitochondrial oxidative phosphorylation" refers to the ability of an environmental influencer (*e.g.*, an epitmetabolic shifter) to induce a shift or change in the metabolic state of a cell from anaerobic glycolysis to aerobic glycolysis or mitochondrial oxidative phosphorylation.

Reference will now be made in detail to preferred embodiments of the invention. While the invention will be described in conjunction with the preferred embodiments, it will be understood that it is not intended to limit the invention to those preferred embodiments. To the contrary, it is intended to cover alternatives, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

II. Environmental influencers

The present invention provides methods of treating metabolic disorders by administration of an Environmental influencer. "Environmental influencers" (Env-influencers) are molecules that influence or modulate the disease environment of a human in a beneficial manner allowing the human's disease environment to shift, reestablish back to or maintain a normal or healthy environment leading to a normal state. Env-influencers include both Multidimensional Intracellular Molecules (MIMs) and Epimetabolic shifters (Epi-shifters) as defined below.

1. Multidimensional Intracellular Molecule (MIM)

The term "Multidimensional Intracellular Molecule (MIM)", is an isolated version or synthetically produced version of an endogenous molecule that is naturally

produced by the body and/or is present in at least one cell of a human. A MIM is characterized by one or more, two or more, three or more, or all of the following functions. MIMs are capable of entering a cell, and the entry into the cell includes complete or partial entry into the cell, as long as the biologically active portion of the molecule wholly enters the cell. MIMs are capable of inducing a signal transduction and/or gene expression mechanism within a cell. MIMs are multidimensional in that the molecules have both a therapeutic and a carrier, e.g., drug delivery, effect. MIMs also are multidimensional in that the molecules act one way in a disease state and a different way in a normal state. For example, in the case of CoQ-10, administration of CoQ-10 to a melanoma cell in the presence of VEGF leads to a decreased level of Bcl2 which, in turn, leads to a decreased oncogenic potential for the melanoma cell. In contrast, in a normal fibroblast, co-administration of CoQ-10 and VEFG has no effect on the levels of Bcl2. Preferably, MIMs selectively act in cells of a disease state, and have substantially no effect in (matching) cells of a normal state. Preferably, MIMs selectively renders cells of a disease state closer in phenotype, metabolic state, genotype, mRNA / protein expression level, etc. to (matching) cells of a normal state.

In one embodiment, a MIM is also an epi-shifter. In another embodiment, a MIM is not an epi-shifter. The skilled artisan will appreciate that a MIM of the invention is also intended to encompass a mixture of two or more endogenous molecules, wherein the mixture is characterized by one or more of the foregoing functions. The endogenous molecules in the mixture are present at a ratio such that the mixture functions as a MIM.

MIMs can be lipid based or non-lipid based molecules. Examples of MIMs include, but are not limited to, CoQ10, acetyl Co-A, palmityl Co-A, L-carnitine, amino acids such as, for example, tyrosine, phenylalanine, and cysteine. In one embodiment, the MIM is a small molecule. In one embodiment of the invention, the MIM is not CoQ10. MIMs can be routinely identified by one of skill in the art using any of the assays described in detail herein.

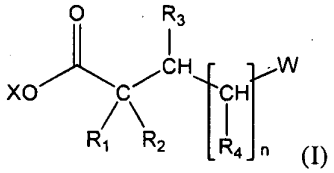
In some embodiments, MIMs include compounds in the Vitamin B family, or nucleosides, mononucleotides or dinucleotides that comprise a compound in the Vitamin B family. Compounds in the vitamin B family include, for example, thiamine (vitamin B1), niacin (also known as nicotinic acid or Vitamin B3), or pyridoxine (vitamin B6) as

well as provitamins such as panthenol (provitamin B5). In some embodiments, the MIM is selected from thiamine, niacin and pyridoxine. Nucleosides, mononucleotides or dinucleotides that comprise a compound in the vitamin B family include, for example, nucleosides, mononucleotides or dinucleotides which include an adenine or a niacin (nicotinic acid) molecule. In some embodiments, the MIM is selected from adenosine, adenosine diphosphate (ADP), flavin adenosine dinucleotide (FAD, which comprises parts of vitamin B2 and ADP) and nicotinic acid dinucleotide.

In other embodiments, the MIMs include amino acids. Examples of amino acids include, for example, tyrosine (e.g., L-tyrosine), cysteine, phenylalanine (*e.g.*, L-phenylalanine) and alanine. In some embodiments, the amino acid is phenylalanine or alanine. In some embodiments, the MIMs include amino acid derivatives such as 4-hydroxyphenylpyruvate or acetylglycine.

In some embodiment, the MIM is a glucose analog, *e.g.*, a glucose molecule wherein one -OH or $-CH_2OH$ substituent has been replaced with a -COOH, a $-COO^-$ or an $-NH_2$ substituent. Examples of glucose analogs include glucosamine, glucuronic acid, glucuronide and glucuronate.

In some embodiments, the MIM is selected from compounds of formula (I):



(I)

wherein

n is an integer of 0 or 1;

R^1, R^2, R^3 and R^4, when present, are each independently selected from hydrogen and hydroxyl or R^1 and R^2 are taken together with the carbon on which they are attached to form a carbonyl (C=O) group;

W is -COOH or $-N(CH_3)_3^+$; and

X is hydrogen, a negative charge or a alkali metal cation, such as Na^+ or.

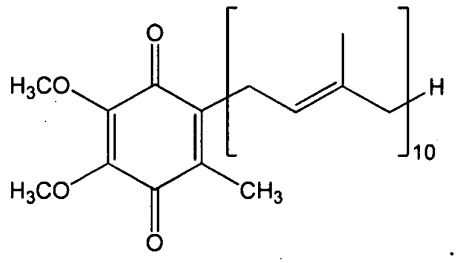
It is to be understood that when n is 0, the CHR^3 group is bonded to the W substituent.

In some embodiments, W is $-N(CH_3)_3^+$. In some embodiments, the MIM is a carnitine, such as L-carnitine.

In some embodiments, the MIM is a dicarboxylic acid. In some embodiments, W is -COOH. In some embodiments, R^3 is hydrogen. In some embodiments, n is 0. In some embodiments, R^1 and R^2 are each independently hydrogen. In some embodiments, W is -COOH, R^3 is hydrogen, n is 0 and R^1 and R^2 are each independently hydrogen. In some embodiments, n is 1. In some embodiments R^1 and R^2 are taken together with the carbon on which they are attached to form a carbonyl (C=O) group. In some embodiments, R^4 is hydrogen. In some embodiments, R^4 is hydroxyl. In some embodiments, W is -COOH, R^3 is hydrogen, n is 1 and R^1 and R^2 are taken together with the carbon on which they are attached to form a carbonyl (C=O) group.

In some embodiments, the MIM is an intermediate of the Krebs Cycle, the excess of which drives the Krebs Cycle towards productive oxidative phosphorylation. Exemplary Krebs Cycle intermediates that are MIMs include succinic acid or succinate, malic acid or malate, and α-ketoglutaric acid or α-ketoglutarate.

In some embodiments, the MIM is a building block of CoQ10, which has the following structure:



Thus, building blocks of CoQ10 include, but are not limited to, phenylalanine, tyrosine, 4-hydroxyphenylpyruvate, phenylacetate, 3-methoxy-4-hydroxymandelate, vanillic acid, 4-hydroxybenzoate, mevalonic acid, farnesyl, 2,3-dimethoxy-5-methyl-p-benzoquinone, as well as the corresponding acids or ions thereof. In some embodiments, the MIM is selected from phenylalanine, tyrosine, 4-hydroxyphenylpyruvate, phenylacetate and 4-hydroxybenzoate.

(i) Methods of Identifying MIMS

The present invention provides methods for identifying a MIM. Methods for identifying a MIM involve, generally, the exogenous addition to a cell of an endogenous molecule and evaluating the effect on the cell, *e.g.*, the cellular microenvironment

profile, that the endogenous molecule provides. Effects on the cell are evaluated at one or more of the cellular, mRNA, protein, lipid, and/or metabolite level to identify alterations in the cellular microenvironment profile. In one embodiment, the cells are cultured cells, *e.g.*, in vitro. In one embodiment, the cells are present in an organism. The endogenous molecule may be added to the cell at a single concentration or may be added to the cell over a range of concentrations. In one embodiment, the endogenous molecule is added to the cells such that the level of the endogenous molecule in the cells is elevated (*e.g.*, is elevated by 1.1 fold, 1.2 fold, 1.3 fold, 1.4 fold, 1.5 fold, 1.6 fold, 1.7 fold, 1.8 fold, 1.9 fold, 2.0 fold, 3.0 fold, 4.0 fold, 5.0 fold, 10 fold, 15 fold, 20 fold, 25 fold, 30 fold, 35 fold, 40 fold, 45 fold, 50 fold or greater) as compared to the level of the endogenous molecule in a control, untreated cell.

Molecules that induce a change in the cell as detected by alterations in, for example, any one or more of morphology, physiology, and/or composition (*e.g.*, mRNA, protein, lipid, metabolite) may be evaluated further to determine if the induced changes to the cellular microenvironment profile are different between a disease cellular state and a normal cellular state. Cells (*e.g.*, cell culture lines) of diverse tissue origin, cell type, or disease state may be evaluated for comparative evaluation. For example, changes induced in the cellular microenvironment profile of a cancer cell may be compared to changes induced to a non-cancerous or normal cell. An endogenous molecule that is observed to induce a change in the microenvironment profile of a cell (*e.g.*, induces a change in the morphology, physiology and/or composition, *e.g.*, mRNA, protein, lipid or metabolite, of the cell) and/or to differentially (*e.g.*, preferentially) induce a change in the microenvironment profile of a diseased cell as compared to a normal cell, is identified as a MIM.

MIMs of the invention may be lipid based MIMs or non-lipid based MIMs. Methods for identifying lipid based MIMs involve the above-described cell based methods in which a lipid based endogenous molecule is exogenously added to the cell. In a preferred embodiment, the lipid based endogenous molecule is added to the cell such that the level of the lipid based endogenous molecule in the cell is elevated. In one embodiment, the level of the lipid based endogenous molecule is elevated by 1.1 fold, 1.2 fold, 1.3 fold, 1.4 fold, 1.5 fold, 1.6 fold, 1.7 fold, 1.8 fold, 1.9 fold, 2.0 fold, 3.0 fold, 4.0 fold, 5.0 fold, 10 fold, 15 fold, 20 fold, 25 fold, 30 fold, 35 fold, 40 fold, 45

fold, 50 fold or greater as compared to the level in an untreated control cell. Formulation and delivery of the lipid based molecule to the cell is dependent upon the properties of each molecule tested, but many methods are known in the art. Examples of formulation and delivery of lipid based molecules include, but are not limited to, solubilization by co-solvents, carrier molecules, liposomes, dispersions, suspensions, nanoparticle dispersions, emulsions, *e.g.*, oil-in-water or water-in-oil emulsions, multiphase emulsions, *e.g.*, oil-in-water-in-oil emulsions, polymer entrapment and encapsulation. The delivery of the lipid based MIM to the cell can be confirmed by extraction of the cellular lipids and quantification of the MIM by routine methods known in the art, such as mass spectrometry.

Methods for identifying non-lipid based MIMs involve the above-described cell based methods in which a non-lipid based endogenous molecule is exogenously added to the cell. In a preferred embodiment, the non-lipid based endogenous molecule is added to the cell such that the level of the non-lipid based endogenous molecule in the cell is elevated. In one embodiment, the level of the non-lipid based endogenous molecule is elevated by 1.1 fold, 1.2 fold, 1.3 fold, 1.4 fold, 1.5 fold, 1.6 fold, 1.7 fold, 1.8 fold, 1.9 fold, 2.0 fold, 3.0 fold, 4.0 fold, 5.0 fold, 10 fold, 15 fold, 20 fold, 25 fold, 30 fold, 35 fold, 40 fold, 45 fold, 50 fold or greater as compared to the level in an untreated control cell. Formulation and delivery of the non-lipid based molecule to the cell is dependent upon the properties of each molecule tested, but many methods are known in the art. Examples of formulations and modes of delivery of non-lipid based molecules include, but are not limited to, solubilization by co-solvents, carrier molecules, active transport, polymer entrapment or adsorption, polymer grafting, liposomal encapsulation, and formulation with targeted delivery systems. The delivery of the non-lipid based MIM to the cell may be confirmed by extraction of the cellular content and quantification of the MIM by routine methods known in the art, such as mass spectrometry.

2. Epimetabolic Shifters (Epi-shifters)

As used herein, an "epimetabolic shifter" (epi-shifter) is a molecule (endogenous or exogenous) that modulates the metabolic shift from a healthy (or normal) state to a disease state and vice versa, thereby maintaining or reestablishing cellular, tissue, organ, system and/or host health in a human. Epi-shifters are capable of effectuating

normalization in a tissue microenvironment. For example, an epi-shifter includes any molecule which is capable, when added to or depleted from a cell, of affecting the microenvironment (*e.g.*, the metabolic state) of a cell. The skilled artisan will appreciate that an epi-shifter of the invention is also intended to encompass a mixture of two or more molecules, wherein the mixture is characterized by one or more of the foregoing functions. The molecules in the mixture are present at a ratio such that the mixture functions as an epi-shifter. Examples of epi-shifters include, but are not limited to, coQ-10; vitamin D3; ECM components such as fibronectin; immunomodulators, such as TNFa or any of the interleukins, e.g., IL-5, IL-12, IL-23; angiogenic factors; and apoptotic factors.

In some embodiments, the epi-shifter is an enzyme, such as an enzyme that either directly participates in catalyzing one or more reactions in the Krebs Cycle, or produces a Krebs Cycle intermediate, the excess of which drive the Krebs Cycle. In some embodiments, the enzyme is an enzyme of the non-oxidative phase of the pentose phosphate pathway, such as transaldolase, or transketolase. In other embodiments, the enzyme is a component enzyme or enzyme complex that facilitates the Krebs Cycle, such as a synthase or a ligase. Exemplary enzymes include succinyl CoA synthase (Krebs Cycle enzyme) or pyruvate carboxylase (a ligase that catalyzes the reversible carboxylation of pyruvate to form oxaloacetate (OAA), a Krebs Cycle intermediate).

In some embodiments, the epi-shifter is a building block of CoQ10. Building blocks of CoQ10 include, but are not limited to, phenylalanine, tyrosine, 4-hydroxyphenylpyruvate, phenylacetate, 3-methoxy-4-hydroxymandelate, vanillic acid, 4-hydroxybenzoate, mevalonic acid, farnesyl, 2,3-dimethoxy-5-methyl-p-benzoquinone, as well as the corresponding acids or ions thereof. In some embodiments, the epi-shifter is selected from phenylalanine, tyrosine, 4-hydroxyphenylpyruvate, phenylacetate and 4-hydroxybenzoate.

In some embodiments, the epi-shifter is a compound in the Vitamin B family. Compounds in the vitamin B family include, for example, riboflavin (vitamin B2), or analogs thereof. Epi-shifters also include any analogs or pro-drugs that may be metabolized *in vivo* to any of the endogenous MIMs, such as those described herein.

In one embodiment, the epi-shifter also is a MIM. In one embodiment, the epi-shifter is not CoQ10. Epi-shifters can be routinely identified by one of skill in the art using any of the assays described in detail herein.

(i) Methods of identifying Epi-shifters

Epimetabolic shifters (epi-shifter) are molecules capable of modulating the metabolic state of a cell, *e.g.*, inducing a metabolic shift from a healthy (or normal) state to a disease state and vice versa, and are thereby capable of maintaining or reestablishing cellular, tissue, organ, system and/or host health in a human. Epi-shifters of the invention thus have utility in the diagnostic evaluation of a diseased state. Epi-shifters of the invention have further utility in therapeutic applications, wherein the application or administration of the epi-shifter (or modulation of the epi-shifter by other therapeutic molecules) effects a normalization in a tissue microenvironment and the disease state.

The identification of an epimetabolic shifter involves, generally, establishing a molecular profile, e.g., of metabolites, lipids, proteins or RNAs (as individual profiles or in combination), for a panel of cells or tissues that display differential disease states, progression, or aggressiveness A molecule from the profile(s) for which a change in level (*e.g.*, an increased or decreased level) correlates to the disease state, progression or aggressiveness is identified as a potential epi-shifter.

In one embodiment, an epi-shifter is also a MIM. Potential epi-shifters may be evaluated for their ability to enter cells upon exogenous addition to a cell by using any number of routine techniques known in the art, and by using any of the methods described herein. For example, entry of the potential epi-shifter into a cell may be confirmed by extraction of the cellular content and quantification of the potential epi-shifter by routine methods known in the art, such as mass spectrometry. A potential epi-shifter that is able to enter a cell is thereby identified as a MIM.

To identify an epi-shifter, a potential epi-shifter is next evaluated for the ability to shift the metabolic state of a cell. The ability of a potential epi-shifters to shift the metabolic state of the cell microenvironment is evaluated by introducing (*e.g.*, exogenously adding) to a cell a potential epi-shifter and monitoring in the cell one or more of: changes in gene expression (e.g., changes in mRNA or protein expression), concentration changes in lipid or metabolite levels, changes in bioenergetic molecule

levels, changes in cellular energetics, and/or changes in mitochondrial function or number. Potential epi-shifters capable of shifting the metabolic state of the cell microenvironment can be routinely identified by one of skill in the art using any of the assays described in detail herein. Potential epi-shifters are further evaluated for the ability to shift the metabolic state of a diseased cell towards a normal healthy state (or conversely, for the ability to shift the metabolic state of a normal cell towards a diseased state). A potential epi-shifter capable of shifting the metabolic state of a diseased cell towards a normal healthy state (or of shifting the metabolic state of healthy normal cell towards a diseased state) is thus identified as an Epi-shifter. In a preferred embodiment, the epi-shifter does not negatively impact the health and/or growth of normal cells.

Epimetabolic shifters of the invention include, but are not limited to, small molecule metabolites, lipid-based molecules, and proteins and RNAs. To identify an epimetabolic shifter in the class of small molecule endogenous metabolites, metabolite profiles for a panel of cells or tissues that display differential disease states, progression, or aggressiveness are established. The metabolite profile for each cell or tissue is determined by extracting metabolites from the cell or tissue and then identifying and quantifying the metabolites using routine methods known to the skilled artisan, including, for example, liquid-chromatography coupled mass spectrometry or gas-chromatography couple mass spectrometry methods. Metabolites for which a change in level (e.g., an increased or decreased level) correlates to the disease state, progression or aggressiveness, are identified as potential epi-shifters.

To identify epimetabolic shifters in the class of endogenous lipid-based molecules, lipid profiles for a panel of cells or tissues that display differential disease states, progression, or aggressiveness are established. The lipid profile for each cell or tissue is determined by using lipid extraction methods, followed by the identification and quantitation of the lipids using routine methods known to the skilled artisan, including, for example, liquid-chromatography coupled mass spectrometry or gas-chromatography couple mass spectrometry methods. Lipids for which a change in level (*e.g.*, an increase or decrease in bulk or trace level) correlates to the disease state, progression or aggressiveness, are identified as potential epi-shifters.

To identify epimetabolic shifters in the class of proteins and RNAs, gene expression profiles for a panel of cells or tissues that display differential disease states,

progression, or aggressiveness are established. The expression profile for each cell or tissue is determined at the mRNA and/or protein level(s) using standard proteomic, mRNA array, or genomic array methods, *e.g.*, as described in detail herein. Genes for which a change in expression (*e.*g., an increase or decrease in expression at the mRNA or protein level) correlates to the disease state, progression or aggressiveness, are identified as potential epi-shifters.

Once the molecular profiles described above are established (e.g., for soluble metabolites, lipid-based molecules, proteins, RNAs, or other biological classes of composition), cellular and biochemical pathway analysis is carried out to elucidate known linkages between the identified potential epi-shifters in the cellular environment. This information obtained by such cellular and/or biochemical pathway analysis may be utilized to categorize the pathways and potential epi-shifters.

The utility of an Epi-shifter to modulate a disease state can be further evaluated and confirmed by one of skill in the art using any number of assays known in the art or described in detail herein. The utility of an Epi-shifter to modulate a disease state can be evaluated by direct exogenous delivery of the Epi-shifter to a cell or to an organism. The utility of an Epi-shifter to modulate a disease state can alternatively be evaluated by the development of molecules that directly modulate the Epi-shifter (*e.g.*, the level or activity of the Epi-shifter). The utility of an Epi-shifter to modulate a disease state can also be evaluated by the development of molecules that indirectly modulate the Epi-shifter (*e.*g., the level or activity of the Epi-shifter) by regulating other molecules, such as genes (*e.g.*, regulated at the RNA or protein level), placed in the same pathway as the Epi-shifter.

The Epimetabolomic approach described herein facilitates the identification of endogenous molecules that exist in a cellular microenvironment and the levels of which are sensed and controlled through genetic, mRNA, or protein-based mechanisms. The regulation response pathways found in normal cells that are triggered by an Epi-shifter of the invention may provide a therapeutic value in a misregulated or diseased cellular environment. In addition, the epimetabolic approach described herein identifies epi-shifters that may provide a diagnostic indication for use in clinical patient selection, a disease diagnostic kit, or as a prognostic indicator.

In certain embodiments, the MIMS and Epi-shifters disclosed herein exclude those that are conventionally used as a dietary supplement. In certain embodiments, these MIMS and/or Epi-shifter that are disclosed herein are of pharmaceutical grade. In certain embodiments, the MIMS and/or Epi-shifter of pharmaceutical grade has a purity between about 95% and about 100% and include all values between 95% and 100%. In certain embodiments, the purity of the MIMS and/or Epi-shifter is 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9%, 99.9 or 100%. In certain embodiments, the MIMS and/or Epi-shifter is free of endotoxins. In other embodiments, the MIMS and/or Epi-shifter is free of foreign protein materials. In certain embodiments, the MIMS and/or Epi-shifter is CoQ10.

III. Assays useful for identifying MIMs/Epi-shifters

Techniques and methods of the present invention employed to separate and identify molecules and compounds of interest include but are not limited to: liquid chromatography (LC), high-pressure liquid chromatography (HPLC), mass spectroscopy (MS), gas chromatography (GC), liquid chromatography/mass spectroscopy (LC-MS), gas chromatography/mass spectroscopy (GC-MS), nuclear magnetic resonance (NMR), magnetic resonance imaging (MRI), Fourier Transform InfraRed (FT-IR), and inductively coupled plasma mass spectrometry (ICP-MS). It is further understood that mass spectrometry techniques include, but are not limited to, the use of magnetic-sector and double focusing instruments, transmission quadrapole instruments, quadrupole ion-trap instruments, time-of-flight instruments (TOF), Fourier transform ion cyclotron resonance instruments (FT-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Quantification of Bioenergetic molecule levels:

Environmental influencers (e.g., MIMs or Epi-shifters) may be identified by changes in cellular bioenergetic molecule levels (*e.g.*, ATP, pyruvate, ADP, NADH, NAD, NADPH, NADP, acetylCoA, FADH2) of cells to which a candidate epi-shifter has been applied. Exemplary assays of bioenergetic molecule levels use colorometric, fluorescence, and/or bioluminescent-based methods. Examples of such assays are provided below.

Levels of ATP within cells can be measured with a number of assays and systems known in the art. For example, in one system, cytoplasmic ATP released from lysed cells reacts with luciferin and the enzyme luciferase to produce light. This bioluminescence is measured by a bioluminometer and the intracellular ATP concentration of the lysed cells can be calculated (EnzyLight™ ATP Assay Kit (EATP-100), BioAssay Systems, Hayward, CA). In another system, for example, both ATP and its dephosphorylated form, ADP, are calculated via bioluminescence; after ATP levels are calculated, ADP is transformed into ATP and then detected and calculated using the same luciferase system (ApoSENSOR™ ADP/ATP Ratio Assay Kit, BioVision Inc., Mountain View, CA).

Pyruvate is an important intermediate in cellular metabolic pathways. Pyruvate may be converted into carbohydrate via gluconeogenesis, converted into fatty acid or metabolized via acetyl CoA, or converted into alanine or ethanol, depending upon the metabolic state of a cell. Thus detection of pyruvate levels provides a measure of the metabolic activity and state of a cell sample. One assay to detect pyruvate, for example, uses both a colorimetric and fluorimetric to detect pyruvate concentrations within different ranges (EnzyChrom™Pyruvate Assay Kit (Cat# EPYR-100), BioAssay Systems, Hayward, CA).

Environmental influencers (e.g., MIMs or Epi-shifters) may influence the process of oxidative phosphorylation carried out by mitochondria in cells, which are involved in the generation and maintenance of bioenergetic molecules in cells. In addition to assays that detect changes in cellular energetics in cell cultures and samples directly (described below), assays exist that detect and quantify the effects of compounds on discrete enzymes and complexes of mitochondria in cells. For example, the MT-OXC MitoTox™ Complete OXPHOS Activity Assay (MitoSciences Inc., Eugene, OR) can detect and quantify the effects of compounds applied directly to complexes I to V extracted from mitochondria. Assays for the detection and quantification of effects on individual mitochondrial complexes such as NADH dehydrogenase (Complex I), cytochrome c oxidase (Complex IV) and ATP synthase (Complex V) are also available (MitoSciences Inc., Eugene, OR).

Measurement of Cellular Energetics:

Environmental influencers (e.g., MIMs or Epi-shifters) may also be identified by changes in cellular energetics. One example of the measurement of cellular energetics are the real-time measures of the consumption of molecular oxygen and/or the change in pH of the media of a cell culture. For example, the ability of a potential epi-shifter to modulate the metabolic state of a cell may be analyzed using, for example, the XF24 Analyzer (Seahorse, Inc.). This technology allows for real time detection of oxygen and pH changes in a monolayer of cells in order to evaluate the bioenergetics of a cell microenvironment. The XF24 Analyzer measures and compares the rates of oxygen consumption (OCR), which is a measure of aerobic metabolism, and extracellular acidification (ECAR), which is a measure of glycolysis, both key indicators of cellular energetics.

Measurement of Oxidative Phosphorylation and Mitochondrial Function

Oxidative Phosphorylation is a process by which ATP is generated via the oxidation of nutrient compounds, carried out in eukaryotes via protein complexes embedded in the membranes of mitochondria. As the primary source of ATP in the cells of most organisms, changes in oxidative phosphorylation activity can strongly alter metabolism and energy balance within a cell. In some embodiments of the invention, environmental influencers (e.g., MIMs or Epi-shifters) may be detected and/or identified by their effects on oxidative phosphorylation. In some embodiments, environmental influencers (e.g., MIMs or Epi-shifters) may be detected and/or identified by their effects on specific aspects of oxidative phosphorylation, including, but not limited to, the electron transport chain and ATP synthesis.

The membrane-embedded protein complexes of the mitochrondria that carry out processes involved in oxidative phosphorylation perform specific tasks and are numbered I, II, III and IV. These complexes, along with the trans-inner membrane ATP synthase (also known as Complex V), are the key entities involved in the oxidative phosphorylation process. In addition to assays that can examine the effects of environmental influencers (e.g., MIMs or Epi-shifters) on mitochondrial function in general and the oxidative phosphorylation process in particular, assays are available that can be used to examine the effects of an epi-shifter on an individual complex separately from other complexes.

Complex I, also known as NADH-coenzyme Q oxidoreductase or NADH dehydrogenase, is the first protein in the electron transport chain. In some embodiments, the detection and quantification of the effect of an epi-shifter on the production of NAD^+ by Complex I may be perfomed. For example, the complex can be immunocaptured from a sample in a 96-well plate; the oxidation of NADH to NAD^+ takes place concurrently with the reduction of a dye molecule which has an increased absorbance at 450 nM (Complex I Enzyme Activity Microplate Assay Kit, MitoSciences Inc., Eugene, OR).

Complex IV, also known as cytochrome c oxidase (COX), is the last protein in the electron transport chain. In some embodiments, the detection and quantification of the effect of an epi-shifter on the oxidation of cytochrome c and the reduction of oxygen to water by Complex IV may be perfomed. For example, COX can be immunocaptured in a microwell plate and the oxidation of COX measured with a colorimetric assay (Complex IV Enzyme Activity Microplate Assay Kit, MitoSciences Inc., Eugene, OR).

The final enzyme in the oxidative phosphorylation process is ATP synthase (Complex V), which uses the proton gradient created by the other complexes to power the synthesis of ATP from ADP. In some embodiments, the detection and quantification of the effect of an epi-shifter on the activity of ATP synthase may be performed. For example, both the activity of ATP synthase and the amount of ATP synthase in a sample may be measured for ATP synthase that has been immunocaptured in a microwell plate well. The enzyme can also function as an ATPase under certain conditions, thus in this assay for ATP synthase activity, the rate at which ATP is reduced to ADP is measured by detecting the simultaneous oxidation of NADH to NAD^+. The amount of ATP is calculated using a labeled antibody to ATPase (ATP synthase Duplexing (Activity + Quantity) Microplate Assay Kit, MitoSciences Inc., Eugene, OR).Additional assays for oxidative phosphorylation include assays that test for effects on the activity of Complexes II and III. For example, the MT-OXC MitoTox™ Complete OXPHOS System (MitoSciences Inc., Eugene, OR) can be used to evaluate effects of a compound on Complex II and III as well as Complex I, IV and V, to provide data on the effects of a compound on the entire oxidative phosphorylation system.

As noted above, real-time observation of intact cell samples can be made using probes for changes in oxygen consumption and pH in cell culture media. These assays

of cell energetics provide a broad overview of mitochondrial function and the effects of potential environmental influencers (*e.g.*, MIMs or Epi-shifters) on the activity of mitochondria within the cells of the sample.

Environmental influencers (*e.g.*, MIMs or Epi-shifters) may also affect mitochondrial permeability transition (MPT), a phenomena in which the mitochondrial membranes experience an increase in permeability due to the formation of mitochondrial permeability transition pores (MPTP). An increase in mitochondrial permeability can lead to mitochondrial swelling, an inability to conduct oxidative phosphorylation and ATP generation and cell death. MPT may be involved with induction of apoptosis. (See, for example, Halestrap, A.P., Biochem. Soc. Trans. 34:232-237 (2006) and Lena, A. *et al.* Journal of Translational Med. 7:13-26 (2009), hereby incorporated by reference in their entirety.)

In some embodiments, the detection and quantification of the effect of an environmental influencer (e.g., MIM or epi-shifter) on the formation, discontinuation and/or effects of MPT and MPTPs are measured. For example, assays can detect MPT throught the use of specialized dye molecules (calcein) that are localized within the inner membranes of mitochondria and other cytosolic compartments. The application of another molecule, $CoCl_2$, serves to squelch the fluorescence of the calcein dye in the cytosol. $CoCl_2$ cannot access, however, the interior of the mitochondria, thus the calcein fluorescence in the mitochondria is not squelched unless MPT has occurred and $CoCl_2$ can access the interior of the mitochondra via MPTPs. Loss of mitochondrial-specific fluorescence signals that MPT has occurred. Flow cytometry can be used to evaluate cellular and organelle fluorescence (MitoProbe™ Transition Pore Assay Kit, Molecular Probes, Eugene, OR). Additional assays utilize a fluorescence microscope for evaluating experimental results (Image-iT™ LIVE Mitochondrial Transition Pore Assay Kit, Molecular Probes, Eugene, OR).

Measurement of Cellular Proliferation and Inflammation

In some embodiments of the invention, environmental influencers (*e.g.*, MIMs or Epi-shifters) may be identified and evaluated by their effects on the production or activity of molecules associated with cellular proliferation and/or inflammation. These molecules include, but are not limited to, cytokines, growth factors, hormones, components of the extra-cellular matrix, chemokines, neuropeptides, neurotransmitters,

neurotrophins and other molecules involved in cellular signaling, as well as intracellular molecules, such as those involved in signal transduction.

Vascular endothelial growth factor (VEGF) is a growth factor with potent angiogenic, vasculogenic and mitogenic properties. VEGF stimulates endothelial permeability and swelling and VEGF activity is implicated in numerous diseases and disorders, including rheumatoid arthritis, metastatic cancer, age-related macular degeneration and diabetic retinopathy.

In some embodiments of the invention, an environmental influencer (e.g., MIM or Epi-shifter) may be identified and characterized by its effects on the production of VEGF. For example, cells maintained in hypoxic conditions or in conditions mimicking acidosis will exhibit increased VEGF production. VEGF secreted into media can be assayed using an ELISA or other antibody-based assays, using available anti-VEGF antibodies (R&D Systems, Minneapolis, MN). In some embodiments of the invention, an Epi-shifter may be identified and/or characterized based on its effect(s) on the responsiveness of cells to VEGF and/or based on its effect(s) on the expression or activity of the VEGF receptor.

Implicated in both healthy immune system function as well as in autoimmune diseases, tumor necrosis factor (TNF) is a key mediator of inflammation and immune system activation. In some embodiments of the invention, an Epi-shifter may be identified and characterized by its effects on the production or the activity of TNF. For example, TNF produced by cultured cells and secreted into media can be quantified via ELISA and other antibody-based assays known in the art. Furthermore, in some embodiments an environmental influencer may be identified and characterized by its effect(s) on the expression of receptors for TNF (Human TNF RI Duoset, R&D Systems, Minneapolis, MN).

The components of the extracellular matrix (ECM) play roles in both the structure of cells and tissues and in signaling processes. For example, latent transforming growth factor beta binding proteins are ECM components that create a reservoir of transforming growth factor beta (TGFβ) within the ECM. Matrix-bound TGFβ can be released later during the process of matrix remodeling and can exert growth factor effects on nearby cells (Dallas, S. Methods in Mol. Biol. 139:231-243 (2000)).

In some embodiments, an environmental influencer (e.g., MIM or Epi-shifter) may be identified or characterized by its effect(s) on the creation of ECM by cultured cells. Researchers have developed techniques with which the creation of ECM by cells, as well as the composition of the ECM, can be studied and quantified. For example, the synthesis of ECM by cells can be evaluated by embedding the cells in a hydrogel before incubation. Biochemical and other analyses are performed on the ECM generated by the cells after cell harvest and digestion of the hydrogel (Strehin, I. and Elisseeff, J. Methods in Mol. Bio. 522:349-362 (2009)).

In some embodiments, the effect of environmental influencer (e.g., MIM or epi-shifter) on the production, status of or lack of ECM or one of its components in an organism may be identified or characterized. Techniques for creating conditional knock-out (KO) mice have been developed that allow for the knockout of particular ECM genes only in discrete types of cells or at certain stages of development (Brancaccio, M. *et al.* Methods in Mol Bio. 522:15-50 (2009)). The effect of the application or administration of an epi-shifter or potential epi-shifter on the activity or absence of a particular ECM component in a particular tissue or at a particular stage of development may thus be evaluated.

Measurement of Plasma Membrane Integrity and Cell Death

Environmental influencers (e.g., MIMs or Epi-shifters) may be identified by changes in the plasma membrane integrity of a cell sample and/or by changes in the number or percentage of cells that undergo apoptosis, necrosis or cellular changes that demonstrate an increased or reduced likelihood of cell death.

An assay for lactate dehydrogenase (LDH) can provide a measurement of cellular status and damage levels. LDH is a stable and relatively abundant cytoplasmic enzyme. When plasma membranes lose physical integrity, LDH escapes to the extracellular compartment. Higher concentrations of LDH correlate with higher levels of plasma membrane damage and cell death. Examples of LDH assays include assays that use a colorimetric system to detect and quantify levels of LDH in a sample, wherein the reduced form of a tetrazolium salt is produced via the activity of the LDH enzyme (QuantiChrom™ Lactate Dehydrogenase Kit (DLDH-100), BioAssay Systems, Hayward, CA; LDH Cytotoxicity Detection Kit, Clontech, Mountain View, CA).

Apoptosis is a process of programmed cell death that may have a variety of different initiating events. A number of assays can detect changes in the rate and/or number of cells that undergo apoptosis. One type of assay that is used to detect and quantify apoptosis is a capase assay. Capases are aspartic acid-specific cysteine proteases that are activated via proteolytic cleavage during apoptosis. Examples of assays that detect activated capases include PhiPhiLux® (OncoImmunin, Inc., Gaithersburg, MD) and Caspase-Glo® 3/7 Assay Systems (Promega Corp., Madison, WI). Additional assays that can detect apoptosis and changes in the percentage or number of cells undergoing apoptosis in comparitive samples include TUNEL/DNA fragmentation assays. These assays detect the 180 to 200 base pair DNA fragments generated by nucleases during the execution phase of apoptosis. Exemplary TUNEL/DNA fragmentation assays include the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) and the DeadEnd™ Colorimetric and Fluorometric TUNEL Systems (Promega Corp., Madison, WI).

Some apoptosis assays detect and quantify proteins associated with an apoptotic and/or a non-apoptotic state. For example, the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega Corp., Madison, WI) uses a single substrate, fluorimetric system to detect and quantify proteases specific to live and dead cells, thus providing a ratio of living cells to cells that have undergone apoptosis in a cell or tissue sample.

Additional assays available for detecting and quantifying apoptosis include assays that detect cell permeability (*e.g.*, APOPercentage™ APOPTOSIS Assay, Biocolor, UK) and assays for Annexin V (*e.g.*, Annexin V-Biotin Apoptosis Detection Kit, BioVision Inc., Mountain View, CA).

IV. Treatment of Metabolic Disorders

In some embodiments, the compounds of the present invention, *e.g.*, the environmental influencers, *e.g.*, MIMs or epi-shifters, described herein, may be used to treat a Coenzyme Q10 responsive state in a subject in need thereof. The language "Coenzyme Q10 responsive state," or "CoQ10 responsive state," includes diseases, disorders, states and/or conditions which can be treated, prevented, or otherwise ameliorated by the administration of Coenzyme Q10. Without wishing to be bound by any particular theory, and as described further herein, it is believed that CoQ10

functions, at least partially, by inducing a metabolic shift to the cell microenvironment, such as a shift towards the type and/or level of oxidative phosphorylation in normal state cells. Accordingly, in some embodiments, CoQ10 responsive states are states that arise from an altered metabolism of cell microenvironment. In one embodiment, the CoQ10 responsive disorder is a metabolic disorder. Coenzyme Q10 responsive states include, for example, metabolic disorders such as obesity, diabetes, pre-diabetes, Metabolic Syndrome, satiety, and endocrine abnormalities. Coenzyme Q10 responsive states further include other metabolic disorders as described herein.

In some embodiments, the compounds of the present invention, *e.g.*, the MIMs or epi-shifters described herein, share a common activity with Coenzyme Q10. As used herein, the phrase "share a common activity with Coenzyme Q10" refers to the ability of a compound to exhibit at least a portion of the same or similar activity as Coenzyme Q10. In some embodiments, the compounds of the present invention exhibit 25% or more of the activity of Coenzyme Q10. In some embodiments, the compounds of the present invention exhibit up to and including about 130% of the activity of Coenzyme Q10. In some embodiments, the compounds of the present invention exhibit about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, 101%, 102%, 103%, 104%, 105%, 106%, 107%, 108%, 109%, 110%, 111%, 112%, 113%, 114%, 115%, 116%, 117%, 118%, 119%, 120%, 121%, 122%, 123%, 124%, 125%, 126%, 127%, 128%, 129%, or 130% of the activity of Coenzyme Q10. It is to be understood that each of the values listed in this paragraph may be modified by the term "about." Additionally, it is to be understood that any range which is defined by any two values listed in this paragraph is meant to be encompassed by the present invention. For example, in some embodiments, the compounds of the present invention exhibit between about 50% and about 100% of the activity of Coenzyme Q10. In some embodiments, the activity shared by Coenzyme Q10 and the compounds of the present invention is the ability to induce a shift in cellular metabolism. In certain embodiments, the activity shared by of CoQ10

and the compounds of the present invention is measured by OCR (Oxygen Consumption Rate) and/or ECAR (ExtraCellular Acidification Rate).

The present invention provides methods for treating, alleviating symptoms of, inhibiting progression of, or preventing a CoQ10 responsive disorder in a mammal, the method comprising administering to the mammal in need thereof a therapeutically effective amount of pharmaceutical composition comprising at least one environmental influencer (env-influencer), wherein the environmental influencer selectively elicits, in a disease cell of the mammal, a cellular metabolic energy shift towards levels of glycolysis and mitochondrial oxidative phosphorylation observed in a normal cell of the mammal under normal physiological conditions.

The present invention further provides methods for treating, alleviating symptoms of, inhibiting progression of, or preventing a metabolic disorder in a mammal, the method comprising administering to the mammal in need thereof a therapeutically effective amount of a pharmaceutical composition comprising at least one environmental influencer (env-influencer), wherein the environmental influencer selectively elicits, in a disease cell of the mammal, a cellular metabolic energy shift towards normalized mitochondrial oxidative phosphorylation.

The present invention further provides methods for selectively augmenting mitochondrial oxidative phosphorylation, in a disease cell of a mammal in need of treatment for a metabolic disorder, the method comprising administering to said mammal a therapeutically effective amount of a pharmaceutical composition comprising at least one env-influencer, thereby selectively augmenting mitochondrial oxidative phosphorylation in said disease cell of the mammal.

The present invention further provides methods of treating or preventing a metabolic disorder in a human, comprising administering an environmental influencer to the human in an amount sufficient to treat or prevent the metabolic disorder, thereby treating or preventing the metabolic disorder.

The present invention further provides methods of treating or preventing an metabolic disorder in a human, comprising selecting a human subject suffering from an metabolic disorder, and administering to said human a therapeutically effective amount of an env-influencer capable of blocking anaerobic use of glucose and augmenting

mitochondrial oxidative phosphorylation, thereby treating or preventing the metabolic disorder.

The present invention still further provides a method for treating or preventing a metabolic disorder in a human, comprising administering an environmental influencer (env-influencer) to the human in an amount sufficient to treat or prevent the metabolic disorder, wherein the environmental influencer (env-influencer) is administered such that it is maintained in its oxidized form during treatment, threby treating or preventing the metabolic disorder.

By "a metabolic disorder" is meant any pathological condition resulting from an alteration in a patient's metabolism. Such disorders include those associated with aberrant whole-body glucose, lipd and /or protein metabolism and pathological consequences arising therefrom. Metabolic disorders include those resulting from an alteration in glucose homeostasis resulting, for example, in hyperglycemia. According to this invention, an alteration in glucose levels is typically an increase in glucose levels by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or even 100% relative to such levels in a healthy individual. Metabolic disorders can detrimentally affect cellular functions such as cellular proliferation, growth, differentiation, or migration, cellular regulation of homeostasis, inter- or intra-cellular communication; tissue function, such as liver function, muscle function, or adipocyte function; systemic responses in an organism, such as hormonal responses (e.g., insulin response). Metabolic disorders include, but are not limited to, obesity, diabetes (also referred to herein as diabetes mellitus) (*e.g.*, diabetes type I, diabetes type II, MODY, and gestational diabetes), pre-diabetes, Metabolic Syndrome, satiety, and endocrine abnormalities, e.g., of aging. Further examples of metabolic disorders include, but are not limited to, hyperphagia, hypophagia, triglyceride storage disease, Bardet-Biedl syndrome, Lawrence-Moon syndrome, Prader-Labhart-Willi syndrome, Kearns-Sayre syndrome, anorexia, medium chain acyl-CoA dehydrogenase deficiency, and cachexia. In some embodiments the metabolic disorder is a Coenzyme Q10 responsive state.

By "treating, reducing, or preventing a metabolic disorder" is meant ameliorating such a condition before or after it has occurred. As compared with an equivalent untreated control, such reduction or degree of prevention is at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, or 100% as measured by any standard technique.

Diabetes mellitus is a heterogeneous group of metabolic diseases which lead to chronic elevation of glucose in the blood (hyperglycemia). Diabetes is characterized by pancreatic islet destruction or dysfunction leading to loss of glucose regulation. The two major types of diabetes mellitus are Type I, also known as "insulin-dependent diabetes" ("IDDM") or "juvenile-onset diabetes", and Type II, also known as "non-insulin dependent" ("NIDDM") or "maturity-onset diabetes".

"Type I diabetes" refers to a condition that results from an autoimmune-mediated destruction of pancreatic .beta. cells with consequent loss of insulin production, which results in hyperglycemia. Type I diabetics require insulin replacement therapy to ensure survival. While medications such as injectable insulin and oral hypoglycemics allow diabetics to live longer, diabetes remains the third major killer, after heart disease and cancer. However, these medications do not control blood sugar levels well enough to prevent swinging between high and low blood sugar levels, with resulting damage to the kidneys, eyes, and blood vessels. Data from the Diabetes Control and Complications Trial (DCCT) show that intensive control of blood glucose significantly delays complications of diabetes, such as retinopathy, nephropathy, and neuropathy, compared with conventional therapy consisting of one or two insulin injections per day. Intensive therapy in the DCCT included multiple injection of insulin three or more times per day or continuous subcutaneous insulin infusion (CSII) by external pump. Insulin pumps are one of a variety of alternative approaches to subcutaneous multiple daily injections (MDI) for approximating physiological replacement of insulin.

"Type 2 diabetes" refers to the condition in which a patient has a fasting blood glucose or serum glucose concentration greater than 125 mg/dl (6.94 mmol/L). Type II diabetes is characterized by hyperglycemia in the presence of higher-than-normal levels of plasma insulin (hyperinsulinemia) and represents over 90% of all cases and occurs most often in overweight adults over 40 years of age. Progression of Type II diabetes is associated with increasing concentrations of blood glucose, coupled with a relative decrease in the rate of glucose-induced insulin secretion. In Type II diabetes, tissue processes which control carbohydrate metabolism are believed to have decreased sensitivity to insulin and therefore occur not from a lack of insulin production, but a decreased sensitivity to increased glucose levels in the blood and an inability to respond by producing insulin. Alternatively, diabetes may result from various defects in the

molecular machinery that mediate the action of insulin on its target cells, such as a lack of insulin receptors on their cell surfaces. Treatment of Type II diabetes therefore frequently does not require administration of insulin but may be based on diet and lifestyle changes, augmented by therapy with oral hypoglycemic agents such as, for example, sulfonylurea.

"Pre-diabetes" refers to a condition where a patient is pre-disposed to the development of type 2 diabetes. Pre-diabetes extends the definition of impaired glucose tolerance to include individuals with a fasting blood glucose within the high normal range.gtoreq.100 mg/dL (Meigs et al., Diabetes 2003 52:1475-1484) and fasting hyperinsulinemia (elevated plasma insulin concentration).

"Obesity" refers to the condition where a patient has a BMI equal to or greater than 30 kg/m.sup.2. "Visceral obesity" refers to a waist to hip ration of 1.0 in male patients and 0.8 in female patients. In another aspect, visceral obesity defines the risk for insulin resistance and the development of pre-diabetes.

"Overweight" refers to a patient with a BMI greater than or 25 kg/m.sup.2 and less than 30 kg/m.sup.2. "Weight gain" refers to the increase in body weight in relationship to behavioral habits or addictions, e.g., overeating or gluttony, smoking cessation, or in relationship to biological (life) changes, e.g., weight gain associated with aging in men and menopause in women or weight gain after pregnancy.

"Metabolic Syndrome" (**MS**), also referred to as Syndrome X, refers to a metabolic disorder that affects other pathways and systems in the body. Originally, Metabolic Syndrome was defined as a cluster of metabolic disorders (including obesity, insulin resistance, hypertension, and dyslipidemia primarily hypertriglyceridemia), that synergize to potentiate cardiovascular disease. More recently (2001), the U.S. National Cholesterol Education Program (NCEP) has classified "Metabolic Syndrome" as meeting any three out of the following five criteria: fasting glucose level of at least 110 mg/dl, plasma triglyceride level of at least 150 mg/dl (hypertriglycerdemia), HDL cholesterol below 40 mg/dl in men or below 50 mg/dl in women, blood pressure at least 130/85 mm Hg (hypertension), and central obesity, with central obesity being defined as abdominal waist circumference greater than 40 inches for men and greater than 35 inches for women. Presently, there are three other internationally recognized definitions for Metabolic Syndrome as follows: 1) World Health Organization 2) American Heart

Association/National Heart, Lung and blood Institute (AHA/NHLBI) and 3) International Diabetes Federation (IDF). The definitions of Metabolic Syndrome by the WHO, AHA/NHLBI and IDF are very similar to the definition of the NECP and all use the same metabolic parameters to define the syndrome, but the WHO also includes assessment of insulin fasting insulin levels (Moebus S et al, Cardiovascular Diabetology, 6: 1-10, 2007; Athyros V G et al, Int. J. Cardiology, 117: 204-210, 2007). Yet subtle differences in the thresholds for these metabolic parameters required to be classified as having the syndrome among these different definitions can result in different classification of a particular subject as having or not having the syndrome according to these different definitions. Also, the prevalence of cardiovascular disease (CVD) with MS varies by the definition used. (Moebus S et al, Cardiovascular Diabetology, 6: 1-10, 2007; Athyros V G et al, Int. J. Cardiology, 117: 204-210, 2007). The American Diabetes Association estimates that 1 in every 5 overweight people suffer from Metabolic Syndrome.

In other aspects, the metabolic syndrome is described by accepted synonyms, which includes, but is not limited to, syndrome X, insulin resistance syndrome, insulin-resistant hypertension, the metabolic hypertensive syndrome, dysmetabolic syndrome. Components of the metabolic syndrome include, but are not limited to, glucose intolerance, impaired glucose tolerance, impaired fasting serum glucose, impaired fasting blood glucose, hyperinsulinemia, pre-diabetes, obesity, visceral obesity, hypertriglyceridemia, elevated serum concentrations of free fatty acids, elevated serum concentrations of C-reactive protein, elevated serum concentrations of lipoprotein(a), elevated serum concentrations of homocysteine, elevated serum concentrations of small, dense low-density lipoprotein (LDL)-cholesterol, elevated serum concentrations of lipoprotein-associated phospholipase (A2), reduced serum concentrations of high density lipoprotein (HDL)-cholesterol, reduced serum concentrations of HDL(2b)-cholesterol, reduced serum concentrations of adiponectin, and albuminuria (see: Pershadsingh HA. Peroxisome proliferator-activated receptor-gamma: therapeutic target for diseases beyond diabetes: quo vadis? Expert Opin Investig Drugs. (2004) 13:215-28, and references cited therein).

The "key elements" of the foregoing metabolic disorders include but are not limited to, impaired fasting glucose or impaired glucose tolerance, increased waist

circumference, increased visceral fat content, increased fasting plasma glucose, increased fasting plasma triglycerides, decreased fasting high density lipoprotein level, increased blood pressure, insulin resistance, hyperinsulinemia, cardiovascular disease (or components thereof such as arteriosclerosis, coronary artery disease, peripheral vascular disease, or cerebrovascular disease), congestive heart failure, elevated plasma norepinephrine, elevated cardiovascular-related inflammatory factors, elevated plasma factors potentiating vascular endothelial dysfunction, hyperlipoproteinemia, arteriosclerosis or atherosclerosis, hyperphagia, hyperglycemia, hyperlipidemia, and hypertension or high blood pressure, increased plasma postprandial triglyceride or free fatty acid levels, increased cellular oxidative stress or plasma indicators thereof, increased circulating hypercoagulative state, hepatic steatosis, hetaptic steatosis, renal disease including renal failure and renal insufficiency.

"Insulin resistance" refers to a condition in which circulating insulin levels in excess of the normal response to a glucose load are required to maintain the euglycemic state (Ford et al., JAMA. 2002, 287:356-9). Insulin resistance and the response of a patient with insulin resistance to therapy, may be quantified by assessing the homeostasis model assessment to insulin resistance (HOMA-IR) score, a reliable indicator of insulin resistance (Katsuki et al., Diabetes Care 2001, 24:362-5). An estimate of insulin resistance by the homeostasis assessment model (HOMA)-IR score may be calculated by a formula disclosed in Galvin et al., Diabet Med 1992, 9:921-8 where HOMA-IR=[fasting serum insulin (.mu.U/mL)].times.[fasting plasma glucose (mmol/L)/22.5].

"Hyperinsulinemia" is defined as the condition in which a subject with insulin resistance, with or without euglycemia, in which the fasting or postprandial serum or plasma insulin concentration is elevated above that of normal, lean individuals without insulin resistance, having a waist-to-hip ration<1.0 (for men) or <0.8 (for women).

The term "impaired glucose tolerance" (IGT) is used to describe a person who, when given a glucose tolerance test, has a blood glucose level that falls between normal and hyperglycemic. Such a person is at a higher risk of developing diabetes although they are not considered to have diabetes. For example, impaired glucose tolerance refers to a condition in which a patient has a fasting blood glucose concentration or fasting serum glucose concentration greater than 110 mg/dl and less than 126 mg/dl (7.00

mmol/L), or a 2 hour postprandial blood glucose or serum glucose concentration greater than 140 mg/dl (7.78 mmol/L) and less than 200 mg/dl (11.11 mmol/L).

The condition of "hyperglycemia" (high blood sugar) is a condition in which the blood glucose level is too high. Typically, hyperglycemia occurs when the blood glucose level rises above 180 mg/dl. Symptoms of hyperglycemia include frequent urination, excessive thirst and, over a longer time span, weight loss.

The condition of "hypoglycemia" (low blood sugar) is a condition in which the blood glucose level is too low. Typically, hypoglycemia occurs when the blood glucose level falls below 70 mg/dl. Symptoms of hypoglycemia include moodiness, numbness of the extremities (especially in the hands and arms), confusion, shakiness or dizziness. Since this condition arises when there is an excess of insulin over the amount of available glucose it is sometimes referred to as an insulin reaction.

(i) Diagnosis of Metabolic Disorders

The methods and compositions of the present invention are useful for treating any patient that has been diagnosed with or is at risk of having a metabolic disorder, such as diabetes. A patient in whom the development of a metabolic disorder (e.g., diabetes or obesity) is being prevented may or may not have received such a diagnosis. One in the art will understand that patients of the invention may have been subjected to standard tests or may have been identified, without examination, as one at high risk due to the presence of one or more risk factors.

Diagnosis of metabolic disorders may be performed using any standard method known in the art, such as those described herein. Methods for diagnosing diabetes are described, for example, in U.S. Pat. No. 6,537,806, hereby incorporated by reference. Diabetes may be diagnosed and monitored using, for example, urine tests (urinalysis) that measure glucose and ketone levels (products of the breakdown of fat); tests that measure the levels of glucose in blood; glucose tolerance tests; and assays that detect molecular markers characteristic of a metabolic disorder in a biological sample (e.g., blood, serum, or urine) collected from the mammal (e.g., measurements of Hemoglobin Alc (HbAlc) levels in the case of diabetes).

A patient who is being treated for a metabolic disorder is one who a medical practitioner has diagnosed as having such a condition. Diagnosis may be performed by

any suitable means, such as those described herein. A patient in whom the development of diabetes or obesity is being prevented may or may not have received such a diagnosis. One in the art will understand that patients of the invention may have been subjected to standard tests or may have been identified, without examination, as one at high risk due to the presence of one or more risk factors, such as family history, obesity, particular ethnicity (e.g., African Americans and Hispanic Americans), gestational diabetes or delivering a baby that weighs more than nine pounds, hypertension, having a pathological condition predisposing to obesity or diabetes, high blood levels of triglycerides, high blood levels of cholesterol, presence of molecular markers (e.g., presence of autoantibodies), and age (over 45 years of age). An individual is considered obese when their weight is 20% (25% in women) or more over the maximum weight desirable for their height. An adult who is more than 100 pounds overweight, is considered to be morbidly obese. Obesity is also defined as a body mass index (BMI) over 30 kg/m.sup.2.

Patients may be diagnosed as being at risk or as having diabetes if a random plasma glucose test (taken at any time of the day) indicates a value of 200 mg/dL or more, if a fasting plasma glucose test indicates a value of 126 mg/dL or more (after 8 hours), or if an oral glucose tolerance test (OGTT) indicates a plasma glucose value of 200 mg/dL or more in a blood sample taken two hours after a person has consumed a drink containing 75 grams of glucose dissolved in water. The OGTT measures plasma glucose at timed intervals over a 3-hour period. Desirably, the level of plasma glucose in a diabetic patient that has been treated according to the invention ranges between 160 to 60 mg/dL, between 150 to 70 mg/dL, between 140 to 70 mg/dL, between 135 to 80 mg/dL, and preferably between 120 to 80 mg/dL.

Optionally, a hemoglobin Alc (HbAlc) test, which assesses the average blood glucose levels during the previous two and three months, may be employed. A person without diabetes typically has an HbAlc value that ranges between 4% and 6%. For every 1% increase in HbAlc, blood glucose levels increases by approximately 30 mg/dL and the risk of complications increases. Preferably, the HbAlc value of a patient being treated according to the present invention is reduced to less than 9%, less than 7%, less than 6%, and most preferably to around 5%. Thus, the HbAlc levels of the patient being

treated are preferably lowered by 10%, 20%, 30%, 40%, 50%, or more relative to such levels prior to treatment.

Gestational diabetes is typically diagnosed based on plasma glucose values measured during the OGTT. Since glucose levels are normally lower during pregnancy, the threshold values for the diagnosis of diabetes in pregnancy are lower than in the same person prior to pregnancy. If a woman has two plasma glucose readings that meet or exceed any of the following numbers, she has gestational diabetes: a fasting plasma glucose level of 95 mg/dL, a 1-hour level of 180 mg/dL, a 2-hour level of 155 mg/dL, or a 3-hour level of 140 mg/dL.

Ketone testing may also be employed to diagnose type I diabetes. Because ketones build up in the blood when there is not enough insulin, they eventually accumulate in the urine. High levels of blood ketones may result in a serious condition called ketoacidosis.

According to the guidelines of the American Diabetes Association, to be diagnosed with Type 2 diabetes, an individual must have a fasting plasma glucose level greater than or equal to 126 mg/dl or a 2-hour oral glucose tolerance test (OGTT) plasma glucose value of greater than or equal to 200 mg/dl (Diabetes Care, 26:S5-S20, 2003).

A related condition called pre-diabetes is defined as having a fasting glucose level of greater than 100 mg/dl but less than 126 mg/dl or a 2-hour OGTT plasma glucose level of greater than 140 mg/dl but less than 200 mg/dl. Mounting evidence suggests that the pre-diabetes condition may be a risk factor for developing cardiovascular disease (Diabetes Care 26:2910-2914, 2003). Prediabetes, also referred to as impaired glucose tolerance or impaired fasting glucose is a major risk factor for the development of type 2 diabetes mellitus, cardiovascular disease and mortality. Much focus has been given to developing therapeutic interventions that prevent the development of type 2 diabetes by effectively treating prediabetes (Pharmacotherapy, 24:362-71, 2004).

Obesity (commonly defined as a Body Mass Index of approximately >30 kg/m.sup.2) is often associated with a variety of pathologic conditions such as hyperinsulinemia, insulin resistance, diabetes, hypertension, and dyslipidemia. Each of these conditions contributes to the risk of cardiovascular disease.

Along with insulin resistance, hypertension, and dyslipidemia, obesity is considered to be a component of the Metabolic Syndrome (also known as Syndrome X) which together synergize to potentiate cardiovascular disease. More recently, the U.S. National Cholesterol Education Program has classified Metabolic Syndrome as meeting three out of the following five criteria: fasting glucose level of at least 110 mg/dl, plasma triglyceride level of at least 150 mg/dl (hypertriglycerdemia), HDL cholesterol below 40 mg/dl in men or below 50 mg/dl in women, blood pressure at least 130/85 mm Hg (hypertension), and central obesity, with central obesity being defined as abdominal waist circumference greater than 40 inches for men and greater than 35 inches for women.

(ii) Assessing Treatment Efficacy of a Metabolic Disorder

The skilled artisan will recognize that the use of any of the above tests or any other tests known in the art may be used to monitor the efficacy of the therapeutic treatments of the invention. Since the measurements of hemoglobin Alc (HbAlc) levels is an indication of average blood glucose during the previous two to three months, this test may be used to monitor a patient's response to diabetes treatment.

The therapeutic methods of the invention are effective in reducing glucose levels or lipid levels in a patient. By "reducing glucose levels" is meant reducing the level of glucose by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to an untreated control. Desirably, glucose levels are reduced to normoglycemic levels, i.e., between 150 to 60 mg/dL, between 140 to 70 mg/dL, between 130 to 70 mg/dL, between 125 to 80 mg/dL, and preferably between 120 to 80 mg/dL. Such reduction in glucose levels may be obtained by increasing any one of the biological activities associated with the clearance of glucose from the blood. Accordingly, an agent having the ability to reduce glucose levels may increase insulin production, secretion, or action. Insulin action may be increased, for example, by increasing glucose uptake by peripheral tissues and/or by reducing hepatic glucose production. Alternatively, the agent of the invention may reduce the absorption of carbohydrates from the intestines, alter glucose transporter activity (e.g., by increasing GLUT4 expression, intrinsic activity, or translocation), increase the amount of insulin-sensitive tissue (e.g., by increasing muscle cell or adipocyte cell differentiation), or alter gene transcription in

adipocytes or muscle cells (e.g., altered secretion of factors from adipocytes expression of metabolic pathway genes). Desirably, the agent of the invention increases more than one of the activities associated with the clearance of glucose. By "reducing lipid levels" is meant reducing the level of lipids by at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to an untreated control.

By "alter insulin signaling pathway such that glucose levels are reduced" is meant to alter (by increasing or reducing) any one of the activities involved in insulin signaling such that the overall result is an increase in the clearance of glucose from plasma. For example, the env-influencer of the invention alters the insulin signaling pathway causing an increase in insulin production, secretion, or action, an increase in glucose uptake by peripheral tissues, a reduction in hepatic glucose production, or a reduction in the absorption of carbohydrates from the intestines.

The ability of an environmental influencer, *e.g.*, epi-shifter, to reduce glucose levels and thereby treat a metabolic disorder may be assessed using standard assays known in the art. For example, cell-based screening assays that identify agents that increase glucose uptake may be employed. In particular, differentiated adipocytes in cell culture can be employed to assess the ability of the epi-shifter to increase glucose uptake upon insulin stimulation, as detected by radiolabeled glucose. In another exemplary assay, human myoblasts obtained by the conditional immortalization of cells derived from a non-diabetic subject can be used to screen the effect of agents on glycogen synthesis, using insulin as a positive control. Prior to treatment, cells are serum-starved, and are then incubated either with the epi-shifter or control for a period of two hours in serum-free media containing radiolabeled glucose, after which, glycogen synthesis is measured. Exemplary assays are further described in the Examples.

V. Therapeutic Targets for Metabolic Disorders

The present invention provides methods for identifying therapeutic targets for metabolic disorders. The invention further provides therapeutic targets identified by such methods. The identification of a therapeutic target involves, generally, the exogenous application of an Env-influencer or candidate Env-influencer to a cell or panel of cell lines, and the subsequent evaluation of changes induced to a treated cell as compared to a control, untreated cell. Induced cellular changes which are monitored

include, but are not limited to, changes to the morphology, physiology or composition, *e.g.*, RNA, protein, lipid or metabolite levels, of the cell. Induced cellular changes as a result of treatment by a candidate Env-influencer can be monitored by using any of the assays described herein. For example, changes in gene expression at the mRNA level can be evaluated by real-time PCR arrays, while changes in gene expression at the protein level can be monitored by using antibody microarrays and 2-D gel electrophoresis. Genes identified as being modulated by the candidate Env-influencer (*e.g.*, at the mRNA and/or protein level) are then evaluated from a Systems Biology perspective using pathway analysis (Ingenuity IPA software) and by a review of the known literature. Genes identified as potential therapeutic targets are next submitted to confirmatory assays such as Western blot analysis, siRNA knock-down, or recombinant protein production and characterization methods. Screening assays can then be used to identify modulators of the targets. Modulators of the therapeutic targets are useful as novel therapeutic agents for metabolic disorders. Modulators of therapeutic targets can be routinely identified using screening assays described in detail herein, or by using routine methodologies known to the skilled artisan.

Genes identified herein as being modulated (*e.g.*, upmodulated or downmodulated, at either the mRNA or protein level) by the MIM/Epi-shifter, CoQ10, are drug targets of the invention. Drug targets of the invention include, but are not limited to, the genes subsequently listed in Tables 2-4 & 6-28 & 63-68 herein. Based on the results of experiments described by Applicants herein, the key proteins modulated by Q10 are associated with or can be classified into different pathways or groups of molecules, including transcription factors, apoptotic response, pentose phosphate pathway, biosynthetic pathway, oxidative stress (pro-oxidant), membrane alterations, and oxidative phosphorylation metabolism. The key proteins modulated by CoQ10, based on the results provided herein, are summarized as follows. A key protein modulated by CoQ10 and which is a transcription factor is HNF4alpha. Key proteins that are modulated by CoQ10 and associated with the apoptotic response include Bcl-xl, Bcl-xl, Bcl-xS, BNIP-2, Bcl-2, Birc6, Bcl-2-L11 (Bim), XIAP, BRAF, Bax, c-Jun, Bmf, PUMA, and cMyc. A key protein that is modulated by CoQ10 and associated with the pentose phosphate pathway is transaldolase 1. Key proteins that are modulated by CoQ10 and associated with a biosynthetic pathway include COQ1, COQ3, COQ6,

prenyltransferase and 4-hydroxybenzoate. Key proteins that are modulated by CoQ10 and associated with oxidative stress (pro-oxidant) include Neutrophil cytosolic factor 2, nitric oxide synthase 2A and superoxide dismutase 2 (mitochondrial). Key proteins that are modulated by CoQ10 and associated with oxidative phosphorylation metabolism include Cytochrome c, complex I; complex II, complex III and complex IV. Further key proteins that are directly or indirectly modulated by CoQ10 include Foxo 3a, DJ-1, IDH-1, Cpt1C and Cam Kinase II.

Accordingly, in one embodiment of the invention, a drug target may include HNF4-alpha, Bcl-xl, Bcl-xS, BNIP-2, Bcl-2, Birc6, Bcl-2-L11 (Bim), XIAP, BRAF, Bax, c-Jun, Bmf, PUMA, cMyc, transaldolase 1, COQ1, COQ3, COQ6, prenyltransferase, 4-hydrobenzoate, neutrophil cytosolic factor 2, nitric oxide synthase 2A, superoxide dismutase 2, VDAC, Bax channel, ANT, Cytochrome c, complex 1, complex II, complex III, complex IV, Foxo 3a, DJ-1, IDH-1, Cpt1C and Cam Kinase II. In a preferred embodiment, a drug target may include HNF4A, Transaldolase, NM23 and BSCv. In one embodiment, the drug target is TNF4A. In one embodiment, the drug target is transaldolase. In one embodiment, the drug target is NM23. In one embodiment, the drug target is BSCv. Screening assays useful for identifying modulators of identified drug targets are described below.

VI. Screening Assays

The invention also provides methods (also referred to herein as "screening assays") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, proteins, peptides, peptidomimetics, peptoids, small molecules or other drugs), which modulate the expression and/or activity of an identified therapeutic target of the invention. Such assays typically comprise a reaction between a therapeutic target of the invention and one or more assay components. The other components may be either the test compound itself, or a combination of test compounds and a natural binding partner of a marker of the invention. Compounds identified *via* assays such as those described herein may be useful, for example, for treating or preventing a metabolic disorder.

The test compounds used in the screening assays of the present invention may be obtained from any available source, including systematic libraries of natural and/or synthetic compounds. Test compounds may also be obtained by any of the numerous

approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, *e.g.*, Zuckermann *et al.*, 1994, *J. Med. Chem.* 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten, 1992, *Biotechniques* 13:412-421), or on beads (Lam, 1991, *Nature* 354:82-84), chips (Fodor, 1993, *Nature* 364:555-556), bacteria and/or spores, (Ladner, USP 5,223,409), plasmids (Cull *et al*, 1992, *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith, 1990, *Science* 249:386-390; Devlin, 1990, *Science* 249:404-406; Cwirla *et al,* 1990, *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici, 1991, *J. Mol. Biol.* 222:301-310; Ladner, *supra.*).

The screening methods of the invention comprise contacting a cell with a test compound and determining the ability of the test compound to modulate the expression and/or activity of a therapeutic target of the invention in the cell. The expression and/or activity of a therapeutic target of the invention can be determined as described herein. The expression and/or activity of a therapeutic target of the invention can also be determined by using routine methods known to the skilled artisan. In one embodiment, a compound is selected based on its ability to increase expression and/or activity of a therapeutic target of the invention. In one embodiment, a compound is selected based on its ability increase expression and/or activity of a therapeutic target selected from the

protein listed in Tables 2-4 & 6-28 & 63-68, wherein the therapeutic target is upmodulated by CoQ10 (*e.g.*, exhibits a positive-fold change). In one embodiment, a compound is selected based on its ability to decrese expression and/or activity of a therapeutic target of the invention. In one embodiment, a compound is selected based on its ability to decrease expression and/or activity of a therapeutic target selected from the proteins listed in Tables 2-4 & 6-28 & 63-68, wherein the therapeutic targetis is downmodulated by CoQ10 (*e.g.*, exhibits a negative-fold change)..

In another embodiment, the invention provides assays for screening candidate or test compounds which are substrates of a therapeutic target of the invention or biologically active portions thereof. In yet another embodiment, the invention provides assays for screening candidate or test compounds which bind to a therapeutic target of the invention or biologically active portions thereof. Determining the ability of the test compound to directly bind to a therapeutic target can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to the drug target can be determined by detecting the labeled marker compound in a complex. For example, compounds (*e.g.*, marker substrates) can be labeled with ^{131}I, ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, assay components can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent capable of modulating the expression and/or activity of a marker of the invention identified as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatment as described above.

VII. Pharmaceutical Compositions and Pharmaceutical Administration

The environmental influencers of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an environmental influencer of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further include minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the environmental influencer.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, creams, lotions, ointments or pasts, drops suitable for administration to the eye, ear, or nose, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application.

The environmental influencers of the present invention can be administered by a variety of methods known in the art. For many therapeutic applications, the preferred route/mode of administration is subcutaneous injection, intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the

art. See, e.g., Sustained and Controlled Release Drug Delivery Systems, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978. In one embodiment, the mode of administration is parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular). In one embodiment, the environmental influencer is administered by intravenous infusion or injection. In another embodiment, the environmental influencer is administered by intramuscular or subcutaneous injection. In a preferred embodiment, the environmental influencer is administered topically.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (i.e., environmental influencer) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile, lyophilized powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and spray-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Techniques and formulations generally may be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton, Pa. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the compounds of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compounds may be formulated in

solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., ationd oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an

added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives in addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the compound(s) of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. A wash solution can be used locally to treat an injury or inflammation to accelerate healing.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

For therapies involving the administration of nucleic acids, the compound(s) of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton,

Pa. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, intranodal, and subcutaneous. For injection, the compound(s) of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compound(s) may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

In one embodiment, the compositions comprising an Environmental influencer are administered topically. It is preferable to present the active ingredient, i.e. Env-influencer, as a pharmaceutical formulation. The active ingredient may comprise, for topical administration, from about 0.001% to about 20% w/w, by weight of the formulation in the final product, although it may comprise as much as 30% w/w, preferably from about 1% to about 20% w/w of the formulation. The topical formulations of the present invention, comprise an active ingredient together with one or more acceptable carrier(s) therefor and optionally any other therapeutic ingredients(s). The carrier(s) should be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

In treating a patient exhibiting a disorder of interest, a therapeutically effective amount of an agent or agents such as these is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the oneogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

The compositions described above may be administered to a subject in any suitable formulation. In addition to treatment of a metabolic disorder with topical formulations of an environmental influencer, e.g., CoQ10, in other aspects of the invention the environmental influencer, e.g., CoQ10, might be delivered by other methods. For example, the environmental influencer, e.g., CoQ10, might be formulated

for parenteral delivery, e.g., for subcutaneous, intravenous, intramuscular, or intratumoral injection. Other methods of delivery, for example, liposomal delivery or diffusion from a device impregnated with the composition might be used. The compositions may be administered in a single bolus, multiple injections, or by continuous infusion (for example, intravenously or by peritoneal dialysis). For parenteral administration, the compositions are preferably formulated in a sterilized pyrogen-free form. Compositions of the invention can also be administered in vitro to a cell (for example, to induce apoptosis in a cancer cell in an in vitro culture) by simply adding the composition to the fluid in which the cell is contained.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18.th ed., Mack Publishing Co., Easton, Pa. (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions. and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of where treatment is required, such as liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear, or nose. Drops according to the present invention may comprise sterile aqueous or oily solutions or suspensions and may be prepared by dissolving the active ingredient in a suitable aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and preferably including a surface active agent. The resulting solution may then be clarified and sterilized by filtration and transferred to the container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for inclusion in the drops are phenylmercuric nitrate or acetate (0.002%), benzalkonium chloride (0.01%) and chlorhexidine acetate

(0.01%). Suitable solvents for the preparation of an oily solution include glycerol, diluted alcohol and propylene glycol.

Lotions according to the present invention include those suitable for application to the skin or eye. An eye lotion may comprise a sterile aqueous solution optionally containing a bactericide and may be prepared by methods similar to those for the preparation of drops. Lotions or liniments for application to the skin may also include an agent to hasten drying and to cool the skin, such as an alcohol or acetone, and/or a moisturizer such as glycerol or an oil such as castor oil or arachis oil.

Creams, ointments or pastes according to the present invention are semi-solid formulations of the active ingredient for external application. They may be made by mixing the active ingredient in finely-divided or powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid, with the aid of suitable machinery, with a greasy or non-greasy basis. The basis may comprise hydrocarbons such as hard, soft or liquid paraffin, glycerol, beeswax, a metallic soap; a mucilage; an oil of natural origin such as almond, corn, arachis, castor or olive oil; wool fat or its derivatives, or a fatty acid such as stearic or oleic acid together with an alcohol such as propylene glycol or macrogels. The formulation may incorporate any suitable surface active agent such as an anionic, cationic or non-ionic surface active such as sorbitan esters or polyoxyethylene derivatives thereof. Suspending agents such as natural gums, cellulose derivatives or inorganic materials such as silicaceous silicas, and other ingredients such as lanolin, may also be included.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing

the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch; gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxy-methylcellulose, and/or polyvinyl pyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coating. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

The composition can include a buffer system, if desired. Buffer systems are chosen to maintain or buffer the pH of compositions within a desired range. The term "buffer system" or "buffer" as used herein refers to a solute agent or agents which, when in a water solution, stabilize such solution against a major change in pH (or hydrogen ion concentration or activity) when acids or bases are added thereto. Solute agent or agents which are thus responsible for a resistance or change in pH from a starting buffered pH value in the range indicated above are well known. While there are countless suitable buffers, potassium phosphate monohydrate is a preferred buffer.

The final pH value of the pharmaceutical composition may vary within the physiological compatible range. Necessarily, the final pH value is one not irritating to human skin and preferably such that transdermal transport of the active compound, i.e.

CoQ10 is facilitated. Without violating this constraint, the pH may be selected to improve CoQ10 compound stability and to adjust consistency when required. In one embodiment, the preferred pH value is about 3.0 to about 7.4, more preferably about 3.0 to about 6.5, most preferably from about 3.5 to about 6.0.

For preferred topical delivery vehicles the remaining component of the composition is water, which is necessarily purified, e.g., deionized water. Such delivery vehicle compositions contain water in the range of more than about 50 to about 95 percent, based on the total weight of the composition. The specific amount of water present is not critical, however, being adjustable to obtain the desired viscosity (usually about 50 cps to about 10,000 cps) and/or concentration of the other components. The topical delivery vehicle preferably has a viscosity of at least about 30 centipoises.

Other known transdermal skin penetration enhancers can also be used to facilitate delivery of CoQ10. Illustrative are sulfoxides such as dimethylsulfoxide (DMSO) and the like; cyclic amides such as 1-dodecylazacycloheptane-2-one (Azone.TM., a registered trademark of Nelson Research, Inc.) and the like; amides such as N,N-dimethyl acetamide (DMA) N,N-diethyl toluamide, N,N-dimethyl formamide, N,N-dimethyl octamide, N,N-dimethyl decamide, and the like; pyrrolidone derivatives such as N-methyl-2-pyrrolidone, 2-pyrrolidone, 2-pyrrolidone-5-carboxylic acid, N-(2-hydroxyethyl)-2-pyrrolidone or fatty acid esters thereof, 1-lauryl-4-methoxycarbonyl-2-pyrrolidone, N-tallowalkylpyrrolidones, and the like; polyols such as propylene glycol, ethylene glycol, polyethylene glycol, dipropylene glycol, glycerol, hexanetriol, and the like; linear and branched fatty acids such as oleic, linoleic, lauric, valeric, heptanoic, caproic, myristic, isovaleric, neopentanoic, trimethyl hexanoic, isostearic, and the like; alcohols such as ethanol, propanol, butanol, octanol, oleyl, stearyl, linoleyl, and the like; anionic surfactants such as sodium laurate, sodium lauryl sulfate, and the like; cationic surfactants such as benzalkonium chloride, dodecyltrimethylammonium chloride, cetyltrimethylammonium bromide, and the like; non-ionic surfactants such as the propoxylated polyoxyethylene ethers, e.g., Poloxamer 231, Poloxamer 182, Poloxamer 184, and the like, the ethoxylated fatty acids, e.g., Tween 20, Myjr 45, and the like, the sorbitan derivatives, e.g., Tween 40, Tween 60, Tween 80, Span 60, and the like, the ethoxylated alcohols, e.g., polyoxyethylene (4) lauryl ether (Brij 30), polyoxyethylene (2) oleyl ether (Brij 93), and the like, lecithin and lecithin derivatives, and the like; the

terpenes such as D-limonene, α.-pinene, β-carene, α-terpineol, carvol, carvone, menthone, limonene oxide, α-pinene oxide, eucalyptus oil, and the like. Also suitable as skin penetration enhancers are organic acids and esters such as salicyclic acid, methyl salicylate, citric acid, succinic acid, and the like.

In one embodiment, the present invention provides CoQ10 compositions and methods of preparing the same. Preferably, the compositions comprise at least about 1% to about 25% CoQ10 w/w. CoQ10 can be obtained from Asahi Kasei N&P (Hokkaido, Japan) as UBIDECARENONE (USP). CoQ10 can also be obtained from Kaneka Q10 as Kaneka Q10 (USP UBIDECARENONE) in powdered form (Pasadena, Texas, USA). CoQ10 used in the methods exemplified herein have the following characteristics: residual solvents meet USP 467 requirement; water content is less than 0.0%, less than 0.05% or less than 0.2%; residue on ignition is 0.0%, less than 0.05%, or less than 0.2% less than; heavy metal content is less than 0.002%, or less than 0.001%; purity of between 98-100% or 99.9%, or 99.5%. Methods of preparing the compositions are provided in the examples section below.

In certain embodiments of the invention, methods are provided for treating or preventing a metabolic disorder in a human by topically administering Coenzyme Q10 to the human such that treatment or prevention occurs, wherein the human is administered a topical dose of Coenzyme Q10 in a topical vehicle where Coenzyme Q10 is applied to the target tissue in the range of about 0.01 to about 0.5 milligrams of coenzyme Q10 per square centimeter of skin. In one embodiment, Coenzyme Q10 is applied to the target tissue in the range of about 0.09 to about 0.15 mg CoQ10 per square centimeter of skin. In various embodiments, Coenzyme Q10 is applied to the target tissue in the range of about 0.001 to about 5.0, about 0.005 to about 1.0, about 0.005 to about 0.5, about 0.01 to about 0.5, about 0.025 to about 0.5, about 0.05 to about 0.4, about 0.05 to about 0.30 , about 0.10 to about 0.25, or about 0.10 to 0.20 mg CoQ10 per square centimeter of skin. In other embodiments, Coenzyme Q10 is applied to the target tissue at a dose of about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, 0.25, 0.26, 0.27, 0.28, 0.29, 0.30, 0.31, 0.32, 0.33, 0.34, 0.35, 0.36, 0.37, 0.38, 0.39, 0.40, 0.41, 0.42, 0.43, 0.44, 0.45, 0.46, 0.47, 0.48, 0.49 or 0.5 mg CoQ10 per square centimeter of skin. In one embodiment,Coenzyme Q10 is applied to the target tissue at a dose of

about 0.12 mg CoQ10 per square centimeter of skin It should be understood that ranges having any one of these values as the upper or lower limits are also intended to be part of this invention, *e.g.*, about 0.03 to about 0.12, about 0.05 to about 0.15, about 0.1 to about 0.20, or about 0.32 to about 0.49 mg CoQ10 per square centimeter of skin.

In another embodiment of the invention, the Coenzyme Q10 is administered in the form of a CoQ10 cream at a dosage of between 0.5 and 10 milligrams of the CoQ10 cream per square centimeter of skin, wherein the CoQ10 cream comprises between 1 and 5% of Coenzyme Q10. In one embodiment, the CoQ10 cream comprises about 3% of Coenzyme Q10. In other embodiments, the CoQ10 cream comprises about 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% of Coenzyme Q10. In various embodiments, the CoQ10 cream is administered at a dosage of about 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 or 10 milligrams of CoQ10 cream per square centimeter of skin. It should be understood that ranges having any one of these values as the upper or lower limits are also intended to be part of this invention, *e.g.*, between about 0.5 and about 5.0, about 1.5 and 2.5, or about 2.5 and 5.5 mg CoQ10 cream per square centimeter of skin.

In another embodiment, the Coenzyme Q10 is administered in the form of a CoQ10 cream at a dosage of between 3 and 5 milligrams of the CoQ10 cream per square centimeter of skin, wherein the CoQ10 cream comprises between 1 and 5% of Coenzyme Q10. In one embodiment, the CoQ10 cream comprises about 3% of Coenzyme Q10. In other embodiments, the CoQ10 cream comprises about 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% or 5% of Coenzyme Q10. In various embodiments, the CoQ10 cream is administered at a dosage of about 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9 or 5.0 milligrams of CoQ10 cream per square centimeter of skin. It should be understood that ranges having any one of these values as the upper or lower limits are also intended to be part of this invention, *e.g.*, between about 3.0 and about 4.0, about 3.3 and 5.3, or about 4.5 and 4.9 mg CoQ10 cream per square centimeter of skin.

Certain aspects of the invention provide methods for treating or preventing a metabolic disorder in a human by topically administering Coenzyme Q10 to the human such that treatment or prevention occurs, wherein the Coenzyme Q10 is topically applied one or more times per 24 hours for six weeks or more.

Certain aspects of the invention provide methods for the prepartion of a Coenzyme Q10 cream 3% which includes the steps of preparing a Phase A, B, C, D and E and combining all the phases such that an oil-in-water emulsion of 3% CoQ10 cream is formed.

In some embodiments, the Phase A ingredients include Alkyl C_{12-15} benzoate NF at 4.00 %w/w, cetyl alcohol NF at 2.00 %w/w, glyceryl stearate/PEG-100 at 4.5 %w/w and stearyl alcohol NF at 1.50 %w/w while the Phase B ingredients include diethylene glycol monoethyl ether NF at 5.00 %w/w, glycerin USP at 2.00 %w/w, propylene glycol USP at 1.50 %w/w, phenoxyethanol NF at 0.475 %w/w, purified water USP at 16.725 %w/w and Carbomer Dispersion 2% at 40.00 %w/w and the Phace C ingredients include lactic acid USP at 0.50 %w/w, sodium lactate solution USP at 2.00 %w/w, trolamine NF at 1.30 %w/w, and purified water USP at 2.50 %w/w. Furthermore in these embodiments the Phase D ingredients include titanium dioxide USP at 1.00 %w/w while the Phase E ingredients include CoQ10 21% concentrate at 15 %w/w.

The term "Trolamine," as used herein, refers to Trolamine NF, Triethanolamine, TEAlan ®, TEAlan 99%, Triethanolamine, 99%, Triethanolamine, NF or Triethanolamine, 99%, NF. These terms may be used interchangeably herein.

In certain other embodiments, the Phase A ingredients include capric/caprylic triglyceride at 4.00 %w/w, cetyl alcohol NF at 2.00 %w/w, glyceril stearate/PEG-100 at 4.5% and stearyl alcohol NF at 1.5 %w/w while the Phase B ingredients include diethylene glycol monoethyl ether NF at 5.00 %w/w, glycerin USP at 2.00 %w/w, propylene glycol USP at 1.50 %w/w, phenoxyethanol NF at 0.475 %w/w, purified water USP at 16.725 %w/w and Carbomer Dispersion 2% at 40.00 %w/w and the Phace C ingredients include lactic acid USP at 0.50 %w/w, sodium lactate solution USP at 2.00 %w/w, trolamine NF at 1.30 %w/w, and purified water USP at 2.50 %w/w. Furthermore in these embodiments the Phase D ingredients include titanium dioxide USP at 1.00 %w/w while the Phase E ingredients include CoQ10 21% concentrate at 15 %w/w.

In certain embodiments of the invention, methods are provided for the preparation of a Coenzyme Q10 cream 3% which include the steps of (1) adding the Phase A ingredients to a suitable container and heating to 70-80 degrees C in a water bath; (2) adding the Phase B ingredients, excluding the Carbomer Dispersion, to a suitable container and mixing to form a mixed Phase B; (3) placing the Phase E

ingredients into a suitable container and melting them at 50-60 degrees C using a water bath to form a melted Phase E; (4) adding the Carbomer Dispersion to a Mix Tank and heating to 70-80 degrees C while mixing; (5) adding the mixed Phase B to the Mix Tank while maintaining the temperature at 70-80 degrees C; (6) adding the Phase C ingredients to the Mix Tank while maintaining the temperature at 70-80 degrees C; (7) adding the Phase D ingredients to the Mix Tank and then continue mixing and homogenizing the contents of the Mix Tank; then (8) stopping the homogenization and cooling the contents of the Mix Tank to 50-60 degrees C; then (9) discontinuing the mixing and adding the melted Phase E to the Mix Tank to form a dispersion; (10) mixing is then resumed until the dispersion is smooth and uniform; then (11) cooling the contents of the Mix Tank to 45-50 degrees C.

In some other embodiments of the invention, a pharmaceutical composition comprising CoQ10 cream 3% is provided. The cream includes a phase A having C_{12-15} alkyl benzoate at 4.00 %w/w of the composition, cetyl alcohol at 2.00 %w/w of the composition, stearyl alcohol at 1.5 %w/w, glyceryl stearate and PEG-100 at 4.5 %w/w; a phase B having glycerin at 2.00 %w/w, propylene glycol at 1.5 %w/w, ethoxydiglycol at 5.0 %w/w, phenoxyethanol at 0.475 %w/w, a carbomer dispersion at 40.00 %w/w, purified water at 16.725 %w/w; a phase C having triethanolamine at 1.300 %w/w, lactic acid at 0.500 %w/w, sodium lactate solution at 2.000 %w/w, water at 2.5 %w/w; a phase D having titanium dioxide at 1.000 %w/w; and a phase E having CoQ10 21% concentrate at 15.000 %w/w. In some embodiments the Carbomer Dispersion includes water, phenoxyethanol, propylene glycol and Carbomer 940.

In some other embodiments of the invention, a pharmaceutical composition comprising CoQ10 cream 3% is provided. The cream includes a phase A having Capric/Caprylic triglyceride at 4.00 %w/w of the composition, cetyl alcohol at 2.00 %w/w of the composition, stearyl alcohol at 1.5 %w/w, glyceryl stearate and PEG-100 at 4.5 %w/w; a phase B having glycerin at 2.00 %w/w, propylene glycol at 1.5 %w/w, ethoxydiglycol at 5.0 %w/w, phenoxyethanol at 0.475 %w/w, a carbomer dispersion at 40.00 %w/w, purified water at 16.725 %w/w; a phase C having triethanolamine at 1.300 %w/w, lactic acid at 0.500 %w/w, sodium lactate solution at 2.000 %w/w, water at 2.5 %w/w; a phase D having titanium dioxide at 1.000 %w/w; and a phase E having CoQ10

21% concentrate at 15.000 %w/w. In some embodiments the Carbomer Dispersion includes water, phenoxyethanol, propylene glycol and Carbomer 940.

In some other embodiments of the invention, a pharmaceutical composition comprising CoQ10 cream 1.5% is provided. The cream includes a phase A having C_{12-15} alkyl benzoate at 5.000 %w/w, cetyl alcohol at 2.000 %w/w, stearyl alcohol at 1.5 %w/w, glyceryl stearate and PEG-100 stearate at 4.500 %w/w; a phase B having glycerin at 2.000 %w/w, propylene at 1.750 %w/w, ethoxydiglycol at 5.000 %w/w, phenoxyethanol at 0.463 %w/w, a carbomer dispersion at 50 %w/w, and purified water at 11.377 %w/w; a phase C having triethanolamine at 1.3 %w/w, lactic acid at 0.400 %w/w, sodium lactate solution at 2.000 %w/w, and water at 4.210 %w/w; a phase D having titanium dioxide at 1.000 %w/w; and a phase E having CoQ10 21% concentrate at 1.500 %w/w.

In some other embodiments of the invention, a pharmaceutical composition comprising CoQ10 cream 1.5% is provided. The cream includes a phase A having Capric/Caprylic triglyceride at 5.000 %w/w, cetyl alcohol at 2.000 %w/w, stearyl alcohol at 1.5 %w/w, glyceryl stearate and PEG-100 stearate at 4.500 %w/w; a phase B having glycerin at 2.000 %w/w, propylene at 1.750 %w/w, ethoxydiglycol at 5.000 %w/w, phenoxyethanol at 0.463 %w/w, a carbomer dispersion at 50 %w/w, and purified water at 11.377 %w/w; a phase C having triethanolamine at 1.3 %w/w, lactic acid at 0.400 %w/w, sodium lactate solution at 2.000 %w/w, and water at 4.210 %w/w; a phase D having titanium dioxide at 1.000 %w/w; and a phase E having CoQ10 21% concentrate at 1.500 %w/w. In some embodiments the Carbomer Dispersion includes water, phenoxyethanol and propylene glycol.

1. Combination Therapies

In certain embodiments, an environmental influencer of the invention and/or pharmaceutical compositions thereof can be used in combination therapy with at least one other therapeutic agent, which may be a different environmental influencer and/or pharmaceutical compositions thereof. The environmental influencer and/or pharmaceutical composition thereof and the other therapeutic agent can act additively or, more preferably, synergistically. In one embodiment, an environmental influencer and/or a pharmaceutical composition thereof is administered concurrently with the

administration of another therapeutic agent. In another embodiment, a compound and/or pharmaceutical composition thereof is administered prior or subsequent to administration of another therapeutic agent.

Examples of other therapeutic agents which can be used with an environmental influencer of the invention include, but are not limited to, diabetes mellitus-treating agents, diabetic complication-treating agents, antihyperlipemic agents, hypotensive or antihypertensive agents, anti-obesity agents, diuretics, chemotherapeutic agents, immunotherapeutic agents immunosuppressive agents, and the like.

Examples of agents for treating diabetes mellitus include insulin formulations (e.g., animal insulin formulations extracted from a pancreas of a cattle or a swine; a human insulin formulation synthesized by a gene engineering technology using microorganisms or methods), insulin sensitivity enhancing agents, pharmaceutically acceptable salts, hydrates, or solvates thereof (e.g., pioglitazone, troglitazone, rosiglitazone, netoglitazone, balaglitazone, rivoglitazone, tesaglitazar, farglitazar, CLX-0921, R-483, NIP-221, NIP-223, DRF-2189, GW-7282TAK-559, T-131, RG-12525, LY-510929, LY-519818, BMS-298585, DRF-2725, GW-1536, GI-262570, KRP-297, TZD18 (Merck), DRF-2655, and the like), alpha-glycosidase inhibitors (e.g., voglibose, acarbose, miglitol, emiglitate and the like), biguanides (e.g., phenformin, metformin, buformin and the like)or sulfonylureas (e.g., tolbutamide, glibenclamide, gliclazide, chlorpropamide, tolazamide, acetohexamide, glyclopyramide, glimepiride and the like) as well as other insulin secretion-promoting agents (e.g., repaglinide, senaglinide, nateglinide, mitiglinide, GLP-1 and the like), amyrin agonist (e.g., pramlintide and the like), phosphotyrosinphosphatase inhibitor (e.g., vanadic acid and the like) and the like.

Examples of agents for treating diabetic complications include, but are not limited to, aldose reductase inhibitors (e.g., tolrestat, epalrestat, zenarestat, zopolrestat, minalrestat, fidareatat, SK-860, CT-112 and the like), neurotrophic factors (e.g., NGF, NT-3, BDNF and the like), PKC inhibitors (e.g., LY-333531 and the like), advanced glycation end-product (AGE) inhibitors (e.g., ALT946, pimagedine, pyradoxamine, phenacylthiazolium bromide (ALT766) and the like), active oxygen quenching agents (e.g., thioctic acid or derivative thereof, a bioflavonoid including flavones, isoflavones, flavonones, procyanidins, anthocyanidins, pycnogenol, lutein, lycopene, vitamins E, coenzymes Q, and the like), cerebrovascular dilating agents (e.g., tiapride, mexiletene

and the like).

Antihyperlipemic agents include, for example, statin-based compounds which are cholesterol synthesis inhibitors (e.g., pravastatin, simvastatin, lovastatin, atorvastatin, fluvastatin, rosuvastatin and the like), squalene synthetase inhibitors or fibrate compounds having a triglyceride-lowering effect (e.g., fenofibrate, gemfibrozil, bezafibrate, clofibrate, sinfibrate, clinofibrate and the like).

Hypotensive agents include, for example, angiotensin converting enzyme inhibitors (e.g., captopril, enalapril, delapril, benazepril, cilazapril, enalapril, enalaprilat, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, trandolapril and the like) or angiotensin II antagonists (e.g., losartan, candesartan cilexetil, olmesartan medoxomil, eprosartan, valsartan, telmisartan, irbesartan, tasosartan, pomisartan, ripisartan forasartan, and the like).

Antiobesity agents include, for example, central antiobesity agents (e.g., dexfenfluramine, fenfluramine, phentermine, sibutramine, amfepramone, dexamphetamine, mazindol, phenylpropanolamine, clobenzorex and the like), gastrointestinal lipase inhibitors (e.g., orlistat and the like), .beta.-3 agonists (e.g., CL-316243, SR-58611-A, UL-TG-307, SB-226552, AJ-9677, BMS-196085 and the like), peptide-based appetite-suppressing agents (e.g., leptin, CNTF and the like), cholecystokinin agonists (e.g., lintitript, FPL-15849 and the like) and the like.

Diuretics include, for example, xanthine derivatives (e.g., theobromine sodium salicylate, theobromine calcium salicylate and the like), thiazide formulations (e.g., ethiazide, cyclopenthiazide, trichloromethiazide, hydrochlorothiazide, hydroflumethiazide, bentylhydrochlorothiazide, penflutizide, polythiazide, methyclothiazide and the like), anti-aldosterone formulations (e.g., spironolactone, triamterene and the like), decarboxylase inhibitors (e.g., acetazolamide and the like), a chlorbenzenesulfonamide formulations (e.g., chlorthalidone, mefruside, indapamide and the like), azosemide, isosorbide, ethacrynic acid, piretanide, bumetanide, furosemide and the like.

Chemotherapeutic agents include, for example, alkylating agents (e.g., cyclophosphamide, iphosphamide and the like), metabolism antagonists (e.g., methotrexate, 5-fluorouracil and the like), anticancer antibiotics (e.g., mitomycin, adriamycin and the like), vegetable-derived anticancer agents (e.g., vincristine,

vindesine, taxol and the like), cisplatin, carboplatin, etoposide and the like. Among these substances, 5-fluorouracil derivatives such as furtulon and neofurtulon are preferred.

Immunotherapeutic agents include, for example, microorganisms or bacterial components (e.g., muramyl dipeptide derivative, picibanil and the like), polysaccharides having immune potentiating activity (e.g., lentinan, sizofilan, krestin and the like), cytokines obtained by a gene engineering technology (e.g., interferon, interleukin (IL) and the like), colony stimulating factors (e.g., granulocyte colony stimulating factor, erythropoetin and the like) and the like, among these substances, those preferred are IL-1, IL-2, IL-12 and the like.

Immunosuppressive agents include, for example, calcineurin inhibitor/immunophilin modulators such as cyclosporine (Sandimmune, Gengraf, Neoral), tacrolimus (Prograf, FK506), ASM 981, sirolimus (RAPA, rapamycin, Rapamune), or its derivative SDZ-RAD, glucocorticoids (prednisone, prednisolone, methylprednisolone, dexamethasone and the like), purine synthesis inhibitors (mycophenolate mofetil, MMF, CellCept(R), azathioprine, cyclophosphamide), interleukin antagonists (basiliximab, daclizumab, deoxyspergualin), lymphocyte-depleting agents such as antithymocyte globulin (Thymoglobulin, Lymphoglobuline), anti-CD3 antibody (OKT3), and the like.

In addition, agents whose cachexia improving effect has been established in an animal model or at a clinical stage, such as cyclooxygenase inhibitors (e.g., indomethacin and the like) [Cancer Research, Vol.49, page 5935-5939, 1989], progesterone derivatives (e.g., megestrol acetate) [Journal of Clinical Oncology, Vol.12, page 213-225, 1994], glucosteroid (e.g., dexamethasone and the like), metoclopramide-based agents, tetrahydrocannabinol-based agents, lipid metabolism improving agents (e.g., eicosapentanoic acid and the like) [British Journal of Cancer, Vol.68, page 314-318, 1993], growth hormones, IGF-1, antibodies against TNF-.alpha., LIF, IL-6 and oncostatin M may also be employed concomitantly with a compound according to the present invention.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references and published patents and patent applications cited throughout the application are hereby incorporated by reference.

EXEMPLIFICATION OF THE INVENTION

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention, as one skilled in the art would recognize from the teachings hereinabove and the following examples, that other assays, cell types, agents, constructs, or data analysis methods, all without limitation, can be employed, without departing from the scope of the invention as claimed.

The contents of any patents, patent applications, patent publications, or scientific articles referenced anywhere in this application are herein incorporated in their entirety.

The practice of the present invention will employ, where appropriate and unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, virology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 3rd Ed., ed. by Sambrook and Russell (Cold Spring Harbor Laboratory Press: 2001); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Using Antibodies, Second Edition by Harlow and Lane, Cold Spring Harbor Press, New York, 1999; Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; and PCR Protocols, ed. by Bartlett et al., Humana Press, 2003.

EXAMPLE 1: Identification of CoQ10 as a MIM

In order to evaluate CoQ10 as a potential MIM, CoQ10 in oxidized form was exogenously added to a panel of cell lines, including both cancer cell lines and normal control cell lines, and the changes induced to the cellular microenvironment profile for each cell line in the panel were assessed. Changes to cell morphology/physiology, and to cell composition, including both mRNA and protein levels, were evaluated and compared for the diseased cells as compared to normal cells. The results of these experiments identified CoQ10 and, in particular, the oxidized form of CoQ10, as a MIM.

In a first set of experiments, changes to cell morphology/physiology were evaluated by examining the sensitivy and apoptotic response of cells to CoQ10. A panel of skin cell lines including a control cell lines (primary culture of keratinocytes and melanocytes) and several skin cancers cell lines (SK-MEL-28, a non-metastatic skin melanoma; SK-MEL-2, a metastatic skin melanoma; or SCC, a squamous cell carcinoma; PaCa2, a pancreatic cancer cell line; or HEP-G2, a liver cancer cell line) were treated with various levels of Coenzyme Q10. The results of these experiments demonstrated that the cancer cell lines exhibited an altered dose dependent response as compared to the control cell lines, with an induction of apoptosis and cell death in the cancer cells only. Exemplary experiments are described in detail in Example 3 below.

Assays were next employed to assess changes in the composition of the cell following treatment with CoQ10. Changes in gene expression at the mRNA level were analyzed using Real-Time PCR array methodology. Exemplary experiments are described in detail in Examples 6 and 9-13 below. In complementary experiments, changes in gene expression at the protein level were analyzed by using antibody microarray methodology, 2-dimensional gel electrophoresis followed by protein identificuation using mass spectrometry characterization, and by western blot analysis. Exemplary experiments are described in detail below in Examples 4, 7 and 8, respectively. The results from these assays demonstrated that significant changes in gene expression, both at the mRNA and protein levels, were induced in the cell lines examined due to the addition of the oxidized form of CoQ10. Genes modulated by CoQ10 treatment were found to be clustered into several cellular pathways, including apoptosis, cancer biology and cell growth, glycolysis and metabolism, molecular transport, and cellular signaling.

Experiments were carried out to confirm the entry of CoQ10 into cells and to determine the level and form of CoQ10 present in the cells. In particular, the level of Coenzyme Q10, as well as the form of CoQ10 (i.e., oxidized or reduced), present in the mitochondria was determined by analyzing mitochondrial enriched preparations from cells treated with CoQ10. The level of Coenzyme Q10 present in the mitochondria was confirmed to increase in a time and dose dependent manner with the addition of exogenous Q10. In a surprising and unexpected result, CoQ10 was determined to be present in the mitochondria primarily in oxidized form. In addition, changes in levels of

proteins from mitochondria enriched samples were analyzed by using 2-D gel electrophoresis and protein identification by mass spectrometry characterization. The results from these experiments demonstrated that the levels of the oxidized form of CoQ10 in the mitochondria over the time course examined correlated with a wide variety of cellular changes, as evidenced by the modulation of mRNA and protein levels for specific proteins related to metabolic and apoptotic pathways. Exemplary experiments are described in detail in Example 5 below.

The results described by Applicants herein identified the endogenous molecule CoQ10 and, in particular, the oxidized form of CoQ10, as a MIM. For example, the results identified CoQ10 as a MIM, since CoQ10 was observed to induce changes in gene expression at both the mRNA and protein level. The results identified CoQ10 as having multidimentional character, since CoQ10 induced differential changes in cell morphology/physiology and cell composition (e.g., differential changes in gene expression at both the mRNA and protein level), in a disease state (e.g., cancer) as compared to a normal (e.g., non-cancerous) state. Moreover, the results identified CoQ10 as having multidimensional character in that CoQ10 was capable of entering a cell, and thus exhibited both therapeutic and carrier effects.

EXAMPLE 2: Methods for Identifying Disease Relevant Processes and Biomarkers for Metabolic Disorders

From the cell based assays in which cell lines were treated with a molecule of interest, the differences in treated vs non-treated cells is evaluated by mRNA arrays, protein antibody arrays, and 2D gel electrophoresis. The proteins identified from comparative sample analysis to be modulated by the MIM or Epi-shifter, are evaluated from a Systems Biology perspective with pathway analysis (Ingenuity IPA software) and a review of the known literature. Proteins identified as potential therapeutic or biomarker targets are submitted to confirmatory assays such as Western blot analysis, siRNA knock-down, or recombinant protein production and characterization methods.

Materials and Methods for Examples 3-8

Coenzyme Q10 stock

A 500 μM Coenzyme Q10 (5% isopropanol in cell growth media) was prepared as follows. A 10 mL 500 μM Coenzyme Q10 stock was made fresh every time.

Molecular Weight: 863.34

(0.0005 mol/L)(0.010 L)(863.34 g/mol) = 0.004317 g

To make 10 mL of 500 μM stock, 4.32 mg Coenzyme Q10 was weighted out in a 15 mL falcon tube, and 500 μL isopropanol was added. The solution was warmed in a 50-60 °C water bath while swirling to dissolve completely. To this solution, 9.5 mL of media (the same media in which the cells are grown) was added.

Cell Culture

Cells were obtained from the American Type Culture Collection or Gibco. Cells were grown in DMEM/F-12 media supplemented with 5% fetal bovine serum, 0.25 ug/mL Amphotericin, 100 ug/mL Streptomycin, and 100 U mL-1 penicillin. Cells were maintained in an atmosphere of 95% air and 5% CO2 at 37 degrees C.

Coenzyme Q10 Treatment and Total Protein Isolation

Cells were grown to 85% confluency prior to exposure with Q10. Supplemented media was conditioned with Q10 to 50 and 100 micro molar concentrations. Flasks were treated with control, 50 μM Q10, and 100 μM Q10 in triplicate. Protein was isolated from the treated and control flask after 4, 8, 12, and 24 hours. For isolation of proteins, cells were washed three times with 5 mL of ice cold PBS at a pH of 7.4. The cells were then scraped in 3 mL of PBS, pelleted by centrifuge, and re-suspended in a lysis buffer at pH 7.4 (80 mM TRIS-HCl, 1% SDS, with protease and phosphotase inhibitors). Protein concentrations were quantified using the BCA method.

Cell Lines

The cell lines listed below were propagated and a cell bank established for each. Large scale production of cells for various assays were performed and the material harvested for analysis. In general, when a cell specific media was not required for maintenance of cell lines, the media used for cell growth was DMEMF-12 with 5% serum. Cells were typically grown to 75-80% confluence (clear spacing) prior to

splitting and use in cell assays and standard practice methods followed. The following cell lines were established for experiments:

SK-MEL-28 (non-metastatic skin melanoma)
SK-MEL-2 (metastatic skin melanoma)
HEKa (kerantinocytes, skin control)
HEMa (melanocyte, skin control)
nFIB (neonatal fibroblasts)
HEP-G2 (liver cancer) [SBH cell line]
SkBr-3 (breast cancer, Her2 overexpressed)
MCF-7 (breast cancer, p53 mutation)
PC-3 (prostate cancer) [SBH cell line]
SkBr-3 (human breast adenocarcinoma)
NCI-ES-0808
SCC (squamous cell carcinoma)
PaCa-2
NIH-3T3

Cell culture:

Cells were obtained for the American Type Culture Collection or Gibco. Cells were grown in DMEM/F-12 media supplemented with 5% fetal bovine serum, 0.25 ug/mL Amphotericin, 100 ug/mL Streptomycin, and 100 U mL-1 penicillin. Cells were maintained in an atmosphere of 95% air and 5% CO2 at 37 degrees C.

Skin malignant melanoma SK-MEL28 cells were grown and maintained in DMEM/F12 with Glutamax (Invitrogen, Carlsbad CA) supplemented with 5% FBS, amphotericin and penicillin/streptomycin. Cells were grown at 37 °C with 5% CO2. Details of additional cell line and growth conditions are outlined in the table below.

Table 1. Cell lines analyzed for sensitivity to Q10.

Cell Line	Description	Growth Conditions
PaCa2	Pancreatic Carcinoma	DMEM/F12 with Glutamax + 10%FBS, 2.5%Horse Serum, amphotericin, penicillin/streptomycin.

HepG2	Hepatocellular Carcinoma	MEM with Earles Salts supplemented with 10% FBS, amphotericin, penicillin/streptomycin, sodium pyruvate and non-essential amino acids.
PC3	Prostate Adenocarcinoma	DMEM/F12 with Glutamax, supplemented with 5%FBS, amphotericin and penicillin/streptomycin.
SKBr3	Breast Cancer	DMEM/F12 with Glutamax supplemented with 5% FBS and amphotericin, penicillin/streptomycin.
MCF-7	Breast Cancer	DMEM/F12 with Glutamax supplemented with 5% FBS and amphotericin, penicillin/streptomycin.

Q10 treatment of SKMEL28 cells:

SK-MEL28 cells were treated with 100 μM Q10 or the control vehicle. The formulation of the Q10 was as follows. In a 15 mL capped tube, 4.32 mg of Q10 (supplied by Cytotech) was transferred and then dissolved by the addition of 500 μL of isopropanol. The resulting solution was warmed in a 65°C water bath and vortexed at high speed. The Q10/isopropanol solution was made to a volume of 10 mL with the addition of equilibrated cell culture media. The stock solution was then vortexed to ensure maximum solubility of Q10. The stock solution was diluted (2 mL of stock with 8 mL of media) to obtain a final concentration of 100 μM Q10. For the control vehicle, 9.5 mL of media was added to 500 μL of isopropanol. The control stock was further diluted (2 mL of stock) with 8 mL of media. Cells were harvested 6, 16, 24, 48 or 72 hours after the start of the treatment.

Q10 treatment of SCC cells:

SCC cells were treated with 100 μM Q10 (prepared as described above) either for 6 hours or 24 hours. The control cells were untreated cells. Cells were harvested and pelleted at the different times after treatment and the pellets were flash frozen and stored at -80 °C until the RNA was isolated at XTAL as described below.

RNA isolation:

Cells were lysed for RNA isolation at different treatment times using the RNeasy Mini kit (Qiagen, Inc., Valencia CA) kit following the manufacturer's instructions. RNA was quantified by measuring Optical Density at 260 nm.

First Strand Synthesis:

First strand cDNA was synthesized from 1 µg of total RNA using the RT2 First Strand Synthesis kit (SABiosciences., Frederick MD) as per manufacturer's recommendations.

Real-time PCR:

Products from the first strand synthesis were diluted with water, mixed with the SYBR green master mix (SABiosciences., Frederick MD) and loaded onto PCR arrays. Real time PCR was run on the PCR Arrays (Apoptosis Arrays, Diabetes Arrays, Oxidative stress and Antioxidant defense Arrays and Heat Shock Protein Arrays.) (SABiosciences, Frederick MD) on a Biorad CFX96.

Determining cell line sensitivity to Coenzyme Q10 by Nexin Assay for Apoptosis:

The percentage of cells in early and late apoptosis was quantified following 24 hours of Coenzyme Q10 treatment. Early and late apoptosis was used as a marker to understand the differences in sensitivity of various cancer cell lines to Coenzyme Q10. The different cell lines tested were PaCa2, HepG2, PC-3, SKBr3, MCF-7 and SK-MEL28. Cells were allowed to adhere overnight in 96-well plates. These cells were treated with either control vehicle, 50 µM Q10 or 100 µM Coenzyme Q10. After 24 hours, the presence of apoptotic cells was estimated on a PCA96 flow cytometer (Guava Technologies, Hayward, CA). In addition, some cells were treated with 4 µM Staurosporine for 2 hours as a positive control for apoptosis. Cells were first washed with PBS and detached with 50 µL of Accumax (Innovative Cell Technologies, San Diego, CA) at room temperature. The dissociation was stopped by addition of culture medium containing 1% Pluronic F-68 (Sigma-Aldrich, St.Louis, MO). Then 100 µL of Nexin reagent (Guava Technologies, Hayward, CA) was added to each of the wells. After 20 minutes of incubation in the dark, the assay was performed in low binding

plates to minimize reattachment of cells to the substrate. The Nexin Reagent contains two dyes. Annexin-V-PE which detects phosphotidyl serine on the outside of a cell; a characteristic of early apoptotic cells. The second dye, 7-AAD permeates only late apoptotic cells while being excluded from live (healthy) and early apoptotic cells. The percentage of four populations of cells; live, early apoptotic, late apoptotic and debris was determined using the Cytosoft 2.5.7 software (Guava Technologies, Hayward, CA).

Immunoblotting

Approximately 50 µg of protein were assayed per sample by immunoblotting. All treatments were run in triplicate with controls. Proteins were separated on 12% TRIS-HCl gels, transferred via electrophoresis to nitro-cellulose membranes and blocked using a 5% milk and TBST solution prior to incubation with primary antibodies. The primary antibodies were incubated overnight at 4 degrees C in a 5% BSA and TBST solution. Secondary antibodies were incubated for one hour at 4 degrees. All antibodies were purchased from Cell Signaling Technology. Antibodies were used at a ratio of 1:1000, with the exception of βActin at a ratio of 1:5000. Blots were developed and results were quantified using the NIH Java based densitometer analysis software Image J. All blots were also probed for and normalized to their respective βActin expression.

Two-Dimensional Electrophoresis

Before isoelectric focusing (IEF), samples were solubilized in 40 mM Tris, 7 M urea, 2 M thiourea, and 1% C7 zwitterionic detergent, reduced with tributylphosphine, and alkylated with 10 mM acrylamide for 90 min at room temperature. After the sample was run through a 10-kDa cutoff Amicon Ultra device with at least 3 volumes of the resuspension buffer, consisting of 7 M urea, 2 M thiourea, and 2% CHAPS to reduce the conductivity of the sample. One hundred micrograms of protein were subjected to IEF on 11-cm pH 3 to 10, pH 4 to 7 or pH 6 to 11 immobilized pH gradient strips (GE, Amersham, USA) to 100,000 volts hour. After IEF, immobilized pH gradient strips were equilibrated in 6 M urea, 2% SDS, 50 mM Tris-acetate buffer, pH 7.0, and 0.01% bromphenol blue and subjected to SDS-polyacrylamide gel electrophoresis on 8 to 16% Tris-HCl Precast Gel, 1 mm (Bio-Rad, USA). The gels were run in duplicate. They

were either fixed, stained in SYPRO Ruby, 80 mL/gel (Invitrogen, USA) and imaged on Fuji FLA-5100 laser scanner or transferred onto PVDF membrane.

Additional information was obtained for a control sample to test the utility of protein identification through the use of methods that utilize dPC (Protein Forest Inc.) selective pI fractionation, followed by trypsin digestion of the dPC plug with mass spec identification and semi-quantization (Nanomate or LC/LTQ/MS). The dPC analysis performed with a control sample demonstrated its utility in identifying a large subset of proteins. The materials produced during the studies were archived so that they may be utilized as a resource should the future need arise

2D Gel Image Analysis:

Analysis of all gel images was performed using Progenesis Discovery and Pro (Nonlinear Dynamics Inc., Newcastle upon Tyne, UK). After spot detection, matching, background subtraction, normalization, and filtering, data for SYPRO Ruby gel images was exported. Pairwise comparisons between groups were performed using the Student's t test in Progenesis Discovery to identify spots whose expression was significantly altered ($p > 0.05$).

Antibody Array:

An antibody microarray (Panorama XP725 Antibody Array, Sigma) was utilized to screen over 700 protein antibodies to assess changes at the protein concentration level in Q10 treated cells (SK-MEL-28, SCC). The expression of a protein in a cell extract is detected when it is bound by a corresponding antibody spotted on the slide. Prior to binding, the proteins are directly labeled with a fluorescent dye which is used for fluorescent visualization and quantitative analysis. The array is used for comparing protein expression profiles of two samples (test versus reference samples), each labeled with a different CyDye (Cy3 or Cy5) and the two samples are applied simultaneously at equal protein concentrations on the array. Fluorescent signal intensity for each sample is then recorded individually at the wavelength corresponding to the dye label of the sample and compared.

High doses of Coenzyme Q10 regulates expression of genes involved in the apoptotic, diabetic and oxidative stress pathways in cultured SKMEL-28 cells.

Experimental details: SKMEL-28 cells (ATCC Catalog # HTB-72) are non metastatic, skin melanoma cells that were cultured in DMEM-F12 containing Glutamax (Invitrogen Cat# 10565-042) supplemented with 5% FBS, Penicillin, Streptomycin and Amphotericin, were treated with the vehicle or 100 uM Coenzyme Q10 for varying amounts of time. Any changes in gene expression consequent to Coenzyme Q10 treatment were quantified using Real time PCR Arrays (Apoptosis Cat #PAHS-12, Diabetes Cat #PAHS-023 and Oxidative Stress Cat #PAHS-065). (SABiosciences, Frederick, MD).

A stock concentration of 500 uM Coenzyme Q10 was prepared by dissolving 4.32 mg in 500ul of isopropanol which was further diluted to 10ml by addition of media. Alternate vortexing and heating to 65 oC dissolved the Coenzyme Q10. 2 ml of the stock solution was diluted to 10 ml with media to get a 100 uM Q10 containing media that was used to treat cells. A vehicle was prepared in parallel with a similar protocol except that the Coenzyme Q10 was not added:

SKMEL-28 cells were plated at a density of $1x10^5$ cells/well in a 6-well plate. After 24 hours, when cells had attached and were at 50% confluence, either the vehicle or 100 uM Q10 was added. Cells were harvested by at 6, 16, 24, 48 or 72 hours after Q10 treatment while the vehicle treated cells were harvested after 24 hours. Cells were lysed for RNA isolation at different treatment times using the RNeasy Mini kit (Qiagen, Inc., Valencia CA Cat #74104) kit following the manufacturer's instructions using a spin column and on-column DNase treatment. RNA was quantified by measuring absorbance at 260 nm.

Real time PCR was preceded by first strand cDNA synthesis using 0.4-1ug of total RNA as the template using the RT2 First Strand Synthesis kit (SABiosciences., Frederick MD Cat# C-03) with a genomic DNA elimination step as per manufacturer's recommendations. Products from the first strand synthesis were diluted with water, mixed with the SYBR green master mix (SABiosciences., Frederick MD Cat#PA-010-12) and loaded onto PCR arrays that contain primer assays for 84 different genes linked within a common pathway, 5 housekeeping genes used for normalization, reverse transcription and PCR controls. Real time PCR was run on a Biorad Cfx96. The amplification was initiated with a hot start to activate the enzyme, followed by 40 cycles each of (95°C-15 second denaturation step and 60°C-1 minute annealing and extension

step) followed by a melting curve program. Ct values, the output from the PCR thermocycler for all treatment groups were organized on an excel spreadsheet and loaded onto the comparative analysis software available at http://www.sabiosciences.com/pcr/arrayanalysis.php.

Purification of Mitochondria Enriched Samples:

Experimental details: SKMEL-28, NCI-ES0808 and NIH-3T3 cells that were treated with 100 μM Q10 for 24 or 48 hours along with cells that were harvested at t=0 were harvested by washing and scraping from T160 flasks. Cells were centrifuged, pelleted, flash frozen and stored at -80 oC until the mitochondria were isolated. Cell pellets were thawed, resuspended and ruptured in Dounce homogenizer. The homogenate was centrifuged and mitochondria were isolated using reagents and the protocol recommended by the Mitochondria Isolation kit for Cultured cells (MitoSciences, Eugene OR, Cat # MS852). The mitochondrial fraction was aliquoted and stored at -80 oC.

Coenzyme Q10 and Ubiquinol-10 Quantification Method:

A method for the simultaneous determination of Coenzyme Q10 (Q10) and the reduced form ubiquinol-10 (Q10H2) was implemented based upon a recently published method (Ruiz-Jimenez, 2007, J. Chromatogr. A, 1175, 242-248) through the use of LC–MS/MS with electrospray ionization (ESI) in the positive ion mode. The highly selective identification and sensitive quantitation of both Q10 and Q10H2 is possible, along with the identification of other selected lipids. An aliquot of the mitochondrial enriched samples from SK-MEL-28 treated with 100 μM Q10 was subjected to a conventional pre-treatment based on protein precipitation (100 μl of packed cells sonicated in 300 μl of 1-propanol), liquid–liquid extraction (add 100 μl of water to supernatant and extract X3 with 200 μl of n-hexane), evaporation of combined hexane extracts to dryness and reconstitution in 50 μl of 95:5 methanol/hexane (v/v). Analysis was by LC-MS/MS on a Waters Quattro II triple quadrupole mass spectrometer with a Prism RP 1 X 100 mm, 5 μm particle size column (Keystone Scientific). Isocratic elution with 4 mM ammonium formate in 20% isopropyl alcohol 80% methanol at a flow rate of 50 μl/min. Ten μl of each sample was injected. MRM analysis was performed using m/z 882.7>197.00

(Q10H2) and m/z 880.80>197.00 (Q10) transitions with cone voltage of 40 and collision energy of 30.

EXAMPLE 3: Sensitivity of Cell Lines to CoQ10

A number of cell lines were tested for their sensitivity to Q10 after 24 hours of application by using a reagent (Nexin reagent) that contains a combination of two dyes, 7AAD and Annexin-V-PE. The 7AAD dye will enter into cells with permeabilized cell membranes; primarily those cells that are in late apoptosis. Annexin-V-PE is a dye that binds to Phosphotidyl serine, which is exposed on the outer surface of the plasma membrane in early apoptotic cells. The Nexin reagent thus can be used to differentiate between different populations of apoptotic cells in a flow cytometer.

PaCa2 cells showed an increase in both early and late apoptotic cells (between 5-10% of gated cells) with 50 μM Q10 and 100 μM Q10 after 24 hours of Q10 application. PC-3 cells also showed an increase in both early and late apoptotic population with 50 μM and 100 μM Q10, although the increase was less when compared to PaCa2 cells. MCF-7 and SK-MEL28 cells showed an increase only in early apoptotic population with 50 μM and 100 μM Q10. HepG2 cells were also sensitive to 50 μM Q10 treatment, where there was an increase of about 20% of the gated populated in the late apoptotic and early apoptotic stages. SKBr3 was the only cell line tested that did not show any significant increases of early and late apoptosis with either 50 μM or 100 μM Q10 treatment. The results are depicted in Figures 1-6.

To provide additional confirmation that Q10 treatment causes an apoptotic response in HepG2 liver cancer cells, a second apoptosis assay was evaluated using the ApoStrand™ ELISA based method that measures single-stranded DNA. The ApoStrand™ ELISA is based on the sensitivity of DNA in apoptotic cells to formamide denaturation and the detection of the denatured DNA with a monoclonal antibody to single-stranded DNA (ssDNA). Treatment of the liver cancer cell line HepG2 with 50 and 100 μM Q10 resulted in detectable apoptosis, with a dose-response of 17% and 32%, respectively (Figure 7). These results are consistent with the observation of Q10 inducing apoptosis in other cancer cell lines from other tissues (e.g., SCC, SKMEL-28, MCF-7, and PC-3).

EXAMPLE 4: Proteomic Analysis of Cells treated with Q10

Cell pellets of samples treated with Q10 were analyzed using proteomic methods. The cell pellets were lysed and treated for use in 2-D gel and Western blot analysis. Three cell types (SKMEL-28, SCC, and nFib) were treated with Q10 and submitted to proteomic characterization by 2-D gel electrophoresis.

Proteomic Analysis of SKMEL-28 Cells Treated with Q10

The first experimental set processed and evaluated by Western blot and 2-D gel electrophoresis was the skin cancer cell line SKMEL-28. This experimental set involved SK-MEL-28 cells treated at 3, 6, 12, and 24 hours with 0, 50 or 100 μM Q10.

The set of Q10 treated SK-MEL-28 samples were subjected to 2-D gel electrophoreses (Figure 8) and were analyzed to identify protein-level changes relative to the control samples. A comparative analysis of 943 spots across all twenty-four gels was performed, comparing the control sample against all of the treated samples. The analysis included the identification of spot changes over the time course due to increase, decrease, or post-translational modification.

The analysis found thirty-two statistically significant differential spot changes. From this, twenty non-redundant spots were excised and submitted for protein identification by trypsin digestion and mass spectrometry characterization. The characterized peptides were searched against protein databases with Mascot and MSRAT software analysis to identify the protein (Table 2).

Table 2. Proteins identified to have a differential response to Q10 treatment in SKMEL-28 cell.

Time (hr)	Q10 Conc. (uM)	2D Spot #	Expression	Difference	Protein	Name	Type
3	50	528	down	1.234	cathepsin D	CTSD	peptidase
3	50	702	down	1.575	chaperonin containing TCP1, subunit 3	CCT3	other
3	50	74	down	1.383	eukaryotic translation initiation factor 3	EIF3G	translation regulator
3	50	829	down	1.074	Ribosomal protein P2	RPLP2	other
3	50	368	down	1.121	transaldolase 1	TALDO1	enzyme
6	50	452	up	-1.464	eukaryotic translation initiation factor 6	EIF6	translation regulator
6	50	175	up	-1.32	Stomatin; HSPC322	STOM	other
6	50	827	up	-1.457	Tyrosine 3/Tryptophan 5-monooxygenase activation protein	YWHAZ	enzyme
6	50	139	up	-1.628	Vimentin	VIM	other
6	50	218	up	-1.416	Vimentin	VIM	other
6	50	218	up	-1.212	Vimentin	VIM	other
6	50	139	up	-1.036	Vimentin	VIM	other
6	50	507	down	1.379	Lamin B1	LMNB1	other
6	50	571	down	1.832	mitochandrial import receptor Tom22	TOMM22	transporter
12	50	166	up	-1.171	ALG-2 interacting protein 1	PDCD6IP	other
12	50	550	up	-1.747	peptidylprolyl isomerase A	PPIA	enzyme
12	50	613	down	1.802	galectin-1	LGALS1	other
12	50	242	down	1.373	Phosphoglycerate mutase; Posphomannomutase 2	PGAM2	phosphatase
24	50	326	down	1.385	glycyl-tRNA synthase	GARS	enzyme
24	50	419	down	1.451	Mago-nashi homolog	MAGOH	other
3	100	528	down	-1.036	cathepsin D	CTSD	peptidase
3	100	702	down	1.151	chaperonin containing TCP1, subunit 3	CCT3	other
3	100	74	down	1.122	eukaryotic translation initiation factor 3	EIF3G	translation regulator
3	100	829	down	1.145	Ribosomal protein P2	RPLP2	other
3	100	368	down	1.209	transaldolase 1	TALDO1	enzyme
6	100	139	up	-1.829	Vimentin	VIM	other
6	100	218	up	-1.761	Vimentin	VIM	other
6	100	452	down	1.134	eukaryotic translation initiation factor 6	EIF6	translation regulator
6	100	252	down	1.4	Sec 13 protein, Keratin II	?	
6	100	827	down	1.12	Tyrosine 3/Tryptophan 5-monooxygenase activation protein	YWHAZ	enzyme
12	100	76	up	-1.679	galectin-1; keratin II	LGALS1	other

A key finding in this experiment was the decrease of Transaldolase 1, which supports the premise that Q10 acts by altering the metabolic state within the cancer cell. Transaldolase 1 is an enzyme in the pentose phosphate pathway (also known as the hexose monophosphate shunt). Transaldolase (EC:2.2.1.2) catalyses the reversible transfer of a three-carbon ketol unit from sedoheptulose 7-phosphate to glyceraldehyde 3-phosphate to form erythrose 4-phosphate and fructose 6-phosphate. This enzyme,

together with transketolase, provides a link between the glycolytic and pentose-phosphate pathways. This is relevant to nucleotide and NADPH synthesis, to facilitate production of reducing equivalents for biosynthetic reactions and maintenance of a reducing environment.

A recent publication (Basta, P., et.al. August 2008, Cancer Detect Prevention, 32, 200-208) provided evidence of genetic polymorphism in Transaldolase and was linked to squamous cell carcinoma of the head and neck. Another recent publication (Qian, Y., et.al. May 2008, Biochem J, 415, 123-134) identified transaldolase deficiency as a modulator of mitochondrial homoeostasis, Ca2+ fluxing and apoptosis.

From these initial results, the other proteins identified by 2-D gel electrophoresis as being modulated by Q10 in SK-MEL-28 were analyzed for known relationships (Figure 9). A functional evaluation of these proteins revealed that there was a group involved in 14-3-3-mediated signaling (PDCP6IP, YWHAZ, and VIM), along with individual proteins linked to a variety of processes [cell cycle; pentose phosphate pathway (TALDO1); ceramide signaling (CTSD); aminoacyl-tRNA biosynthesis (GARS), and mitochondrial protein import (TOM22)].

Proteomic Analysis of SCC Cells Treated with Q10

Another skin cancer cell line, Squamous Cell Carcinoma (SCC), was also prepared and analyzed by 2-D gel electrophoreses as a follow-up experiment the previous SK-MEL-28 analysis The SCC cells were treated with 100 µM Q10 for 6 hour or 24 hours before harvesting. A control of untreated cells was also harvested. The cell pellets were lysed and the samples were subjected to 2-D electrophoresis (in duplicate). Analysis of over six hundred protein spots in the comparative study was performed, comparing the control sample against the six hour and twenty-four hour treatments.

The top twenty-five statistically significant differential spot changes were evaluated from the comparative analysis of the 2-D electrophoresis gels. From this, twelve spots were excised and submitted for identification by trypsin digestion and mass spectrometry characterization (results summarized in Table 3 below).

Table 3. Proteins identified to have a differential response to 100 µM Q10 treatment in SCC cells at 6 and 24 hours.

Spot #	Protein	Name	Cellular localization	Function	Response (fold change)
331	Transaldolase 1	TALDO1	Cytoplasm	Enzyme	Decrease

					(1.5) at 6 and 14 hr
23	Human BSCv (chromosome 20 reading frame 3)	C20ORF3	Plasma membrane	strictosidine synthase	Decrease (2.1) at 6 and 24 hr
54	NM23 protein	NME1	Nucleus, (mitochondria?)	Kinase	Increase (-1.2) at 6 hr, decrease at 24 hr
116	two Human ESTs from MCF7 breast cancer cell line (HSP 70)			HSP70	Decrease (2.6) at 6 hr, further decrease at 24 hr
176	Heat shock 27kDa protein 1	HSPB1	Cytoplasm	Response to environmental stresses	Increase (-1.9) at 6 and 24 hr
135	Keratin I	KRT1	Cytoplasm	intermediate filaments	Decrease (2.3) at 6 and 24 hr
50	Keratin 14	KRT14	Cytoplasm	intermediate filaments	Increase (-1.6) at 6 and 24 hr
68	Keratin 13	KRT13	Cytoplasm	intermediate filaments	Increase (-1.5) at 6 and 24 hr
49	Proteasome Beta 7	PSMB7	Cytoplasm	Proteasome subunit	Decrease (1.6) at 24 hr only
93	Proteasome activator subunit 3	PSME3	Cytoplasm	peptidase	Decrease (1.3) at 24 hr only
66	Rho GDP dissociation inhibitor (GDI) alpha	ARHGDIA	Cytoplasm	Inhibitor	Decrease (1.5) at 6 hr only
1	Unknown?				Decrease (9.5)

Transaldolase 1: As previously observed in the SKMEL-28 cells treated with Q10, the enzyme Transaldolase 1 was modulated with a decrease in levels. This provides an independent confirmation of the previously observation of a linkage between Q10 and alterations in transaldolase (and thus the metabolic state of the cell).

Transaldolase is an enzyme in the non-oxidative phase of the pentose phosphate pathway (Figure 10). The pentose phosphate pathway is critical in the metabolic state of cells for the generation of nicotinamide adenine dinucleotide phosphate (reduced

NADH), for reductive biosynthesis, and in the formation of ribose which is an essential component of ATP, DNA, and RNA. Transaldolase also links the pentose phosphate pathway to glycolysis. Glycolysis is the metabolic pathway by which cancer cells obtain the energy needed for cell survival, as the mitochondrial process of oxidative phosphorylation is not utilized. Q10 is an essential coenzyme factor required for oxidatative phosphorylation and mitochondrial ATP production.

BSCv: Spot 23 was a novel human protein from Chromosome 20 named BSCv. BSCv protein is also known as Adipocyte plasma membrane-associated protein (Gene names: APMAP or C20orf3) and is predicted to be a single-pass type II membrane protein with sequence similarity to the strictosidine synthase family of proteins. Q10 treatment caused a reduction in the levels of this protein. This protein is not well characterized, nor has its homology with strictosidine synthases been confirmed. Interestingly, this protein has been associated with a role in adipocyte differentiation (Albrektsen et al., 2001). Recent proteomic studies of human omental adipose tissue identified BSCv as one of nine proteins with differential expression for polcystic ovary syndrome (PCOS) from morbidly obese women (Corton, 2008 Hum. Reprod. 23: 651-661). As a cell surface protein that responds to Q10, an antibody against BSCv would be useful as a biomarker. Based on the current results and the literature available, BSCv may a have a potential role in cancer and diabetes.

NM23A: Non-metastatic cells 1, protein (NM23A, also known as NME1) is thought to be a metastasis suppressor. This gene (NME1) was identified because of its reduced mRNA transcript levels in highly metastatic cells. The protein has activity as a nucleoside diphosphate kinase (NDK) and exists as a hexamer composed of 'A' (encoded by this gene) and 'B' (encoded by NME2) isoforms. Mutations in this gene have been identified in aggressive neuroblastomas. NDK activities maintain an equilibrium between the concentrations of different nucleoside triphosphates such as, for example, when GTP produced in the citric acid (Krebs) cycle is converted to ATP. The NDK complex is associated with p53 through interaction with STRAP. It is noteworthy that STRAP is linked to HNF4A. Thus, NM23A is a potential protein involved in pathways important for cell control and disease treatment.

Rho GDP dissociation inhibitor (GDI) alpha: GDI Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them. The protein is upregulated in cancer cells.

EXAMPLE 5: Mitochondrial Enrichment Analysis

Several lines of evidence suggested that a closer evaluation of the role of mitochondrial proteins and cancer biology and Q10 response was warranted. First, there is the essential role of Q10 in the mitochondrial oxidative phosphorylation process for energy production in normal cells. However, the metabolic shift that occurs in cancer cells is to energy production through the alternative pathway of glycolysis, which does not require Q10. Second, the apoptotic response of cells requires mitochondrial proteins to occur. Q10 has been established as stimulating apoptosis in cancer cells (Bcl-2 family proteins, cytochrome c). Finally, new mitochondrial proteins were identified as being modulated by Q10 treatment, as exemplified by the modulation in protein levels of the mitochondrial import receptor protein TOM22 (see experiments described herein).

Production of Mitochondrial Enriched Samples

The skin cancer SKMEL-28 cells were treated with 100 μM Q10 or a mock vehicle for 6, 19, or 48 hours. The cells were harvested by washing and scraping the cells from T-160 flasks (4 for each time point). The cells were collected by centrifugation and the pellets flash frozen and stored at -80 °C. The cell pellets were resuspended and ruptured using a 2 mL Dounce homogenizer. The reagents and method were obtained from a Mitochondria Isolation Kit for Cultured Cells (MitoSciences, Cat# MS852). The resultant mitochondria samples were divided into 75 μL aliquots (4-5 aliquots per sample) and stored at -80 °C.

Proteomic Analysis of Mitochondria Enriched Samples Isolated from SK-MEL-28 Cells Treated with Q10

2-D gel electrophoresis was performed on proteins solubilized from two aliquots of the SK-MEL-28 mitochondria enriched samples treated with 100 μM Q10 for 6, 19, and 48 hours (along with the corresponding mock vehicle controls). The samples were subjected to 2-D electrophoresis (in duplicate). Analysis of 525 protein spots in the

comparative study was performed, comparing the control samples against the other time point samples (Figure 11).

The nine statistically significant differential spot changes were selected from the comparative analysis of the 2-D electrophoresis gels. From these, 9 spots were excised and submitted for identification by trypsin digestion and mass spectrometry characterization

Table 4. Proteins identified to have a differential response to Q10 treatment in SKMEL-28 mitochondria.

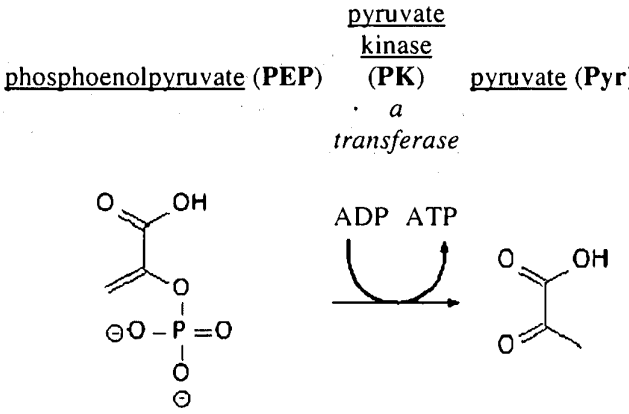
Spot #	Protein	Name	Function	Response (fold change)
11	Unknown protein	?	?	Up (1.3) at 6 hr, drop to low levels after this
131	Unknown, same as spot #11, modified	?	?	Down (1.3) at 6 hr, drops more for 19 and 48 hr
279	acyl-CoA thioesterase 7 isoform hBACHb	ACOT7	Cleaves fatty acyl-CoA's into free fatty acids and CoA	Down (1.3) at 6 hr, back to normal at 48 hr
372	Pyruvate kinase	PKM2	catalyzes the production of phosphoenolpyruvate from pyruvate and ATP	Up (1.5) at 6 hr, back to normal at 48 hr
110	ER60 protein	PDIA3	Protein disulfide isomerase	Up at 19 and 48 hr
185	Keratin 10	KRT10	intermediate filament	Up only at 19 hr
202	Beta-Actin		Structural protein	Up only at 19 hr
246	Malectin	MLEC	carbohydrate-binding protein of the endoplasmic reticulum and a candidate player in the early steps of protein N-glycosylation	Up only at 19 hr
75	Coiled-coil domain containing 58	CCDC58	Conserved hypothetical protein – nuclear pore forming	Up at 48 hr

Acyl-CoA thioesterase 7: Acyl-CoA thioesterase 7 (ACOT7) is a member of the enzyme family that catalyzes the hydrolysis of fatty acyl-CoA to free fatty acid and CoA. This enzyme thus has a role in the regulation of lipid metabolism and cellular signaling. ACOT7 has a preference for long-chain acyl-CoA substrates with fatty acid chains of 8-16 carbon atoms (C8-C16). The exact cellular function is ACOT7 is not fully

understood. The transcription of this gene is activated by sterol regulatory element-binding protein 2, thus suggesting a function in cholesterol metabolism.

The results in this Example indicate that ACOT7 is potentially involved in the metabolism of Q10, either directly or indirectly. Thus, targeting ACOT7 could facilitate modulation of intercellular levels of Q10 and thus impact cellular Q10 effects.

Pyruvate kinase: Pyruvate kinase is an enzyme involved in the last step of glycolysis. It catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP.



The protein is presumably that of PKM2, the type 2 isoform, as this was identified from the mitochondria enriched SK-MEL-28 sample. This isoform is well known to be involved in tumor cell formation and regulation.

Quantification of Q10 Levels in Mitochondria

A method for the simultaneous determination of Coenzyme Q10, (Q10) and the reduced form ubiquinol-10 (Q10H2) was implemented based upon a recently published method (Ruiz-Jimenez, 2007, J. Chroma A, 1175, 242-248) through the use of LC–MS–MS with electrospray ionization (ESI) in the positive mode. The highly selective identification and sensitive quantitation of both Q10 and Q10H2 is possible, along with the identification of other selected lipids. An aliquot of the mitochondrial enriched samples from SK-MEL-28 treated with 100 μM Q10 were subject to a conventional pre-

treatment based on protein precipitation, liquid–liquid extraction, evaporation to dryness and reconstitution with 95:5 methanol/hexane (v/v).

In this analysis, Q10, Q10H2, and Q9 were quantitated (Table 5). The levels of the related molecule Q9 were low, and near the level of detection. The level of the untreated samples were relatively consistent, with the 6 hour Q10 treated sample having this same level. To control for sample variance in total material, the levels of cholesterol was also measured to confirm that the differences were not due to sample size errors. When the Q10 levels were corrected against total protein values obtained by protein extraction other aliquots of the same mitochondrial preps, the relative ratios were comparative. Thus, a significant increase in Q10 levels was obtained at 19 hours (~3-fold) with an even larger increase by the 48 hour time point (~ 6-fold) (Figure 12).

Table 5. HPLC-MS Quantification results for the levels of Q10 present in mitochondrial enriched samples from SK-MEL-28 cells treated with 100 μM Q10 in the media.

			Peak Area		ng/Sample			μg/sample
File	Sample	Injection	Q9	Q10	Q9	Q10	$Q10H_2$	Cholesterol
081204-05	100 ng Std		245,342	352792				
081204-06	6 hr mock#1	10%	2560	32649	1.04	9.25		
081204-07	Solvent Blank#1	5 ul	3781	3174	1.54	0.9		
081204-08	Solvent Blank#2	5 ul	2396	4399	0.98	1.25		
081204-09	6 hr mock#2	20%	1572	36328	0.64	10.3		
081204-10	Solvent Blank#3	10 ul	1722	2504	0.7	0.71		
081204-11	48 hr Q10 treated	20%	4879	164496	1.99	[illegible]	0.28	13.86
081204-12	48 hr mock	20%	2412	25552	0.98	7.24	[illegible]	[illegible] 13.04
081204-13	6 hr Q10 treated	20%	692	25427	0.28	7.21		
081204-14	19 hr Q10 treated	20%	1161	59164	0.47	16.77		
081204-15	19 hr mock	20%	901	19999	0.37	5.67		

A surprising result from this study was the finding that the Q10 was supplied to the cells as the oxidized form. For the 48 hour samples, the reduced form Q10H2 was also measured and found to be present in significantly lower amounts (0.28 ng/sample of CoQ10H2 as compared to 46.63 ng/sample of CoQ10). There was a general increase (3-fold) in the levels of Q10H2 in the Q10 treated 48 hour sample, although the levels were near the presumed detection limit of the assay. Interestingly, the oxidized form (Q10)

can act as a pro-oxidant in biological systems. According to the literature, when human plasma was evaluated for Q10 and Q10H2, the majority (90%) of the molecule was found in the reduced form of Q10H2 (Ruiz-Jimenez, 2007, J. Chroma A, 1175, 242-248) which can act as an anti-oxidant.

Thus, these results confirm and quantitate that the levels of Q10 increase in the mitochondria upon the exogenous addition of Q10 to the media. A surprising and unexpected discovery was that Q10 was maintained in the supplied oxidized form (pro-oxidant) and not converted to the reduced (anti-oxidant) form of Q10H2 in any significant amounts.

EXAMPLE 6: Real-Time PCR Arrays

Experiment 1: Apoptosis Array

As discussed above in Example 3, exposure of cancer cells to Q10 induces a portion of these cells to die due to apoptotic processes. To identify proteins that were involved in the Q10 response, real-time polymerase chain reaction (RT-PCR) methods were employed to identify changes in the level of mRNA for genes/proteins involved in targeted pathway arrays for apoptosis.

Using PCR arrays as a screening tool, a spectrum of molecular targets that would potentially offer an insight to the mode of biological action of Q10 within the cells were thus evaluated. Changes in mRNA levels were evaluated using real-time PCR quantification to assess mRNA levels in pre-selected subsets containing 80 pathway specific targets.

For the interpretation of mRNA results, the genes that were altered in their mRNA transcription by a two-fold level were identified and evaluated. The level of gene transcription to produce mRNA only provides a rough estimate of potential changes in the level of the expressed protein. The skilled artisan will appreciate that each mRNA may have different rates at which it is degraded or its translation inefficiently, thus resulting in differing amounts of protein.

SkBr-3 cells treated with 50um Q10 for 24 hours

The assay method of RT-PCR was utilized to provide a measure of mRNA level changes to a total of 84 apoptotic pathway related proteins. The experiments with the real-time PCR apoptosis analysis on SkBr3 with Q10 (24 hr) identified the following mRNA's being affected: Bcl2, Bcl2L1, Bcl2L11, Birc6, Bax, Xiap, Hprt1, Apaf1, Abl1, Braf. These results again provided supporting evidence for the apoptotic response of cancer cells to Q10 treatment.

Table 6A

Symbol	Up-Down Regulation	Unigene	Refseq	Description	Gname
BCL2L1	13.1957	Hs.516966	NM_138578	BCL2-like 1	BCL-XL/S
BNIP2	6.3291	Hs.646490	NM_004330	BCL2/adenovirus E1B 19kDa interacting protein 2	BNIP-2/NIP2
BCL2	5.4717	Hs.150749	NM_000633	B-cell CLL/lymphoma 2	Bcl-2
BIRC6	4.7966	Hs.150107	NM_016252	Baculoviral IAP repeat-containing 6 (apollon)	APOLLON/BRUCE
BCL2L11	4.6012	Hs.469658	NM_006538	BCL2-like 11 (apoptosis facilitator)	BAM/BIM
XIAP	4.3832	Hs.356076	NM_001167	X-linked inhibitor of apoptosis	API3/BIRC4
BRAF	4.3832	Hs.550061	NM_004333	V-raf murine sarcoma viral oncogene homolog B1	B-raf 1/BRAF1
BAX	3.896	Hs.631546	NM_004324	BCL2-associated X protein	Bax zeta
APAF1	2.6244	Hs.708112	NM_001160	Apoptotic peptidase activating factor 1	CED4/DKFZp781B1145
HPRT1	-160.6748	Hs.412707	NM_000194	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	HGPRT/HPRT

Results that are consistent from three independent experiments from SK-MEL-28 cells are summarized below in Table 6B. Many genes are regulated in SCC cells as well with 100 μM Q10 treatment. The genes in the Apoptosis array that appear to be regulated in SCC cells are described in Table 7. We find that many genes are regulated at 6 hours, both in SK-MEL-28 cells and in SCC cells. By 24 hours, the regulation is decreased. Genes that appear to be regulated in both SK-MEL-28 cells and in SCC cells are described in Table 8.

Table 6B. Genes in SK-MEL-28 cells regulated by 100 μM Q10 treatment when analyzed by the Apoptosis Array.

Symbol	Description	Regulation	Location	Possible Functions
ABL1	C-abl oncogene 1, receptor tyrosine kinase	Down Regulated at 72 hours	Nucleus	Tyrosine Kinase
BAG1	BCL2-associated athanogene	Up Regulated at 48 hours	Cytoplasm	Anti-apoptotic, glucocorticoid receptor pathway
BCL2	B-cell CLL/lymphoma 2	Down Regulated at 48 hours	Cytoplasm	Cell death
BCL2A1	BCL2-related protein A1	Down Regulated at 48 hours	Cytoplasm	Regulates Caspases, phosphorylates TP73
BCL2L1	BCL2-like 1	Down Regulated at 72 hours	Cytoplasm	Caspase Inhibitor
BCL2L10	BCL2-like 10 (apoptosis facilitator)	Down Regulated at 48 hours	Cytoplasm	Caspase Activator
BCL2L11	BCL2-like 11 (apoptosis facilitator)	Down Regulated at 48 hours	Cytoplasm	Pro-Apoptotic, Caspase3 Activator
BIRC3	Baculoviral IAP repeat-containing 3	Down Regulated at 6 hours	Cytoplasm	Anti-apoptotic
BIRC8	Baculoviral IAP repeat-containing 8	Down Regulated at 48 hours	Cytoplasm	Activates Caspase
CARD8	Caspase recruitment domain family, member 8	Down Regulated at 48 hours	Nucleus	Caspase Activator
CASP14	Caspase 14, apoptosis-related cysteine peptidase	Down Regulated at 48 hours	Cytoplasm	Apoptosis related cysteine peptidase
CASP5	Caspase 5, apoptosis-related cysteine peptidase	Down Regulated at 48 hours	Cytoplasm	Apoptosis related cysteine peptidase
CD40LG	CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)	Down Regulated at 48 hours	Extracellular Space	CD40 receptor binding
CIDEA	Cell death-inducing DFFA-like effector a	Up Regulated at 48 hours	Cytoplasm	Pro-Apoptotic
FADD	Fas (TNFRSF6)-associated via death domain	Down Regulated at 6 hours	Cytoplasm	Pro-Apoptotic
FAS	Fas (TNF receptor superfamily, member 6)	Up Regulated at 48 hours	Plasma Membrane	Pro-Apoptotic

FASLG	Fas ligand (TNF superfamily, member 6)	Down Regulated at 48 hours	Extracellular Space	Pro-Apoptotic
GADD45A	Growth arrest and DNA-damage-inducible, alpha	Up Regulated at 48 hours	Nucleus	Growth Arrest
HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	Down Regulated at 48 hours	Cytoplasm	Pro-Apoptotic
PYCARD	PYD and CARD domain containing	Down Regulated at 6 hours	Cytoplasm	Apoptotic Protease Activator
TNF	Tumor necrosis factor (TNF superfamily, member 2)	Up Regulated at 48 hours then down regulated	Extracellular Space	TNF receptor binding
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	Up Regulated at 48 hours then down regulated	Plasma Membrane	Caspase Activator
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	Down Regulated at 72 hours	Plasma Membrane	p53 signaling, caspase activation.
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Down Regulated at 72 hours	Plasma Membrane	Pro-apoptotic
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	Down Regulated at 48 hours	Plasma Membrane	Activates Caspase
CD27	CD27 molecule	Down Regulated at 48 hours	Plasma Membrane	Caspase Inhibitor
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	Down Regulated at 48 hours	Plasma Membrane	Pro-apoptotic
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	Upregulated at 48 hours	Extracellular Space	Pro-apoptotic
TP73	Tumor protein p73	Down Regulated at 48 hours	Nucleus	Transcription factor
TRAF3	TNF receptor-associated factor 3	Down Regulated at 48 hours	Cytoplasm	Zinc-finger domain
TRAF4	TNF receptor-associated factor 4	Down Regulated at 48 hours	Cytoplasm	Zinc-finger domain

Table 7. Genes in SCC cells that are regulated by 100 μM Q10 treatment when analyzed by the Apoptosis Array.

Symbol	Description	Regulation.
AKT1	V-akt murine thymoma viral oncogene homolog 1	Down regulated at 6 hours and then up regulated at 24 hours.
BAG4	BCL2-associated athanogene 4	Up regulated at 24 hours.
BAX	BCL2-associated X protein	Up regulated at 24 hours.
BCL2	B-cell CLL/lymphoma 2	Up regulated at 24 hours.
BCL2L1	BCL2-like 1	Down regulated at 6 hours and then up regulated at 24 hours.
BIRC3	Baculoviral IAP repeat-containing 3	Down regulated at 6 hours.
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	Down regulated at 24 hours.
CARD6	Caspase recruitment domain family, member 6	Down regulated at 6 hours.
CASP6	Caspase 6, apoptosis-related cysteine peptidase	Up regulated at 24 hours.
CASP7	Caspase 7, apoptosis-related cysteine peptidase	Up regulated at 24 hours.
CD40	CD40 molecule, TNF receptor superfamily member 5	Down regulated at 6 hours.
FADD	Fas (TNFRSF6)-associated via death domain	Up regulated at 24 hours.
GADD45A	Growth arrest and DNA-damage-inducible, alpha	Up regulated at 24 hours.
HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	Up regulated at 24 hours.
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	Down regulated at 6 hours.
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	Down regulated at 6 hours and then up regulated at 24 hours.
CD27	CD27 molecule	Down regulated at 6 hours.
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	Down regulated at 6 hours.
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	Up regulated at 24 hours.
CD70	CD70 molecule	Down regulated at 6 hours.
TP53	Tumor protein p53	Up regulated at 24 hours.
TP73	Tumor protein p73	Down regulated at 6 hours and then up regulated at 24 hours.
TRAF2	TNF receptor-associated factor 2	Up regulated at 24 hours.

5

Table 8. Genes from the apoptosis array regulated with 100 μM Q10 treatment in both SK-MEL-28 and SCC cells.

Symbol	Description
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1 (Bcl-xl)
BIRC3	Baculoviral IAP repeat-containing 3
FADD	Fas (TNFRSF6)-associated via death domain
GADD45A	Growth arrest and DNA-damage-inducible, alpha
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21
CD27	CD27 molecule
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
TP73	Tumor protein p73
TRAF2	TNF receptor-associated factor 2

Interestingly, the altered mRNA levels showed a significant up-regulation in a series of apoptitic proteins, with Bcl-xl one of the highest. This was also observed in the protein array experiments on SK-MEL-28 cells.

Bcl-xl is a transmembrane molecule in the mitochondria (Bcl-xl stands for "Basal cell lymphoma-extra large"). It is involved in the signal transduction pathway of the FAS-L and is one of several anti-apoptotic proteins which are members of the Bcl-2 family of proteins. It has been implicated in the survival of cancer cells. However, it is known that alternative splicing of human Bcl-x mRNA may result in at least two distinct Bcl-x mRNA species, Bcl-xL and Bcl-xS. The predominant protein product (233 amino acids) is the larger Bcl-x mRNA, Bcl-xL, which inhibits cell death upon growth factor withdrawal (Boise et al., 1993 . Cell 74, 597-608). Bcl-xS, on the other hand, inhibits the ability of Bcl-2 to inhibit cell death and renders cells more susceptible to apoptotic cell death. The employed assays utilized do not distinguish which isoform of Bcl-x is being upregulated. The Bcl-x isoform being upregulated by CoQ10 in these studies may be determined by routine methods known in the art, e.g., by using RT-PCR methods to evaluate the ratio of the two mRNA splicing isoforms (Bcl-xL vs Bcl-sL).

From the survey of apoptotic related proteins it was observed multiple pro- and anti-apoptotic factors were in the BCL-2 family or that interact with these factors have modulated expression levels (BCL2L11, BNIP2, BAG1, HRK, BAK1, BCL2, BCL2L1). These proteins govern mitochondrial outer membrane permeabilization.

An early marker for apoptotic response is observed with the upregulation of Caspase-9 (16 hour) which is consistent with previous observations of apoptosis with

caspase 3/7 proteins. Induction of stress signaling pathways causes release of cytochrome c from mitochondria and activation of apaf-1 (apoptosome), which in turn cleaves the pro-enzyme of caspase-9 into the active form. Once intiated caspase-9 goes on to cleave procaspase-3 & procaspase-7 to trigger additional apoptotic pathways.

There is also a consistent linkage to the tumor necrosis factor receptor family of proteins being modulated.

A strong down regulation of tumor protein p73 is also noted. Analyses of many tumors typically found in humans including breast and ovarian cancer show a high expression of p73 when compared to normal tissues in corresponding areas. Recent finding are suggesting that deregulated over expression of transcription factors within the body involved in cell cycle regulation and synthesis of DNA in mammalian cells (i.e.: E2F-1), induces the expression of p73. The suggestion is that p73 may be an oncoprotein, but may involve different mechanism that the related p53 protein. A schematic showing mapping of the apoptosis pathway is provided in Figure 13.

SKMEL-28 Cells

From the survey of apoptotic related proteins it was observed multiple pro- and anti-apoptotic factors were in the BCL-2 family or that interact with these factors have modulated expression levels (BCL2L11, BNIP2, BAG1, HRK, BAK1, BCL2, BCL2L1). These proteins govern mitochondrial outer membrane permeabilization.

An early marker for apoptotic response is observed with the upregulation of Caspase-9 (16 hour) which is consistent with previous observations of apoptosis with caspase 3/7 proteins. Induction of stress signaling pathways causes release of cytochrome c from mitochondria and activation of apaf-1 (apoptosome), which in turn cleaves the pro-enzyme of caspase-9 into the active form. Once intiated caspase-9 goes on to cleave procaspase-3 & procaspase-7 to trigger additional apoptotic pathways.

Table 9. Changes in mRNA levels for SKMEL-28 cells treated with 100 μM A10, evaluated by RT-PCR arrays focused around apoptotic pathways.

Refseq	Description	Symbol	6 hr Q10	16 hr Q10	24 hr Q10	72 hr Q10
NM_006538	BCL2-like 11 (apoptosis facilitator)	BCL2L11	2.13	2.41	1.92	2.51
NM_000875	Insulin-like growth	IGF1R	1.77	1.09	1.33	1.25

	factor 1 receptor					
NM_004048	Beta-2-microglobulin	B2M	1.74	1.76	1.58	3.11
NM_003921	B-cell CLL/lymphoma 10	BCL10	1.55	1.87	1.48	-3.11
NM_004330	BCL2/adenovirus E1B 19kDa interacting protein 2	BNIP2	1.46	1.51	1.57	-1.61
NM_005157	C-abl oncogene 1, receptor tyrosine kinase	ABL1	1.42	2.77	-1.22	-2.03
NM_004323	BCL2-associated athanogene	BAG1	1.41	1.44	-1.61	-2.45
NM_001229	Caspase 9, apoptosis-related cysteine peptidase	CASP9	1.32	3.96	1.83	1.14
NM_003806	Harakiri, BCL2 interacting protein (contains only BH3 domain)	HRK	1.18	4.52	2.73	-1.14
NM_001924	Growth arrest and DNA-damage-inducible, alpha	GADD45A	1.07	3.34	1.13	-2.36
NM_001188	BCL2-antagonist/killer 1	BAK1	1.06	2.73	-1.00	-4.54
NM_004295	TNF receptor-associated factor 4	TRAF4	-1.91	2.63	-1.58	-740.66
NM_003842	Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	-2.07	1.53	-1.81	-710.49
NM_000633	B-cell CLL/lymphoma 2	BCL2	-2.98	-1.63	-2.82	-11.36
NM_001242	CD27 molecule	CD27	-3.40	-2.38	-1.35	-12.72
NM_014430	Cell death-inducing DFFA-like effector b	CIDEB	-3.48	1.56	-3.69	-2.59
NM_001065	Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	-4.53	2.28	-3.30	1.22
NM_005427	Tumor protein p73	TP73	-4.66	-9.80	-8.71	-26.96
NM_003844	Tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	-4.84	-5.26	-4.33	-11.84
NM_138578	BCL2-like 1	BCL2L1	-4.94	-1.80	-6.17	-7.04
NM_001165	Baculoviral IAP repeat-containing 3	BIRC3	-13.68	-1.98	-2.42	-3.42

There is a consistent linkage to the tumor necrosis factor receptor family of proteins being modulated.

A strong down regulation of tumor protein p73 is also noted. Analyses of many tumors typically found in humans including breast and ovarian cancer show a high expression of p73 when compared to normal tissues in corresponding areas. Recent finding are suggesting that deregulated over expression of transcription factors within

the body involved in cell cycle regulation and synthesis of DNA in mammalian cells (i.e.: E2F-1), induces the expression of p73. The suggestion is that p73 may be an oncoprotein, but may involve different mechanism that the related p53 protein

Experiment 2: Real-time PCR Arrays using Oxidative Stress and Antioxidant defense Array

To identify proteins that were involved in the Q10 response, real-time polymerase chain reaction (RT-PCR) methods were employed to identify changes in the level of mRNA's for genes/proteins involved in targeted pathway arrays for oxidative stress and antioxidant defense.

Table 10 below lists the genes that are regulated in SK-MEL28 cells with 100 μM Q10 treatment. Results are given only for those genes that are regulated in two independent experiments. Although there is a significant amount of gene regulation seen at 6 hours, most significant changes in RNA levels are seen at 48 hours.

Table 10. Genes in SK-MEL-28 cells that are regulated by 100 μM Q10 treatement as seen in the Oxidative Stress and Antioxidant Defense Arrays.

Symbol	Description	Regulation	Location	Possible Functions.
ALB	Albumin	Down Regulation at 48 hours	Extracellular space	Carrier protein, anti-apoptotic
AOX1	Aldehyde oxidase 1	Up regulation from 16 hours	Cytoplasm	Produces free radicals, drug metabolic process.
APOE	Apolipoprotein E	Down Regulation at 48 hours	Extracellular space	Lipid metabolism
ATOX1	ATX1 antioxidant protein 1 homolog (yeast)	Down Regulation at 48 hours	Cytoplasm	Copper metabolism
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	Down Regulation at 48 hours	Cytoplasm	Anti-apoptotic
CSDE1	Cold shock domain containing E1, RNA-binding	Down Regulation at 48 hours	Cytoplasm	Transcriptional regulation.
CYBA	Cytochrome b-245, alpha polypeptide	Down Regulation at 48 hours	Cytoplasm	Apoptotic,
CYGB	Cytoglobin	Down Regulation at 48 hours	Cytoplasm	Peroxidase, Transporter.
DHCR24	24-dehydrocholesterol reductase	Down Regulation at 6 hours	Cytoplasm	Electron carrier, binds to TP53, involved in apoptosis.
DUOX1	Dual oxidase 1	Up Regulation at 48 hours	Plasma Membrane	Calcium ion binding, electron carrier.
DUOX2	Dual oxidase 2	Down Regulation at 48 hours	Unknown	Calcium ion binding.
EPHX2	Epoxide hydrolase 2, cytoplasmic	Down Regulation at 48 hours	Cytoplasm	Arachidonic acide metabolism.
EPX	Eosinophil peroxidase	Down Regulation at 48 hours	Cytoplasm	Phenyl alanine metabolism, apoptosis.

GPX2	Glutathione peroxidase 2 (gastrointestinal)	Down Regulation at 48 hours	Cytoplasm	Electron carrier, binds to TP53, involved in apoptosis.
GPX3	Glutathione peroxidase 3 (plasma)	Up Regulation at 48 hours	Extracellular space	Arachidonic acid metabolims, up regulated in carcinomas.
GPX5	Glutathione peroxidase 5 (epididymal androgen-related protein)	Up Regulation at 48 hours	Extracellular space	Arachidonic acid metabolism.
GPX6	Glutathione peroxidase 6 (olfactory)	Down Regulation at 48 hours	Extracellular space	Arachidonic acid metabolism.
GSR	Glutathione reductase	Down Regulation at 48 hours	Cytoplasm	Glutamate and glutathione metabolism, apoptosis.
GTF2I	General transcription factor II, i	Down Regulation at 6 hours	Nucleus	Transcriptional activator, transcription of fos.
KRT1	Keratin 1 (epidermolytic hyperkeratosis)	Up Regulation at 48 hours	Cytoplasm	Sugar Binding.
LPO	Lactoperoxidase	Down Regulation at 48 hours	Extracellular space	Phenyl alanine metabolism.
MBL2	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	Down Regulation at 48 hours	Extracellular space	Complement signaling, pattern recognition in receptors.
MGST 3	Microsomal glutathione S-transferase 3	Upregulation at 16 hours	Cytoplasm	Xenobiotic metabolism.
MPO	Myeloperoxidase	Down Regulation at 48 hours	Cytoplasm	Anti-apoptotic, phenyl alanine metabolism.
MPV1 7	MpV17 mitochondrial inner membrane protein	Down Regulation at 6 hours	Cytoplasm	Maintenance of mitochondrial DNA.
MT3	Metallothionein 3	Down Regulation at 48 hours	Cytoplasm	Copper ion binding.
NCF1	Neutrophil cytosolic factor 1, (chronic granulomatous disease, autosomal 1)	Down Regulation from 6 hours	Cyoplasm	Produces free radicals.
NCF2	Neutrophil cytosolic factor 2 (65kDa, chronic granulomatous disease, autosomal 2)	Up Regulation at 48 hours	Cytoplasm	Electron carrier.
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	Down Regulation at 48 hours	Unknown	Kinase, Purine and pyrimidine metabolism.
NOS2 A	Nitric oxide synthase 2A (inducible, hepatocytes)	Down Regulation at 48 hours	Cytoplasm	Glucocorticoid receptor signaling, apoptosis.
OXR1	Oxidation resistance 1	Down Regulation at 48 hours	Cytoplasm	Responds to oxidative stress.
PDLIM 1	PDZ and LIM domain 1 (elfin)	Up Regulation at 48 hours	Cytoplasm	Transcriptional activator.
PIP3-E	Phosphoinositide-binding protein PIP3-E	Down Regulation at 48 hours	Cytoplasm	Peroxidase.
PRDX 2	Peroxiredoxin 2	Down Regulation at 6 hours	Cytoplasm	Role in phenyl alanine metabolism. Role in cell death.
PRDX 4	Peroxiredoxin 4	Down Regulation from 24 hours	Cytoplasm	Thioredoxin peroxidase.
PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	Down Regulation at 48 hours	Cytoplasm	Forms oxygen free radicals.
PRG3	Proteoglycan 3	Down Regulation at 48 hours	Extracellular space	Role in cell death.
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Down Regulation at 48 hours	Cytoplasm	arachidonic acid metabolism, prostaglandin synthesis.

PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Up Regulation at 48 hours	Cytoplasm	arachidonic acid metabolism, prostaglandin synthesis.
PXDN	Peroxidasin homolog (Drosophila)	Up Regulation at 48 hours	Unknown	binds to TRAF4, calcium ion binding, iron ion binding.
PXDNL	Peroxidasin homolog (Drosophila)-like	Down Regulation at 48 hours	Unknown	peroxidase, calcium ion binding, iron ion binding.
RNF7	Ring finger protein 7	Up Regulation at 16 hours	Nucleus	apoptotic, copper ion binding, ubiquitin pathway.
SGK2	Serum/glucocorticoid regulated kinase 2	Down Regulation at 48 hours	Cytoplasm	Kinase, potasium channel regulator.
SIRT2	Sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae)	Up regulation at 16 hours	Nucleus	Transcription factor.
SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	Up Regulation at 16 hours	Cytoplasm	Apoptotic, Caspase Activator.
SOD2	Superoxide dismutase 2, mitochondrial	Up regulation at 16 hours	Cytoplasm	Apoptotic, Regulated by TNF.
SOD3	Superoxide dismutase 3, extracellular	Down Regulation at 48 hours	Extracellular space	Pro-apoptotic
SRXN1	Sulfiredoxin 1 homolog (S. cerevisiae)	Down Regulation at 48 hours	Cytoplasm	DNA binding, oxidoreductase
TPO	Thyroid peroxidase	Down Regulation at 48 hours	Plasma Membrane	iodination of thyroglobulin, tyrosine metabolism, phenylalanine metabolism.
TTN	Titin	Down Regulation at 48 hours	Cytoplasm	Actin cytoskeleton signaling, integrin signaling
TXNDC2	Thioredoxin domain-containing 2 (spermatozoa)	Down Regulation at 48 hours	Cytoplasm	Pyrimidine metabolism

The Neutrophil cytosolic factor 2 (NCF2, 65kDa, chronic granulomatous disease, autosomal 2) was one of the initial top induced mRNA's (observed at 6 hours). Subsequently at the 16 hour time point and onward, Neutrophil cytosolic factor 1 (NCF1) (chronic granulomatous disease, autosomal 1) was induced at very high levels after an initial lag phase.

Neutrophil cytosolic factor 2 is the cytosolic subunit of the multi-protein complex known as NADPH oxidase commonly found in neutrophils. This oxidase produces a burst of superoxide which is delivered to the lumen of the neutrophil phagosome.

The NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) is a membrane-bound enzyme complex. It can be found in the plasma membrane as well as in the membrane of phagosome. It is made up of six subunits. These subunits are: a Rho guanosine triphosphatase (GTPase), usually Rac1 or Rac2 (Rac stands for Rho-related C3 botulinum toxin substrate)

- Five "phox" units. (Phox stands for phagocytic oxidase.)
  - P91-PHOX (contains heme)
  - p22phox
  - p40phox
  - p47phox (NCF1)
  - p67phox (NCF2)

It is noted that another NADPH oxidase levels do not change. The enzyme is NOX5, which is a novel NADPH oxidase that generates superoxide and functions as a H+ channel in a Ca(2+)-dependent manner

In addtition Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1(PREX1) was also upregulated. This protein acts as a guanine nucleotide exchange factor for the RHO family of small GTP-binding proteins (RACs). It has been shown to bind to and activate RAC1 by exchanging bound GDP for free GTP. The encoded protein, which is found mainly in the cytoplasm, is activated by phosphatidylinositol-3,4,5-trisphosphate and the beta-gamma subunits of heterotrimeric G proteins.

The second major early induced protein was Nitric oxide synthase 2A (inducible, hepatocytes) (NOS2A). Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. This gene encodes a nitric oxide synthase which is expressed in liver and is inducible by a combination of lipopolysaccharide and certain cytokines.

Superoxide dismutase 2, mitochondrial (SOD2) is a member of the iron/manganese superoxide dismutase family. It encodes a mitochondrial protein that forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. Mutations in this gene have been associated with idiopathic cardiomyopathy (IDC), premature aging, sporadic motor neuron disease, and cancer.

An example of a down regulated protein is Forkhead box M1 (FOXM1), which is known to play a key role in cell cycle progression where endogenous FOXM1 expression peaks at S and G2/M phases. Recent studies have shown that FOXM1, regulates expression of a large array of G2/M-specific genes, such as Plk1, cyclin B2,

Nek2 and CENPF, and plays an important role in maintenance of chromosomal segregation and genomic stability. The FOXM1 gene is now known as a human proto-oncogene. Abnormal upregulation of FOXM1 is involved in the oncogenesis of basal cell carcinoma (BCC). FOXM1 upregulation was subsequently found in the majority of solid human cancers including liver, breast, lung, prostate, cervix of uterus, colon, pancreas, and brain. Further studies with BCC and Q10 should evaluate FOXM1 levels.

SKMEL-28 Cells

Further experiments were carried out using SKMEL-28 cells. The level of mRNA present in SKMEL-28 cells treated with 100 μM Q10 were compared to the levels in untreated cells at various time points using real-time PCR methods (RT-PCR). The PCR array (SABiosciences) is a set of optimized real-time PCR primer assays on 96-well plates for pathway or disease focused genes as well as appropriate RNA quality controls. The PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray.

Table 11. Listing and classification of mRNA levels evaluated in the Oxidative Stress and Antioxidant Defense PCR Array. After six hours of treatment with 100 μM Q10 on SKMEL-28 cells, the largest changes to the mRNA levels are indicated by highlighting the protein code (increased – bold; decreased – underlined; or no change – grey).

Antioxidants:

Glutathione Peroxidases (GPx): GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GSTZ1.

Peroxiredoxins (TPx): PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, PRDX6.

Other Peroxidases: CAT, CSDE1, CYGB, DUOX1, DUOX2, EPX, **GPR156**, LPO, MPO, PIP3-E, PTGS1, PTGS2, PXDN, PXDNL, TPO, TTN.

Other Antioxidants: ALB, APOE, GSR, MT3, SELS, SOD1, SOD3, SRXN1, TXNDC2, TXNRD1, TXNRD2.

Genes Involved in Reactive Oxygen Species (ROS) Metabolism:

Superoxide Dismutases (SOD): SOD1, SOD2, SOD3.

Other Genes Involved in Superoxide Metabolism: ALOX12, CCS, CYBA, DUOX1, DUOX2, EIF2A, MT3, NCF1, **NCF2, NOS2A**, NOX5, PREX1, PRG3.

Other Genes Involved in ROS Metabolism: AOX1, BNIP3, EPHX2, MPV17, SFTPD.

Oxidative Stress Responsive Genes: ANGPTL7, APOE, ATOX1, CAT, CCL5, CSDE1, CYGB, DGKK, DHCR24, DUOX1, DUOX2, DUSP1, EPX, FOXM1, GLRX2, **GPR156**, GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GSS, KRT1, LPO, MBL2, MPO, MSRA, MTL5, NME5, NUDT1, OXR1, OXSR1, PDLIM1, PIP3-E, PNKP, PRDX2, PRDX5, PRDX6, PRNP, RNF7, SGARA3, SELS, SERP1, SGK2, SIRT2, SOD1, SOD2, SRXN1, STK25, TPO, TTN, TXNRD2.

Table 12. Time course evaluation of 100 μM treatment of SKMEL-28. The mRNA level changes were monitored by RT-PCR methods and oxidative stress and antioxidant defense proteins array was evaluated.

Refseq	Symbol	Description	6 hr Q10	16 hr Q10	24 hr Q10	48 hr Q10	72 hr Q10
NM_000265	NCF1	Neutrophil cytosolic factor 1, (chronic granulomatous disease, autosomal 1)	0	high	3.3829	15.7838	31.5369
NM_012423	RPL13A	Ribosomal protein L13a	-0.9025	3.1857	2.5492	4.9253	7.82
NM_020820	PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	-3.2971	2.867	0.3222	6.3719	7.476
NM_012237	SIRT2	Sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae)	-0.9025	4.0829	4.4766	5.7166	6.6257
NM_005125	CCS	Copper chaperone for superoxide dismutase	-0.6206	3.0077	3.452	2.9801	6.1539
NM_181652	PRDX5	Peroxiredoxin 5	-2.995	3.0454	3.5381	4.7955	6.0169
NM_016276	SGK2	Serum/glucocorticoid regulated kinase 2	0	0	0	0.5995	5.937
NM_003551	NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	-0.6652	3.1138	3.3694	3.1549	5.782
NM_004417	DUSP1	Dual specificity phosphatase 1	-0.6998	0.5902	2.7713	3.321	5.5375
NM_001752	CAT	Catalase	-0.8589	2.8424	0.1046	3.8557	5.3988
NM_000041	APOE	Apolipoprotein E	-0.8212	3.2069	-0.9543	3.7694	5.3315
NM_000101	CYBA	Cytochrome b-245, alpha polypeptide	-0.3945	4.3475	3.9208	6.2452	5.0762
NM_000433	NCF2	Neutrophil cytosolic factor 2 (65kDa, chronic granulomatous disease, autosomal 2)	1.2266	3.0077	0.0954	5.476	0
NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-0.6912	2.7046	2.6552	4.0553	-3.3022
NM_183079	PRNP	Prion protein (p27-30) (Creutzfeldt-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	-0.2144	3.5236	2.9086	5.0837	-3.9396
NM_004052	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-2.9376	3.3288	4.312	-18.2069	-4.8424
NM_000242	MBL2	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	-0.3622	-1.9072	-3.0142	-1.1854	-6.4544
NM_021953	FOXM1	Forkhead box M1	-0.8135	0.068	-0.9216	3.3655	-10.0953

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- a Rho guanosine triphosphatase (GTPase), usually Rac1 or Rac2 (Rac stands for Rho-related C3 botulinum toxin substrate)
- Five "phox" (phagocytic oxidase) units.
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mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. This gene encodes a nitric oxide synthase which is expressed in liver and is inducible by a combination of lipopolysaccharide and certain cytokines.

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Experiment 3: Real-Time PCR Arrays using Heat Shock Array

Heat Shock Arrays were run for SCC cells and the data of regulated genes is summarized below in Table 13.

Table 13. Genes from the Heat Shock Protein array regulated with 100 μM Q10 treatment in SCC cells.

Symbol	Description	Regulation.	Location.	Possible functions.
CCT6B	Chaperonin containing TCP1, subunit 6B (zeta 2)	Down regulated at 24 hours	Cytoplasm	Protein folding and protein complex assembly.
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	Up regulated at 6 hours.	Nucleus	Responds to DNA damage and changes in protein folding.
DNAJB13	DnaJ (Hsp40) related, subfamily B, member 13	Down regulated at 6 hours.	Unknown	Protein folding and apoptosis.
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	Down regulated at 6 hours.	Unknown	Binds to HSP, involved in protein folding and in protein complex assembly.
DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	Down regulated at 6 hours.	Unknown	Binds to HSP, involved in protein folding and in protein complex assembly.
DNAJC4	DnaJ (Hsp40) homolog, subfamily C, member 4	Down regulated at 6 hours.	Cytoplasm	Binds to HSP, involved in protein folding and in protein complex assembly.

DNAJC5B	DnaJ (Hsp40) homolog, subfamily C, member 5 beta	Down regulated at 6 hours.	Unknown	Involved in protein folding responds to changes in protein folding.
HSPA8	Heat shock 70kDa protein 8	Up regulated at 6 hours.	Cytoplasm	Regulates TNF, binds BAG1, STUB1, TP53, involved in apoptosis.
HSPH1	Heat shock 105kDa/110kDa protein 1	Up regulated at 6 hours.	Cytoplasm	Binds to HSPA8, important for protein folding, responds to protein unfolding and stress.

Experiment 4: Real-Time PCR Arrays using Diabetes Array

The experiments described in this example were performed to test the overall hypothesis that Q10 would have an impact on multiple genes and alter the metabolic state of a cell. The mRNA from SKMEL-28 cells treated with 100 μM Q10 was evaluated by RT-PCR against a panel of target proteins involved in diabetes and related pathways. Results from this experiment demonstrate that several proteins involved in glycolyic pathways and insulin processing are altered in their mRNA expression levels (summarized in Table 14).

Table 14. Major mRNA level changes to SKMEL-28 cells treated with 100 μM Q10 for 16 hours.

Refseq	Description	Symbol	Fold Change after 16 hours (100 μM Q10)
NM_000162	Glucokinase (hexokinase 4)	GCK	8.5386
NM_178849	Hepatocyte nuclear factor 4, alpha	HNF4A	8.421
NM_005249	Forkhead box G1	FOXG1	4.6396
NM_000599	Insulin-like growth factor binding protein 5	IGFBP5	2.2721
NM_001101	Actin, beta	ACTB	-2.0936
NM_002863	Phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	PYGL	-2.65
NM_001065	Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	-2.8011
NM_021158	Tribbles homolog 3 (Drosophila)	TRIB3	-2.8011
NM_003749	Insulin receptor substrate 2	IRS2	-2.9404
NM_004578	RAB4A, member RAS oncogene	RAB4A	-3.1296

	family		
NM_004176	Sterol regulatory element binding transcription factor 1	SREBF1	-3.5455
NM_004969	Insulin-degrading enzyme	IDE	-4.4878
NM_005026	Phosphoinositide-3-kinase, catalytic, delta polypeptide	PIK3CD	-6.8971
NM_000208	Insulin receptor	INSR	-8.6099
NM_003376	Vascular endothelial growth factor A	VEGFA	-15.5194
NM_001315	Mitogen-activated protein kinase 14	MAPK14	-74.3366

The results of this initial experiment show that the mRNA levels for a variety of insulin related proteins were modulated in both directions. The results indicate that Q10 would have an impact on diabetic disease treatment and/or evaluation.

Further experiments werenext conducted to confirm the results above obtained from SK-MEL-28 cells treated with Q10. Many of the genes in SK-MEL-28 cells are regulated as early as 6 hours after Q10 treatment. However, the initial regulation becomes less evident by 16 and 24 hours. Around 48 hours, we find that many of the genes in the Diabetes array are again strongly regulated. Results that are consistent from two or more or independent experiments are summarized below in Table 15. SCC cells also appeared to exhibit regulation in some genes, both at 6 and 24 hours after Q10 treatment. These results from SCC cells are summarized in Table 16 while genes that are regulated both in SK-MEL-28 cells and in SCC cells are summarized in Table 17.

Table 15. Genes in SK-MEL-28 cells regulated by 100 μM Q10 treatment when analyzed by the Diabetes Array.

Symbol	Description	Regulation.	Location	Possible Function
ADRB3	Adrenergic, beta-3-, receptor	Down Regulated at 48 hours	Plasma membrane	cAMP signaling, G-protein signaling
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	Down Regulated at 48 hours	Extracellular space	Anti-apoptotic, positive regulation of angiogenesis.
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	Up regulated at 48 hours	Nucleus	Glucocorticoid receptor signaling, VDR/RXR activation.
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	Down Regulated at 48 hours	Plasma Membrane	T cell receptor signaling, activates CASP8.
DUSP4	Dual specificity phosphatase 4	Down Regulated at 48 hours	Nucleus	Phosphatase
ENPP1	Ectonucleotide	Down Regulated	Plasma	Negative regulator

	pyrophosphatase/phosphodiesterase 1	at 48 hours	membrane	of the insulin receptor pathway
FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	Down Regulated at 48 hours	Nucleus	Anti-apoptotic, transcription factor
G6PD	Glucose-6-phosphate dehydrogenase	Up regulated at 48 hours, then down regulated	Cytoplasm	Pentose Phosphate Pathway, Glutathione metabolism.
HMOX1	Heme oxygenase (decycling) 1	Down Regulated at 48 hours	Cytoplasm	Heme oxygenase decycling
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	Down Regulated at 48 hours	Plasma membrane	Regulated by atorvastatin, processes some caspases.
IL4R	Interleukin 4 receptor	Down Regulated at 48 hours	Plasma membrane	Up regulation by TP73, binds to IRS1 and IRS2
IRS1	Insulin receptor substrate 1	Up regulated at 48 hours then down regulated	Plasma membrane	Binds Insulin receptor
IRS2	Insulin receptor substrate 2	Down Regulated at 48 hours	Plasma membrane	IGF-1 signaling
NSF	N-ethylmaleimide-sensitive factor	Down Regulated at 48 hours	Cytoplasm	GABA signaling
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	Down Regulated at 48 hours	Cytoplasm	Kinase
PPARG	Peroxisome proliferator-activated receptor gamma	Down Regulated at 48 hours	Nucleus	Transcriptional factor
PRKCB 1	Protein kinase C, beta 1	Down Regulated at 48 hours	Cytoplasm	PKC family
SELL	Selectin L (lymphocyte adhesion molecule 1)	Down Regulated at 48 hours	Plasma membrane	Activates RAS, MAPK
SREBF 1	Sterol regulatory element binding transcription factor 1	Up regulated at 48 hours then down regulated	Nucleus	Transcriptional factor
STXBP 1	Syntaxin binding protein 1	Down Regulated at 48 hours	Cytoplasm	Present in myelin enriched fraction.
TGFB1	Transforming growth factor, beta 1	Up regulated at 48 hours then down regulated	Extracellular space	Pro-apoptotic
NKX2-1	NK2 homeobox 1	Down Regulated at 48 hours	Nucleus	Transcriptional activator
TNF	Tumor necrosis factor (TNF superfamily, member 2)	Up regulated at 48 hours	Extracellular space	Pro-apoptotic
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Down Regulated at 72 hours	Plasma membrane	Pro-apoptotic
VEGFA	Vascular endothelial growth factor A	Up regulated at 58 hours then down regulated	Cytoplasm	Kinase

Table 16. Genes in SCC cells regulated by 100 μM Q10 treatment when analyzed by the Diabetes Array.

Symbol	Description	Regulation.
G6PD	Glucose-6-phosphate dehydrogenase	Down regulated at 6 hours.
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	Down regulated at 6 hours.
INPPL1	Inositol polyphosphate phosphatase-like 1	Down regulated at 6 hours.
NOS3	Nitric oxide synthase 3 (endothelial cell)	Down regulated at 6 hours.
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	Down regulated at 6 hours.
PPARA	Peroxisome proliferative activated receptor, alpha	Down regulated at 6 hours.
PYGL	Phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	Down regulated at 6 hours.
SREBF1	Sterol regulatory element binding transcription factor 1	Down regulated at 6 hours.
STXBP2	Syntaxin binding protein 2	Down regulated at 6 hours.
TNF	Tumor necrosis factor (TNF superfamily, member 2)	Down regulated at 6 hours.
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Down regulated at 6 and 24 hours.
VEGFA	Vascular endothelial growth factor A	Down regulated at 6 hours.

Table 17. Genes from the diabetes array regulated with 100 μM Q10 treatment for both SK-MEL-28 and SCC cells.

Symbol	Description.
G6PD	Glucose-6-phosphate dehydrogenase
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide
SREBF1	Sterol regulatory element binding transcription factor 1
TNF	Tumor necrosis factor (TNF superfamily, member 2)
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
VEGFA	Vascular endothelial growth factor A

The mRNA levels for a variety of insulin related proteins were modulated in both directions. Q10 has an impact on regulation of cellular metabolism, and thus influences metabolic disregluation diseases such as diabetes. Two proteins that were significantly modulated are further discussed below.

Mitogen-activated protein kinase 14 (MAPK14): Mitogen-activated protein kinase 14 (MAPK14) is a member of the MAP kinase family. MAP kinases act as an

integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. Results from this experiment show that the MAPK14 was significantly down-regulated.

Hepatocyte nuclear factor 4, alpha (HNF4A): HNF4 (Hepatocyte Nuclear Factor 4) is a nuclear receptor protein mostly expressed in the liver, gut, kidney, and pancreatic beta cells that is critical for liver development. In humans, there are two isoforms of NHF4, alpha and gamma encoded by two separate genes HNF4A and HNF4G respectively. (See, e.g., Chartier FL, Bossu JP, Laudet V, Fruchart JC, Laine B (1994). "Cloning and sequencing of cDNAs encoding the human hepatocyte nuclear factor 4 indicate the presence of two isoforms in human liver". *Gene* **147** (2): 269–72.)

HNF4 was originally classified as an orphan receptor. However HNF4 was found later to be constitutively active by virtue of being continuously bound to a variety of fatty acids. (See, e.g., Sladek F (2002). "Desperately seeking...something". *Mol Cell* **10** (2): 219–221 and Jump DB, Botolin D, Wang Y, Xu J, Christian B, Demeure O (2005). "Fatty acid regulation of hepatic gene transcription". *J Nutr* **135** (11)). The ligand binding domain of HNF4, as with other nuclear receptors, adopts a canonical alpha helical sandwich fold (see, e.g., Wisely GB, Miller AB, Davis RG, Thornquest AD Jr, Johnson R, Spitzer T, Sefler A, Shearer B, Moore JT, Miller AB, Willson TM, Williams SP (2002). "Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids". *Structure* **10** (9): 1225–34 and Dhe-Paganon S, Duda K, Iwamoto M, Chi YI, Shoelson SE (2002). "Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand". *J Biol Chem* **277** (41): 37973–6) and interacts with co-activator proteins. (See, e.g., Duda K, Chi YI, Shoelson SE (2004). "Structural basis for HNF-4alpha activation by ligand and coactivator binding". *J Biol Chem* **279** (22): 23311–6). Mutations in the HNF4-α gene have been linked to maturity onset diabetes of the young (MODY). (See, e.g., Fajans SS, Bell GI, Polonsky KS (2001). "Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young". *N Engl J Med* **345** (13): 971–80.)

Hepatocyte nuclear factor 4 (HNF4) is a tissue-specific transcription factor known to regulate a large number of genes in hepatocytes and pancreatic cells. Although HNF4 is highly expressed in some sections of the kidney, little is known about its role in

this organ and about HNF4-regulated genes in the kidney cells. The abundance and activity of HNF4 are frequently reduced in renal cell carcinoma (RCC) indicating some tumor suppressing function of HNF4 in renal cells. Interestingly, many of the genes regulated by HNF4 have been shown to be deregulated in RCC microarray studies. These genes (ACY1, WT1, SELENBP1, COBL, EFHD1, AGXT2L1, ALDH5A1, THEM2, ABCB1, FLJ14146, CSPG2, TRIM9 and HEY1) are good candidates for genes whose activity is changed upon the decrease of HNF4 in RCC.

In the structure of the ligand binding domain of HNF4alpha (1M7W.pdb; Dhe-Paganon (2002) JBC, 277, 37973); a small lipid was observed and which co-purified from *E. coli* production. The crystal contains two conformations of the protein, where the elongated helix 10 and short helix 12 have alternate conformations. Upon examination of the lipid binding region, it was interesting to observe that there are two exits regions. One exit region holds the small lipids head group, and it is noted that several pocket regions are co-localized with this exit port. A hypothesis would be that Q10 binds specifically to this transcription factor. When Q10 in modeled into this lipid binding tunnel, the Q10 ring would fit into the surface pocket (Figure 28). A known loss-of-function mutation (E276Q) would have the potential to order the residues lining this surface pocket, and thus have a negative impact on the putative Q10 binding.

In addition, with this Q10 binding model, the hydrophobic tail would extend out of the internal cavity and would then interact with the elongated helix 10. Thus, this interaction could potential alter the conformation of the helix 10/12 group. This may then alter the activation/inactivation equilibrium of the transcription factor activity.

EXAMPLE 7: Antibody MicroArray Analysis

The evaluation of protein concentration due to the presence of Q10 was evaluated through the utilization of antibody microarray methods. The microarray contained antibodies for over 700 proteins, sampling a broad range of protein types and potential pathway markers.

An initial experiment to assess changes at the protein concentration level in cells treated with Q10 was conducted with an antibody microarray (Panorama XP725 Antibody Array, Sigma) and SK-MEL-28 cells treated for 6 or 24 hour. The cells were

harvested and extracted to obtain a soluble protein supernatant. Two portions of protein (~1 mg total) from each sample (at 1 mg/mL) were each label with fluorescent dye (Cy3 and Cy5, respectively). The excess dye was removed from the protein and the material utilized for the microarray incubations. To compare two time point samples, equal amounts of protein were mixed, with each sample being of the different label type (e.g., 3 hour extract labeled with Cy3 was mixed with the 24 hour extract labeled with Cy5). After incubation with the microarray chip (according to manufactures recommended protocols), the chips were washed and dried. The microarrays were scanned with a fluorescent laser scanner to measure the relative fluorescence intensity of the Cy3 and Cy5 dyes.

Table 18. Proteins with increased levels in SK-MEL-28 cells after 24 hour treatment with 50 μM Q10

Name	Ratio
Cdk1	0.1
DcR1	0.1
Protein Kinase Cb2	0.1
Tumor Necrosis Factor Soluble Receptor II	0.1
BAD	0.1
Caspase13	0.2
FBI1 PAKEMON	0.2
Zyxin	0.2
Cdc25A	0.3
PIASx	0.3
Nerve Growth Factor b	0.3
Protein Tyrosine Phosphatase PEST	0.3
hBRM hSNF2a	0.4
GRP94	0.4
Calmodulin	0.4
Serine Threonine Protein Phosphatase 2C a b	0.4
ARC	0.4
NeurabinII	0.4
Nitric Oxide Synthase bNOS	0.4
Serine Threonine Protein Phosphatase 1b	0.4

Name	Ratio
Heat Shock Protein 110	0.4
Serine Threonine Protein Phosphatase 1g1	0.4
COX II	0.5
HSP70	0.5
BLK	0.5
Cytokeratin 8 12	0.5
BUBR1	0.5
FOXC2	0.5
Serine Threonine Protein Phosphatase 2 A Bg	0.5
MSH6	0.5
DR6	0.5
Rad17	0.5
BAF57	0.5
Transforming Growth Factorb pan	0.5
BTK	0.5
SerineThreonine Protein Phosphatase 2 A/B pan2	0.5
CNPase	0.5
SynCAM	0.5
Proliferating Cell Nuclear Antigen	0.5

Table 19. Proteins with increased levels in SK-MEL-28 cells after 24 hour treatment with 50 µM Q10

Name	Ratio	Name	Ratio
BclxL	4.2	Claspin	2.1
BID	3.7	GRP75	2.1
Bmf	3.7	Caspase 6	2.1
PUMA bbc3	3.0	ILP2	2.1
Zip Kinase	2.8	aActinin	2.1
Bmf	2.8	Vitronectin	2.1
DcR2	2.7	DRAK1	2.1
E2F1	2.7	PTEN	2.1
FAK pTyr577	2.5	Grb2	2.1
FKHRL1 FOXO3a	2.5	HDAC4	2.0
MTBP	2.5	HDAC7	2.0
Connexin 32	2.5	Nitric Oxide Synthase bNOS	2.0
Annexin VII	2.4	HDAC2	2.0
p63	2.4	p38 MAPK	2.0
SUMO1	2.4	Reelin	2.0
IAfadin	2.3	Protein Kinase Cd	2.0
MDMX	2.3	cerbB3	2.0
Pyk2	2.3	hSNF5 INI1	2.0
RIP Receptor Interacting Protein	2.3	Protein Kinase Ca	2.0
RICK	2.3	Glutamate receptor NMDAR 2a	2.0
IKKa	2.3	Leptin	2.0
Bclx	2.3	Dimethyl Histone H3 diMeLys4	2.0
Afadin	2.2	BID	2.0
Proliferating Cell Protein Ki67	2.2	MeCP2	2.0
Histone H3 pSer28	2.2	Nerve growth factor receptor p75	2.0
CASK LIN2	2.2	Myosin Light Chain Kinase	2.0
Centrin	2.2	cRaf pSer621	2.0
TOM22	2.1	GRP78 BiP	2.0
Nitric Oxide Synthase Endothelial eNOS	2.1	cMyc	2.0
Protein Kinase Ba	2.1	Raf1	2.0
Laminin	2.1	MTA2 MTA1L	2.0
Myosin Ib Nuclear	2.1	Sir2	2.0
Caspase 7	2.1	ATF2 pThr69 71	2.0
MAP Kinase 2 ERK2	2.1	Protein Kinase C	2.0
KIF17	2.1	Protein Kinase Cb2	2.0

In order to confirm the previously observed apoptosis proteins, and to expand the evaluation into a larger number of pro-apoptosis and anti-apoptosis proteins, two assay methods were chosen which were capable of screening the broad family of proteins potentially involved.

First, an antibody micro array (Panorama XP725 Antibody Array, Sigma) was utilized to screen over 700 protein antibodies to assess changes at the protein concentration level in SK-MEL-28 cells treated for 24 hours with 50 μM Q10.

From the Antibody array experiments, on SKMEL-28 with Q10 (24 hr), the following are some of the identified proteins with altered levels: Bcl-xl, Bmf, BTK, BLK, cJun (pSer63), Connexin 32, PUMA bbc3, BID, Par4, cCbl. The key conclusion from this initial study was that the expected pro-apoptosis proteins are altered.

Antibody Microarray for SK-MEL-28

An antibody micro array (Panorama XP725 Antibody Array, Sigma) was utilized to screen over 700 protein antibodies to assess changes at the protein concentration level in SK-MEL-28 cells treated for 24 hours with 50 μM Q10.

Table 20. Changes in protein levels in SKMEL-28 treated with 50 μM Q10

Name	Antibody Number (Sigma)	SKMEL28 Q10/ SKMEL28 control	SKMEL28 / HEKa control	HEKa Q10/ HEKa control
BclxL	B9429	2.46	1.04	1.83
PUMA bbc3	P4743	2.31	1.14	2.14
Bmf	B1559	2.23	1.12	2.11
Bmf	B1684	2.09	1.13	1.74
cJun pSer63	J2128	1.99	1.14	1.85
BLK	B8928	1.94	1.05	1.51

From the Antibody array experiments, on SKMEL-28 with Q10 (24 hr), the following are some of the identified proteins with altered levels: Bcl-xl, Bmf, BTK, BLK, cJun (pSer63), Connexin 32, PUMA bbc3, BID, Par4, cCbl. These data confirm that the levels of pro-apoptosis proteins are altered upon incubation with elevated levels of exogenously added Q10.

Bcl-xl ("Basal cell lymphoma-extra large") is a transmembrane molecule in the mitochondria. It is involved in the signal transduction pathway of the FAS-L and is one of several anti-apoptotic proteins which are members of the Bcl-2 family of proteins. It has been implicated in the survival of cancer cells. However, it is known that alternative

splicing of human Bcl-x mRNA may result in at least two distinct Bcl-x mRNA species, Bcl-xL and Bcl-xS. The predominant protein product (233 amino acids) is the larger Bcl-x mRNA, Bcl-xL, which inhibits cell death upon growth factor withdrawal (Boise et al., 1993 . *Cell* 74, 597-608). Bcl-xS, on the other hand, inhibits the ability of Bcl-2 to inhibit cell death and renders cells more susceptible to apoptotic cell death.

Table 21. Proteins with increased levels in SCC cells after 24 hour treatment with 100 μM Q10.

Name	Ratio
PUMA bbc3	3.81
HDAC7	3.21
BID	3.12
MTBP	3.00
p38 MAP Kinase NonActivated	2.93
PKR	2.87
TRAIL	2.86
DR5	2.86
Cdk3	2.82
NCadherin	2.71
Reelin	2.68
p35 Cdk5 Regulator	2.63
HDAC10	2.60
RAP1	2.59
PSF	2.56
cMyc	2.55
methyl Histone H3 MeLys9	2.54
HDAC1	2.51
F1A	2.48
ROCK1	2.45
Bim	2.45
FXR2	2.44
DEDAF	2.44
DcR1	2.40
APRIL	2.40
PRMT1	2.36
Pyk2 pTyr580	2.34
Vitronectin	2.33
Synaptopodin	2.32
Caspase13	2.30
Syntaxin 8	2.29
DR6	2.29
BLK	2.28
ROCK2	2.28

Name	Ratio
Sir2	2.25
DcR3	2.24
RbAp48 RbAp46	2.21
OGlcNAc Transferase	2.21
GRP78 BiP	2.20
Sin3A	2.20
p63	2.20
Presenilin1	2.19
PML	2.18
PAK1pThr212	2.17
HDAC8	2.16
HDAC6	2.15
Nitric Oxide Synthase Inducible iNOS	2.15
Neurofibromin	2.15
Syntaxin 6	2.13
Parkin	2.12
Rad17	2.11
Nitric Oxide Synthase bNOS	2.10
TIS7	2.09
OP18 Stathmin (stathmin 1/oncoprotein 18)	2.08
phospho-b-Catenin pSer45	2.07
NeurabinII	2.07
e Tubulin	2.07
PKB pThr308	2.07
Ornithine Decarboxylase	2.07
P53 BP1	2.06
Pyk2	2.05
HDAC5	2.05
Connexin 43	2.05
a1Syntrophin	2.04
MRP1	2.04
cerbB4	2.03
S Nitrosocysteine	2.03
SGK	2.02
Rab5	2.01

Ubiquitin Cterminal Hydrolase L1	2.01
Myosin Ib Nuclear	2.00
Par4 Prostate Apoptosis Response 4	2.00

Table 22. Proteins with reduced levels in SCC cells after 24 hour treatment with 100 μM Q10.

Name	Ratio
AP1	0.68
Centrin	0.55
CUGBP1	0.67
Cystatin A	0.69
Cytokeratin CK5	0.60
Fibronectin	0.63
gParvin	0.70
Growth Factor Independence1	0.63
Nerve Growth Factor b	0.60
ProCaspase 8	0.72
Rab7	0.62
Rab9	0.73
Serine Threonine Protein Phosphatase 1g1	0.71
Serine Threonine Protein Phosphatase 2 A Bg	0.73
SKM1	0.70
SLIPR MAGI3	0.67
Spectrin a and b	0.70
Spred2	0.66
TRF1	0.74

EXAMPLE 8: Western Blot Analysis

The first experiment processed and evaluated by Western blot and 2-D gel electrophoresis was carried out on the skin cancer cell line SKMEL-28. This experimental set involved SK-MEL-28 cells treated at 3, 6, 12, and 24 hours with 50 or 100 μM Q10.

A variety of cell types were evaluated by Western blot analysis against an antibody for Bcl-xL (Figure 14), an antibody for Vimentin (Figure 15), a series of antibodies for mitochondrial oxidative phosphorylation function (Figures 16-21) and against a series of antibodies related to mitochondrial membrane integrity (Figures 22-

27). The results from these experiments demonstrated that several of the examined proteins were upregulated or downregulated as a result of cell treatment with Q10.

EXAMPLE 9: Diabetes related genes identified as being modulated at the mRNA level by treatment of pancreatic cancer cells (PaCa2) with 100 um Q10

Diabetes arrays were run for samples treated with 100uM Q10 at various times after treatment. Experiments were carried out essentially as described above. The various genes found to be modulated upon Q10 treatment are summarized in Table 23 below. The results showed that the following genes are modulated by Q10 treatment: ABCC8, ACLY, ADRB3, CCL5, CEACAM1, CEBRA, FOXG1, FOXP3, G6PD, GLP1R, GPD1, HNF4A, ICAM1, IGFBP5, INPPL1, IRS2, MAPK14, ME1, NFKB1, PARP1, PIK3C2B, PIK3CD, PPARGC1B, PRKAG2, PTPN1, PYGL, SLC2A4, SNAP25, HNF1B, TNRFSF1A, TRIB3, VAPA, VEGFA, IL4R and IL6.

Table 23: Genes from the diabetes array whose expression is regulated with 100 μM Q10 and their possible functions in a cell. Up-regulated (grey) and down-regulated (white).

Gene Name	Gene Function.
ADRB	cAMP signaling, G-protein signaling
CCL5	Natural ligands for CCR5 and is regulated by TNF.
CEACAM1	Anti-apoptotic, positive regulation of angiogenesis.
GLPR1	Increases Insulin and decreases glucagon secretion from the pancreas.
GPD1	Carbohydrate metabolism, NADH oxidation.
ICAM1	Regulated by atorvastatin, processes some caspases.
MAPK14	DNA damage checkpoint, angiogenesis, glucose metabolic process.
PARP1	DNA repair, regulates TP53, NOS2A, NFKB,telomere maintenance.
PIK3C2B	Phosphoinositide mediated signaling, regulates AKT and AKT1.
PIK3CD	Kinase
PYGL	carbohydrate metabolism, regulates glycogen and glycogen synthase.

SLC2A4	regulates glucose and is regulated by INS and insulin.
SNAP25	regulation of insulin secretion; nerotransmitter uptake.
CEBPA	Glucocorticoid receptor signaling, VDR/RXR activation.
FOXP3	Regulates IL4, IL2.
G6PD	Pentose Phosphate Pathway, Glutathione metabolism.
IGFBP5	Regulation of cell growth, regulated by IGF1
INPPL1	Regulates Akt and glycogen.
IRS2	IGF-1 signaling
ME1	Regulates malic acid and is regulated by T3.
NFKB1	Regulates IL6 and TNF.
PPARGC1B	Regulated by MAPK14
PRKAG2	Fatty acid, cholesterol biosynthesis.
PTPN1	dephosphorylates JAK2 and EGFreceptor kinase.
VEGFA	Kinase, angiogenesis.
IL4R	Up regulation by TP73, binds to IRS1 and IRS2
HNF1B	HNF4A
TNFRSF1A	Pro-apoptotic
TRIB3	Regulates AKT1 and negative regulator of NFkB.
VAPA	Regulates NFkB, vesicle trafficking.

EXAMPLE 10: Angiogenesis related genes identified as being modulated at the mRNA level by treatment of pancreatic cancer cells (PaCa2) with 100 μM Q10

Angiogenesis arrays were run for samples treated with 100uM Q10 at various times after treatment. Experiments were carried out essentially as described above. The various genes found to be modulated upon Q10 treatment are summarized in Table 24 below. The results showed that the following genes are modulated by Q10 treatment: AKT1, ANGPTL4, ANGPEP, CCL2, CDH4, CXCL1, EDG1, EFNA3, EFNB2, EGF, FGF1, ID3, IL1B, IL8, KDR, NRP1, PECAM1, PROK2, SERPINF1, SPHK1, STAB1, TGFB1, VEGFA and VEGFB.

Table 24: A list of genes from the angiogenesis array whose expression is regulated with 100 μM Q10 and their possible functions in a cell. Up-regulated (grey) and down-regulated (white).

Gene	Gene Function.
ANGPTL4	antiangiogenesis, negative regulator of apoptosis, lipid metabolism.
CDH5	blood vessel maturation, cell-adhesion, negative regulator of cell proliferation.
FGF1	Cell adhesion, cell proliferation.
AKT1	carbohydrate metabolic process, glycogen biosynthetic process, glucose metabolic process, insulin receptor signaling pathway, activation of pro-apoptotic gene products, apoptotic mitochondrial changes
ANPEP	proteolysis, multicellular organismal development, cell differentiation
CCL2	chemotaxis,anti-apoptosis,JAK-STAT cascade, organ morphogenesis, viral genome replication
CXCL1	chemotaxis, inflammatory response, immune response,negative regulation of cell proliferation,actin cytoskeleton organization and biogenesis.
EDG1	positive regulation of cell proliferation, transmission of nerve impulse, regulation of cell adhesion, neuron differentiation, positive regulation of cell migration, positive regulation of Ras
EFNB2	cell-cell signaling, regulated by VEGFA.
EGF	activation of MAPKK activity, positive regulation of mitosis, DNA replication
ILIB	response to glucocorticoid stimulus, apoptosis, signal transduction, cell-cell signaling, negative regulation of cell proliferation
IL8	cell cycle arrest
KDR	VEGF pathway, regulated by AKT.
NRP1	cell adhesion, signal transduction, cell-cell signaling, cell proliferation, regulated by VEGFA
PECAM1	cell adhesion, regulated by TNF.
PROK2	activation of MAPK, anti-apoptosis, cell proliferation, regulates AKT,
SPHK1	anti-apoptosis, cell proliferation, regulates mitosis, cell migration.
STAB1	inflammatory response, cell adhesion, receptor-mediated endocytosis, cell-cell signaling, negative regulation of angiogenesis, defense response to bacterium
VEGFA	anti-apoptosis, regulates TNF, regulated by HIF1.

EXAMPLE 11: Apoptosis related genes identified as being modulated at the mRNA level by treatment of pancreatic cancer cells (PaCa2) with 100 μM Q10

Apoptosis arrays were run for samples treated with 100uM Q10 at various times after treatment. Experiments were carried out essentially as described above. The various genes found to be modulated upon Q10 treatment are summarized in Table 25 below. The results showed that the following genes are modulated by Q10 treatment: ABL1, AKT1, Bcl2L1, BclAF1, CASP1, CASP2, CASP6, CIDEA, FADD, LTA, TNF, TNFSF10A and TNFSF10.

Table 25: A list of genes from the apoptosis array whose expression is regulated with 100 μM Q10 and their possible functions in a cell. Up-regulated (Grey) and down-regulated (white).

Gene	Gene Function.
CASP1	Pro-Apoptotic, Regulates IL1B, regulated by TNF.
CASP6	Pro-Apoptotic, regulates PARP, MCL1, APP
TNF	cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation
TNFSF10	Pro-Apoptotic, regulates caspases.
ABL1	Regulates Bcl2L1, TP53, Pro-apoptotic, actin cytoskeleon organization and biogenesis.
AKT1	Prop-apoptotic, apoptotic mitochondrial changes, carbohydrate transport, response to heat, glucose metabolism, IGF signaling pathway.
BclAF1	Pro-Apoptotic.
Bcl2L1	Anti-Apoptotic, release of cytochrome c from mitochondria, regulates Caspases, binds to BAD, BAX, BCl2L11
CASP2	Anti-Apoptotic.
CIDEA	Pro-Apoptotic
FADD	Pro-Apoptotic
LTA	Pro-Apoptotic
TNFSF10A	Caspase Activator

EXAMPLE 12: PCR Diabetes Arrays on Liver Cancer (HepG2) Cells

HepG2 (liver cancer) cells were treated with either the vehicle for 24 hours or 100 μM Q10 for different times. The treatment was initiated on 1x105 cells per well, following the procedure utilized in the PaCa2 cells (above, Examples 9-11). However, the total amount of RNA that was extracted from these samples was lower than expected. Reverse transcription is normally done using 1 μg of total RNA (determined by measurement at 260 nm). The maximum volume that can be used per reverse transcription is 8 μl. Since the RNA concentration was low, the RT-PCR array analysis using the vehicle, and Q10 treated samples from 16 hours and 48 hours was performed using 0.44 μg of RNA. The arrays provided an initial analysis of trends and patterns in HepG2 gene regulation with 100 μM Q10 treatment, as summarized in Table 26 below. The results showed that each of the genes PPARGC1A, PRKAA1 and SNAP25 were downregulated at 16 hours following treatment (by approximately 20 fold, 6 fold and 5 fold, respectively). At 48 hours following treatment, PPARGC1A and PRKAA1 had normalized or were slightly upregulated, while SNAP25 was downregulated by approximately 2 fold.

Table 26: List of genes regulated in the Diabetes Arrays when HepG2 cells were treated with 100 μM Q10.

Gene	Gene name	Gene Function.
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Involved in cell death, proliferation, cellular respiration and transmembrane potential.
PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit	Regulates TP53 and is involved in apoptosis, regulates glycolysis, regulates metabolic enzyme activities.
SNAP25	synaptosomal-associated protein, 25kDa	Plays in transport, fusion, exocytosis and release of molecules.

EXAMPLE 13: PCR Angiogenesis Array on Liver Cancer (HEPG2) Cells

HepG2 (liver cancer) cells were treated with either the vehicle for 24 hours or 100 μM Q10 for different times. The treatment was initiated on 1x105 cells per well, following the procedure utilized in the PaCa2 cells (above Examples 9-11). However, the total amount of RNA that was extracted from these samples was lower than expected. Reverse transcription is normally done using 1 μg of total RNA (determined by measurement at 260 nm). The maximum volume that can be used per reverse transcription is 8 μl. Since the RNA concentration was low, the RT-PCR array analysis using the vehicle, and Q10 treated samples from 16 hours and 48 hours was performed using 0.44 μg of RNA. The arrays provided an initial analysis of trends and patterns in HepG2 gene regulation with 100 μM Q10 treatment, as summarized in Table 27 below. The various genes found to be modulated upon Q10 treatment are summarized in Table 27 below. The results showed that each of the genes ANGPTL3, ANGPTL4, CXCL1, CXCL3, CXCL5, ENG, MMP2 and TIMP3 were upregulated at 16 hours following treatment (by approximately 5.5, 3, 3, 3.2, 3, 3, 1 and 6.5 fold, 6 fold and 5 fold, respectively, over that of control). ID3 was downregulated at 16 hours following Q10 treatment, by approximately 5 fold over control. At 48 hours following treatment, ANGPTL3, CXCL1, CXCL3, ENG and TIMP3 were still upregulated (by approximately 3.5, 1.5, 3.175, 2 and 3 fold, respectively, over control), while ANGPTL4, CXCL5, ID3 and MMP2 were downregulated by approximately 1, 1, 2 and 18 fold, respectively, over control.

Table 27: List of genes regulated in the Angiogenesis Arrays when HepG2 cells were treated with 100 μM Q10.

Gene	Gene Name.	Gene Function.
ANGPTL3	angiopoietin-like 3	Predominantly expressed in live, role in cell migration and adhesion, regulates fatty acid and glycerol metabolism.
ANGPTL4	angiopoietin-like 4	Regulated by PPARG, apoptosis inhibitor for vascular endothelial cells, role lipid and glucose metabolism and insulin sensitivity.
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Role in cell proliferation and migration
CXCL3	chemokine (C-X-C motif) ligand 3	Chemokine activation, hepatic stellar cell activation, migration, proliferation.

CXCL5	chemokine (C-X-C motif) ligand 5	Produced along with IL8 when stimulated with IL1 or TNFA. Role in chemotaxis, migration, proliferation.
ENG	endoglin	Binds to TGFBR and is involved in migration, proliferation, attachment and invasion.
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	Regulates MMP2, Regulated by TGFB1, Vitamin D3, Retinoic acid, VEGFA, involved in apoptosis, proliferation, differentiation, migration.
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Hepatic stellate cell activation, HIF□ signaling, binds to TIMP3, involved in tumorigenesis, apoptosis, proliferation, invasiveness, migration and chemotaxis.
TIMP3	TIMP metallopeptidase inhibitor 3	Regulates MMP2, ICAM1. Regulated by TGFB, EGF, TNF, FGF and TP53. Involved in apoptosis, cell-cell adhesion and malignancy.

Proteins known to be involved in the process of angiogenesis were components in the RT-PCR array. Angiogenesis is a critical process by which cancer cells become malignant. Some of these proteins are also implicated in diabetes.

ANGPTL3 and ANGPTL4: The literature related to ANGPTL3 connects this protein to the regulation of lipid metabolism. In particular, the literature (Li, C. *Curr Opin Lipidol.* 2006 Apr;17(2):152-6) teaches that both angiopoietins and angiopoietin-like proteins share similar domain structures. ANGPTL3 and 4 are the only two members of this superfamily that inhibit lipoprotein lipase activity. However, ANGPTL3 and 4 are differentially regulated at multiple levels, suggesting non-redundant functions in vivo. ANGPTL3 and 4 are proteolytically processed into two halves and are differentially regulated by nuclear receptors. Transgenic overexpression of ANGPTL4 as well as knockout of ANGPTL3 or 4 demonstrate that these two proteins play essential roles in lipoprotein metabolism: liver-derived ANGPTL3 inhibits lipoprotein lipase activity primarily in the fed state, while ANGPTL4 plays important roles in both fed and fasted states. In addition, ANGPTL4 regulates the tissue-specific delivery of lipoprotein-derived fatty acids. ANGPTL4 is thus an endocrine or autocrine/paracarine inhibitor of lipoprotein lipase depending on its sites of expression.

Lipoprotein lipase is an enzyme that hydrolyzes lipids in lipoproteins, such as those found in chylomicrons and very low-density lipoproteins (VLDL), into three free fatty acids and one glycerol molecule. Lipoprotein lipase activity in a given tissue is the

rate limiting step for the uptake of triglyceride-derived fatty acids. Imbalances in the partitioning of fatty acids have major metabolic consequences. High-fat diets have been shown to cause tissue-specific overexpression of LPL, which has been implicated in tissue-specific insulin resistance and consequent development of type 2 diabetes mellitus.

The results in this Example indicate that Q10 is modulating proteins involved in lipid metabolism and thus warrants further investigation of ANGPTL3/ANGPTL4 and their related pathways. For example, ANGPTL3/ANGPTL4 have been implicated to play a role in the following pathways: Akt, cholesterol, fatty acid, HDL-cholesterol, HNF1A, ITGA5, ITGA5, ITGAV, ITG83, L-trilodothynonine, LIPG, LPL, Mapk, Nrth, NR1H3, PPARD, PTK2, RXRA, triacylglerol and 9-cis-retinoic acid.

EXAMPLE 14: PCR Apoptosis Array on Liver Cancer (HEPG2) Cells

Apoptosis arrays were run for samples treated with 100uM Q10 for 16 and 48 hours as described above. However, the array for 48 hours was run choosing FAM as the fluorophore instead of SYBR. Both FAM and SYBR fluoresce at the same wavelength.

The various genes found to be modulated upon Q10 treatment are summarized in Table 28 below. The results showed that CASP9 was upregulated at 16 hours following Q10 treatment, by approximately 61 fold over control, while BAG1 and TNFRSF1A were downregulated at 16 hours following treatment by approximately 6 and 4 fold, respectively, over that of control. At 48 hours following treatment, CASP9, BAG1 and TNFRSF1A were upregulated by approximately 55, 1 and 1 fold, respectively, over control.

Table 28: List of genes regulated in the Apoptosis Arrays when HepG2 cells were treated with 100 μM Q10.

Gene	Gene Name	Gene Function.
BAG1	BCL2-associated athanogene	Involved with Apoptosis
CASP9	caspase 9, apoptosis-related cysteine peptidase	Apoptosis through release of cytochrome c.
TNFRSF1A	tumor necrosis factor receptor	anti-apoptosis, binds many cell

	superfamily, member 1A	death factors, regulates ICAM1

EXAMPLE 15: Assessing Ability of Epi-shifter to Treat Metabolic Disorder

In order to determine if a selected Epi-shifter, *e.g.*, CoQ10, is capable of treating a metabolic disorder, e.g., diabetes, cell based assays that monitor an increase in insulin-stimulated glucose uptake in vitro are employed. In particular, differentiated mouse adipocytes are used to identify agents that have the ability to increase glucose uptake upon insulin stimulation, as detected by scintillation counting of radiolabelled glucose (using, for example, the Perkin Elmer 1450 Microbeta JET reader). These assays are conducted as follows.

Materials and Methods:

Prees Media

Complete media, also referred to as "Prees" media, is prepared as follows. Dulbecco's Modified Eagle's Medium (DMEM) is supplemented with L-glutamine, penicillin-G and streptomycin (pen/strep), and heat-inactivated fetal bovine serum (FBS) (heat inactivated at 65.degree. C. for 30 minutes). Because serum can affect the growth, adherence, and differentiation of cells, any new lot of serum was first tested prior to use. Media was equilibrated in the incubator (5% CO.sub.2) until the pH was within the proper range (.about.7), as indicated by the red/orange color of the indicator dye. If the media became pink (indicating a high pH), we discarded the media as basic conditions can affect cells and denature the insulin used in the differentiation medium-1 (DM1) and the differentiation medium-2 (DM2).

Differentiation Medias

Differentiation media-1 (DM1) was prepared by supplementing DMEM with 10% FBS, L-glutamine, pen/strep, IBMX (375 .mu.M), insulin (120 nM), and dexamethasone (188 nM). Differentiation media-2 (DM2) was prepared by supplementing DMEM with 10% FBS, L-glutamine, pen/strep, and insulin (120 nM).

Preparation of Gelatinized Plates

Cell culture plates are gelatinized as follows. Gelatin (1% w/v in distilled water) was autoclaved and stored at room temperature. The bottom of each cell culture well was covered uniformly in the gelatin solution, ensuring that no bubbles are formed. This solution was removed leaving behind a thin film of gelatin. These plates are left to dry under the tissue culture hood. Plates are next washed with PBS, after which a 0.5% glutaric dialdehyde solution (glutaraldehyde in distilled water) was added to the cell culture wells. After ten minutes, wells are washed twice with DMEM containing pen-strep. Each washing step should last for approximately five minutes.

Cell Culture

3T3-L1 pre-adipocyte cells are split approximately every 2-3 days or upon reaching a confluence of approximately 60%. Overconfluency may affect the ability of these cells to differentiate into adipocytes.

Other Reagents

D-(+)-glucose ("cold" glucose, not radiolabeled) was added to DPBS mix to a final concentration of 10 mM.

Lysis buffer, a mixture of a base (e.g., sodium hydroxide at a final concentration of 0.5N) and a detergent (e.g., sodium dodecyl sulphate (SDS) diluted to a final concentration of 0.1% w/v) was freshly prepared each time (within one to two hours of use). Prior to use, lysis buffer was warmed up to a temperature exceeding that of room temperature for a period of approximately 30 minutes to avoid precipitation of the buffer.

Determination of Glucose Uptake

Pre-adipocyte 3T3-L1 cells are plated at a density of approximately 5000 cells/well (in black NUNC 96 well plate). These cells are differentiated into adipocytes in two separate steps. Initially, cells are cultured in differentiation medium-1 (DM1) (day 1 of adipocyte differentation) for a period of two to three days. DM1 prevents proliferation and induces the expression of adipocyte-specific genes. Cells are next cultured in differentiation medium-2 (DM2) for 3 to 4 days, after which the culture media is replaced by fresh DM2. The glucose uptake assay is performed at day 9-15 of

differentiation.

Two days prior to the experiment (at day 7-13 of differentiation), DM2 is removed and replaced with fresh Prees media. Candidate compounds are added at this time, allowing an incubation period of approximately 48 hours. On the day of the experiment, cells (now at day 9 to 15 of differentiation) are serum starved for three hours in DPBS, magnesium sulfate (0.8 mM), and Hepes (10 mM) at pH .about.7. After this incubation period, fresh DPBS containing insulin (10 nM) is added to the adipocytes. Fresh DPBS without any insulin are placed on cells that served as a negative control. Following an incubation period of 25 minutes at 37.degree. C., radioactive glucose (labeled with .sup.14C, at a final concentration of 0.04 mM, .about.0.26 .mu.Ci .sup.14C-glucose in each well) is added to the media for a period of 15 minutes at room temperature. Media is next removed and cells are washed thoroughly and lysed. Upon lysis, cells form a small, cloudy mass, detached from the well bottom. 10% glacial acetic acid is added to each well to neutralize the lysis reaction. Scintillation fluid is next added to the wells and the incorporation of glucose is determined by measuring the amount of radioactivity in each well using the MicroBeta plate reader.

Using the foregoing experimental protocol, an Epi-shifter is identified as capable of treating a metabolic disorder, e.g., diabetes, when the Epi-shifter enhances, increases or augments insulin-stimulated glucose uptake in the cells in vitro.

EXAMPLE 16: Identification of a MIM associated with an Metabolic Disorder

In order to evaluate a candidate molecule (e.g., environmental influencer) as a potential MIM, the selected candidate MIM is exogenously added to a panel of cell lines, including both diseased (cancer) cell lines and normal control cell lines, and the changes induced to the cellular microenvironment profile for each cell line in the panel are assessed. Changes to cell morphology, physiology, and/or to cell composition, including for example, mRNA and protein levels, are evaluated and compared for the diseased cells as compared to normal cells.

Changes to cell morphology/physiology are evaluated by examining the sensitivy and apoptotic response of cells to the candidate MIM. These experiments are carried out as described in detail in Example 3. Briefly, a panel of cell lines consisting of at least one control cell line and at least one cancer cell line are treated with various

concentrations of the candidate MIM. The sensitivity of the cell lines to the potential MIM are evaluated by monitoring cell survival at various times, and over the range of applied concentrations. The apoptoic response of the cell lines to the potential MIM are evaluated by using, for example, Nexin reagent in combination with flow cytometry methodologies. Nexin reagent contains a combination of two dyes, 7AAD and Annexin-V-PE, and allows quantification of the population of cells in early and late apoptosis. An additional apoptosis assay that measures single-stranded DNA may be used, using for example ApostrandTM ELISA methodologies. The sensitivity and apoptotic response of the disease and control cell lines are evaluated and compared. A molecule that displays differential cytotoxicity and/or that differentially induces the apoptotic response in the diseased cells as compared to the normal cells is identified as a MIM.

Changes in the composition of cells following treatment with the candidate MIM are evaluated. Changes in gene expression at the mRNA level are analyzed using Real-Time PCR array methodology. These experiments are carried out as described in detail in Examples 6 and 9-13. Briefly, the candidate MIM is exogenously added to one or more cell lines including, for example a diseased cell and a normal control cell line, and mRNA is extracted from the cells at various times following treatment. The level of mRNAs for genes involved in specific pathways are evaluated by using targeted pathway arrays, including, for example, arrays specific for apoptosis, oxidative stress and antioxidate defense, angiogenesis, heat shock or diabetes. The genes that are altered in their mRNA transcription by a two-fold level or greater are identified and evaluated. A molecule that induces changes in mRNA levels in cells and/or that induces differential changes in the level of one or more mRNAs in the diseased cells as compared to the normal cells is identified as a MIM.

In complementary experiments, changes in gene expression at the protein level are analyzed by using antibody microarray methodology, 2-dimensional gel electrophoresis followed by protein identificuation using mass spectrometry characterization, and by western blot analysis. These experiments are carried out as described in detail in Examples 7, 4 and 8, respectively. Briefly, the candidate MIM is exogenously added to one or more cell lines, including, for example a diseased cell and a normal control cell line, and soluble protein is extracted from the cells at various times, *e.g.*, 6 hours or 24 hours, following treatment. Changes induced to protein levels by the

candidate MIM are evaluated by using an antibody microarray containing antibodies for over 700 proteins, sampling a broad range of protein types and potential pathway markers. Further complementary proteomic analysis can be carried by employing 2-dimensional (2-D) gel electrophoresis coupled with mass spectrometry methodologies. The candidate MIM is exogenously added to one or more cell lines, including, for example a diseased cell and a normal control cell line, and cell pellets are lysed and subjected to 2-D gel electrophoresis. The gels are analyzed to identify changes in protein levels in treated samples relative to control, untreated samples. The gels are analyzed for the identification of spot changes over the time course of treatment due to increased levels, decreased levels or post-translational modification. Spots exhibiting statistically significant changes are excised and submitted for protein identification by trypsin digestiona dn mass spectrometry characterization. The characterized peptides are searched against protein databases with, for example, Mascot and MSRAT software analysis to identify the proteins. In addition to the foregoing 2-D gel analysis and antibody microarray experiments, potential changes to levels of specific proteins induced by the candidate MIM may be evaluated by Western blot analysis. In all of the proteomic experiments, proteins with increased or decreased levels in the various cell lines are identified and evaluated. A molecule that induces changes in protein levels in cells and/or that induces differential changes in the level of one or more proteins in the diseased cells as compared to the normal cells is identified as a MIM.

Genes found to be modulated by treatment with a candidate MIM from the foregoing experiments are subjected to cellular and biochemical pathway analysis and can thereby be categorized into various cellular pathways, including, for example apoptosis, cancer biology and cell growth, glycolysis and metabolism, molecular transport, and cellular signaling.

Experiments are carried out to confirm the entry of a candidate MIM into cells, to determine if the candidate MIM becomes localized within the cell, and to determine the level and form of the candidate MIM present in the cells. These experiments are carried out, for example, as described in detail in Example 5. For example, to determine the level and the form of the candidate MIM present in the mitochondria, mitochondrial enriched preparations from cells treated with the candidate MIM are prepared and analyzed. The level of the candidate MIM present in the mitochondria can thereby be

confirmed to increase in a time and dose dependent manner with the addition of exogenous candidate MIM. In addition, changes in levels of proteins from mitochondria enriched samples are analyzed by using 2-D gel electrophoresis and protein identification by mass spectrometry characterization, as described above for total cell protein samples. Candidate MIMs that are found to enter the cell and to be present at increased levels, *e.g.*, in the mitochondria, are identified as a MIM. The levels of the candidate MIM in the cell, or, for example, specifically in the mitochondria, over the time course examined can be correlated with other observed cellular changes, as evidenced by, for example, the modulation of mRNA and protein levels for specific proteins.

Candidate MIMs observed to induce changes in cell composition, *e.g.*, to induce changes in gene expression at the mRNA or protein level, are identified as a MIM. Candidate MIMs observed to induce differential changes in cell morphology, physiology or cell composition (*e.g.*, differential changes in gene expression at the mRNA or protein level), in a disease state (*e.g.*, diabetes or obestity) as compared to a normal state are identified as a MIM and, in particular, as having multidimensional character. Candidate MIMs found to be capable of entering a cell are identified as a MIM and, in particular, as having multidimensional character since the candidate MIM thereby exhibits a carrier effect in addition to a therapeutic effect.

EXAMPLE 17: Identification of CoQ10 As An Epi-shifter Associated With A Metabolic Disorder

A panel of skin cell lines consisting of a control cell lines (primary culture of keratinocytes and melanocytes) and several skin cancers cell lines (SK-MEL-28, a non-metastatic skin melanoma; SK-MEL-2, a metastatic skin melanoma; or SCC, a squamous cell carcinoma; PaCa2, a pancreatic cancer cell line; or HEP-G2, a liver cancer cell line) were treated with various levels of Coenzyme Q10. The cancer cell lines exhibited an altered dose dependent response when compared to the control cell lines, with an induction of apoptosis and cell death in the cancer cells only. Detailed exemplary experiments are presented in, *e.g.*, Example 3 herein.

Assays were employed to assess changes in the mRNA and protein levels composition of the above-identified cells following treatment with CoQ10. Changes in mRNA expression were analyzed using real-time PCR microarrays specific for each of apoptosis, oxidative stress and antioxidants, angiogenesis and diabetes. Changes in protein expression were analyzed using antibody microarray analysis and western blot analysis. The results from these assays demonstrated that significant changes in gene expression, both at the mRNA and protein levels, were occurring in the cell lines due to the addition of the Coenzyme Q10. Numerous genes known to be associated with or involved in cellular metabolic processes were observed to be modulated as a result of treatment with CoQ10. For example, expression of the nuclear receptor protein HNF4A was found to be upmodulated in cells following Q10 treatment. Expression of transaldolase 1 (TAL) was also modulated in cells treated with Q10. TAL balances the levels of NADPH and reactive oxygen intermediate, thereby regulating the mitochondrial trans-membrande potentional, which is a critical checkpoint of ATP synthesis and cell survival. Of particular relevance to metabolic disorders, numerous genes known to be associated with, e.g., diabetes, were identified as being regulated by Q10. Detailed exemplary experiments are presented in, *e.g.*, Examples 4, 6, 7, 8 and 9 herein.

Q10 is an essential cofactor for exidative phosphorylation processes in the mitochondria for energy production. The level of Coenzyme Q10, as well as the form of CoQ10, present in the mitochondria was determined by analyzing mitochondrial enriched preparations from cells treated with CoQ10. The level of Coenzyme Q10 present in the mitochondria was confirmed to increase in a time and dose dependent manner with the addition of exogenous Q10. The time course correlated with a wide variety of cellular changes as observed in modulation of mRNA and protein levels for specific proteins related to metabolic and apoptotic pathways. Detailed exemplary experiments are presented in, *e.g.*, Example 5 herein.

The results described herein identified the endogenous molecule CoQ10 as an epi-shifter. In particular, the results identified CoQ10 as inducing a shift in the metabolic state, and partially restoration of mitochondrial function, in cells. These conclusions are based on the following interpretation of the data described herein and the current knowledge in the relevant art.

Q10 is known to be synthesized, actively transported to, enriched in, and utilized in the mitochondrial inner membrane. Q10 is also known to be an essential cofactor for oxidative phosphorylation processes in the mitochondrial for energy production. However, most cancer cells predominantly produce energy by glycolysis followed by lactic acid fermentation in the cytosol, rather than by oxidation of pyruvate in mitochondria like most normal cells. The oxidative phosphorylation involves the electron transport complexes and cytochrome c. Apoptosis involves the disruption of the mitochondria, with permiabilization of the inter mitochondrial membrane by pro-apoptitic factors. By utilizing a different metabolic energy synthesis pathway, cancer cells are able to mitigate the normal apoptosis response to abnormalities in the cell. While not wishing to be bound by theory, Applicants propose that Q10 is functioning by upregulating the oxidative phosphorylation pathway proteins, thus switching the mitochondrial function back to a state that would recognize the oncogenic defects and trigger apoptosis. Thus, Q10 is acting as an Epi-shifter by shifting the metabolic state of a cell.

EXAMPLE 18: Identification of An Epi-shifter Associated With a Metabolic Disorder

A panel of skin cell lines consisting of control cell lines (e.g., primary culture of keratinocytes and melanocytes) and cancer cell lines (e.g., SK-MEL-28, a non-metastatic skin melanoma; SK-MEL-2, a metastatic skin melanoma; or SCC, a squamous cell carcinoma; PaCa2, a pancreatic cancer cell line; or HEP-G2, a liver cancer cell line) are treated with various levels of a candidate Epi-shifter. Changes to cell morphology/physiology are evaluated by examining the sensitivy and apoptotic response of cells to the candidate Epi-shifter. These experiments are carried out as described in detail in Example 3. Briefly, the sensitivity of the cell lines to the candidate Epi-shifter are evaluated by monitoring cell survival at various times, and over a range of applied concentrations. The apoptoic response of the cell lines to the candidate Epi-shifter are evaluated by using, for example, Nexin reagent in combination with flow cytometry methodologies. Nexin reagent contains a combination of two dyes, 7AAD and Annexin-V-PE, and allows quantification of the population of cells in early and late

apoptosis. An additional apoptosis assay that measures single-stranded DNA may be used, using for example ApostrandTM ELISA methodologies. The sensitivity and apoptotic response of the disease and control cell lines are evaluated and compared. Candidate Epi-shifters are evaluated based on their ability to inhibit cell growth preferentially or selectively in cancer cells as compared to normal or control cells. Candidate Epi-shifters are further evaluated based on their ability to preferentially or selectively induce apoptosis in cancer cells as compared to normal or control cells.

Assays are employed to assess changes in the mRNA and protein level composition of the above-identified cells following treatment with the candidate Epi-shifter. Changes in mRNA levels are analyzed using real-time PCR microarrays. These experiments are carried out as described in detail in Examples 6 and 9-13. Briefly, mRNA is extracted from the cells at various times following treatment. The level of mRNAs for genes involved in specific pathways are evaluated by using targeted pathway arrays, including, arrays specific for apoptosis, oxidative stress and antioxidate defense, angiogenesis, heat shock or diabetes. The genes that are altered in their mRNA transcription by a two-fold level or greater are identified and evaluated.

Changes in protein expression are analyzed using antibody microarray analysis, 2-D gel electrophoresis analysis coupled with mass spectrometry characterization, and western blot analysis. These experiments are carried out as described in detail in Examples 7, 4 and 8, respectively. Briefly, soluble protein is extracted from the cells at various times, *e.g.*, 6 hours or 24 hours, following treatment with the candidate Epi-shifter. Changes induced to protein levels by the candidate Epi-shifter are evaluated by using an antibody microarray containing antibodies for over 700 proteins, sampling a broad range of protein types and potential pathway markers. Further complementary proteomic analysis can be carried out by employing 2-dimensional (2-D) gel electrophoresis coupled with mass spectrometry methodologies. The candidate Epi-shifter is exogenously added to the cell lines and cell pellets are lysed and subjected to 2-D gel electrophoresis. The gels are analyzed to identify changes in protein levels in treated samples relative to control, untreated samples. The gels are analyzed for the identification of spot changes over the time course of treatment due to increased levels, decreased levels or post-translational modification. Spots exhibiting statistically significant changes are excised and submitted for protein identification by trypsin

digestion and mass spectrometry characterization. The characterized peptides are searched against protein databases with, for example, Mascot and MSRAT software analysis to identify the proteins. In addition to the foregoing 2-D gel analysis and antibody microarray experiments, potential changes to levels of specific proteins induced by the candidate MIM may be evaluated by Western blot analysis. In all of the proteomic experiments, proteins with increased or decreased levels in the various cell lines are identified and evaluated.

Candidate Epi-shifters are evaluated based on changes induced to gene expression, at the mRNA and/or protein levels, in the cell lines due to the addition of the candidate Epi-shifter. In particular, candidate Epi-shifters are evaluated based on their ability to modualate genes known to be associated with or involved in cellular metabolic processes. Of particular relevance to metabolic disorders, candidate Epi-shifters are evaluated based on their ability to modulate genes known to be associated with, for example, diabetes or obesity.

The level of the candidate Epi-shifter, as well as the form of the candidate Epi-shifter, present in the cell or a particular cell location is determined using routine methods known to the skilled artisan. For example, the level of the candidate Epi-shifter in mitochondria over time and over a range of doses is determined by analyzing mitochondrial enriched preparations from cells treated with the candidate Epi-shifter. The levels of the candidate Epi-shifter in the mitochondria over the time course can be compared and correlated with other cellular changes observed, such as modulation of mRNA and protein levels for specific proteins related to metabolic and apoptotic pathways.

Candidate Epi-shifters observed to induce a shift in the metabolic state of a cell based on the results obtained from the foregoing experiments are identified as Epi-shifters. For example, a candidate Epi-shifter that enhances, increases or augments insulin-stimulated glucose uptake in cells is identified as an Epi-shifter.

EXAMPLE 19: Identification of Vitamin D3 as an Epi-shifter

Vitamin D3, or 1α, 25-dihydroxyvitamin D3 (also known as calcitriol), is a vitamine D metabolite that is synthesized from vitamin D by a two-step enzymatic process. Vitamin D3 interacts with its ubiquitous nuclear vitamin D receptor (VDR) to regulate the transcription of a wide spectrum of genes involved in calcium and phosphate homeostasis as well as in cell division and differentiation. Vitamin D3 has been reported to have anticancer effects in numerous model systems, including squamous cell carcinoma, prostate adenocarcinoma, cancers of the ovary, breast and lung (reviewed in Deeb *et al.* 2007 *Nature Reviews Cancer* 7:684-700).

The anticancer effects of vitamin D3 are reported to involve multiple mechanisms, including growth arrest at the G1 phase of the cell cycle, apoptosis, tumor cell differentiation, disruption of growth factor-mediated cell survival signals, and inhibition of angiogenesis and cell adhesion (reviewed in Deeb *et al.* 2007 *Nature Reviews Cancer* 7:684-700). For example, with particular respect to apoptosis, Vitamin D3 has been reported to induce apoptosis by regulating key mediators of apoptosis, such as repressing the expression of the anti-apoptotic, pro-survival proteins BCL2 and BCL-XL, or inducing the expression of pro-apoptotic proteins (e.g., BAX, BAK and BAD) (Deeb *et al.* 2007).. In a further example, with particular respect to angiogenesis, Vitamin D3 has been reported to inhibit the proliferation of some tumor-derived endothelial cells and to inhibit the expression of vascular endothelial growth factor (VEGF) that induces angiogenesis in tumors (reviewed in Masuda and Jones, 2006 *Mol. Cancer Ther.* 5(4): 797-8070). In another example, with particular respect to cell cycle arrest, Vitamin D3 has been reported to induce gene transcription of the cyclin-dependent kinase inhibitor p21WAFI/CIPI and to induce the synthesis and/or stabilization of the cyclin-dependent kinase inhibiotor p27KIPI protein, both of which are critical for induction of G1 arrest. (Deeb *et al.* 2007)..

Based on the foregoing observations, Vitamin D3 is identified as an Epi-shifter, *i.e.*, owing to its ability to shift the metabolic state of a cell. Vitamin D3 is an Epi-shifter owing to its ability to induce apoptosis in a cell and, in particular, based on its ability to differentially inhibit cell growth and induce the apoptotic response in diseased (cancer) cells as compared to normal cells (*e.g.*, differentially modulate expression of

proteins, such as BCL-2, BCL-XL, and BAX, involved in apoptosis in cancer cells as compared to normal cells).

EXAMPLE 20: Western analysis of cells treated with Coenzyme Q10

Over the past five decades enormous volume of information has been generated implicating endogenous/exogenous factors influencing specific processes as the underlying cause of malignant transformations. Clinical and basic literature provides evidence that changes in the DNA structure and function play a significant role in the initiation and progression of cancer, defining cancer as a genetic disease (Wooster, 2010; Haiman, 2010). In the early 1920s, Otto Warburg and other investigators involved in characterizing fundamental changes in etiology of oncogenesis described two major observations (a) the ability of cells to transport and utilize glucose in the generation of ATP for energy production in the presence of oxygen – also known as Warburg Effect and (b) alterations in the mitochondrial structure and function – including changes in the electron transport leading to a decrease in the production of mitochondrial ATP. The past few years has seen a resurgence in the investigating the central role of cellular bioenergetics in the etiology of cancer i.e. viewing cancer as a metabolic disease.

Historically, although mutations in genes has been thought to be responsible for changes in gene expression, there is accumulating literature in support of epigenetic processes playing a critical role in influencing gene expression in supporting carcinogenesis. This is evidenced by the observation that mutation rate for most genes is low and cannot account for the numerous (spectrum of) mutations found in the cancer cells. Epigenetic alteration is regulated by methylation and modification of histone tails, both changes inherently linked to the energy (nutrient) status of the cells since they require the availability of co-factors e.g. acetyl CoA requirement for histone acetylation (ref). The biosynthesis of acetyl CoA depends on glycolysis and Kreb's Cycle, directly linking the intracellular energy status to regulation of gene expression and activity.

In normal cells, mitochondrial oxidative phosphorylation generates sufficient ATP to meet the energy demands for maintaining normal physiological activities and cell survival. A consequence of mitochondrial energy production is the generation of

reactive oxygen species (ROS), aberrant production of which leads to damage of mitochondria (refs). It is well established that chronic ROS generation by the mitochondria leads to cumulative accumulation of genetic mutations, a phenomenon that has been implicated in the etiology of carcinogenesis. It has been suggested that cancer cells decrease mitochondrial respiration to minimize ROS generation, and switch to glycolysis to sustain energy production. Thus, a progressive shift of energy generation from oxidative phosphorylation to glycolysis would be essential for a cell to maintain energy production to maintain physiological functions and could be associated with the progression of a normal cell phenotype to that of a cancer cell. The progressive shift in cellular energy (bioenergetic) profile in tandem with accumulated alteration (mutations) in mitochondrial genetic make-up alters the cellular metabolome. Changes in the whole cell metabolomic profile as a consequence of mitochondrial phosphorylation to glycolysis transition corresponds to an abnormal bioenergetic induced metabolomic profile and is the underlying cause supporting carcinogenesis. Targeted intervention using an endogenous molecule to elicit a cellular metabolomic shift towards conditions of a non-cancerous normal mitochondrial oxidative phosphorylation associated cellular bioenergetic state represents a therapeutic endpoint in the treatment of cancer.

Coenzyme Q10 as a MIM causing an Epi-Metabolomic Shift

The data presented herein demonstrates that treatment of normal and cancer cells with Coenzyme Q10 is associated with changes in the expression of proteins that regulate key biochemical terminals within the glycolysis – mitochondrial oxidative stress continuum. The combination of data describing assessment of protein expression by western blotting and oxygen consumption rates demonstrates that in normal cells, there is no significant alteration in normal glycolytic and mitochondrial respiration rates following exposure to Coenzyme Q10. Thus, the values for expression of the proteins and mitochondrial respiration rates in normal cell lines e.g. HDFa (normal human adult fibroblast), HASMC (normal human aortic smooth muscle cell), nFib (normal fibroblast) and HeKa (normal human keratinocytes) can be considered as representatives of baseline physiological state. Any deviation in expression of proteins and mitochondrial respiration rates in cancer cell lines, e.g. HepG2 (liver cancer), PaCa-2 (pancreatic cancer), MCF7 (breast cancer), SK-MEL (melanoma) and SCC-25 (squamous cell

carcinoma), is representative of alteration due to initiation/progression of the disease, in this case cancer. The experimental evidence provides support to the hypothesis that exposure of Coenzyme Q10 to cancer cells is associated with cellular pathophysiological reorganization that is reminiscent of normal cells. Specifically, the data provided herein demonstrates that Coenzyme Q10 exposure in cancer cells is associated with a shift in the glycolytic pathways and mitochondrial oxidative phosphorylation responsible for induction of global reorganization of cellular architecture to that observed in normal cells.

In normal cells, the end-points of glycolytic output are linked to mitochondrial oxidative phosphorylation (OXPHOS), i.e. generation of pyruvate from glucose via the glycolytic pathway for the entry into the Kreb's Cycle (also known as Tricarboxylic acid cycle, TCA, or Citric Acid Cycle) to generate reducing equivalents to support the mitochondrial OXPHOS for ATP production. Thus, in normal cells the expression and functional orientation of gene products involved in glycolysis is primed towards adequate generation of pyruvate and its entry into the Kreb's Cycle. Dysregulated expression and function of key proteins participating in glycolysis and Kreb's Cycle pathways in cancer cells results in enhanced glycolysis with a significant decrease in mitochondrial function. Exposure of cancer cells to Coenzyme Q10, an endogenous molecule that selectively influences the mitochondrial respiratory chain, alters (normalizes) expression of proteins of the glycolyis and Kreb's Cycle pathways to facilitate a bioenergetic shift such that energy production (i.e. ATP generation) is restored to the mitochondria.

EXPERIMENTAL PROCEDURE

Western Blot Experiment 1

The cells that were used for the experiment were HDFa, and MCF-7 cells that were treated or not with Coenzyme Q10 at two different concentrations, 50 μM and 100 μM, and harvested after 24 hours of treatment. The whole cell pellets were resuspended one at a time in 1 mL of C7 buffer and transferred to labeled 15 mL tubes. The samples were then sonicated in the cold room on ice using 6 sonic pulses with the setting at #14. The samples were spun for a short time to 2500 g after sonication and the samples

transferred to 2 ml tubes. The pH was verified of each sample (pH should be 9.0) using the foam remaining in the 50 mL sample tubes.

Alkylation and reduction of samples was performed for each sample by adding 10 ul of 1M acrylamide, 25 ul of tributylphoshene and incubation for 90 mins with intermittent mixing. After incubation, 10 ul of 1M DTT was added and the tubes were spun at 20,000 g at 20 deg C for 10 minutes and transferred the supernatant to labeled Amicon Ultra centrifugal filter units with a 10 k cut off (Millipore catalog # UFC 801024). The samples were spun for 15 minutes at 2500 g in 2 intervals. The conductivity was measured for Chaps alone as well as the samples using a conductivity meter. If the conductivity of samples is high, then 1 ml of chaps was added for buffer exchange and spun again at 2500 g until the volume was down to 250 ul. When the conductivity was 200 or less the samples were spun in 5 min intervals at 2500 g until the volume of the supernatant was between 150-100 ul. The sample supernatants were transferred to eppendorf tubes and Bradford assay was performed using BSA as standard.

The samples were processed as per standard protocols as described above and the amount of protein in each of the samples was determined by Bradford assay. Sample volumes equivalent to 10 ug of protein were prepared as shown below with Lamelli Loading dye (LDS) and MilliQ water were run on a 4-12% Bis-Tris Novex NuPAGE gel (Invitrogen, cat # NP0323Box)

The gels were run for 50 minutes using 1X MOPS buffer using a NOVEX Xcell Surelock system at 200 V. The gels were then transferred for 1 hour using a NOVEX Xcell Surelock wet transfer protocol at 30 V. The blots were stained with Simply Blue Safestain from Invitrogen (LC6065).

IDH1 and ATP Citrate Lyase levels in HDFa and MCF-7 samples.

After transfer each of the blots was placed in between 2 Whatman Filter papers and dried for 15-20 minutes. After drying the blots were labeled with the date, the type of samples and either blot 1 or blot 2 using a HB pencil. The molecular weight markers were outlined with the pencil and with single lines for the blue and a doublet for the colored markers. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with

5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each). Blot 1 was probed with the primary antibody for IDH1 (Cell Signaling # 3997) in TBST with 5% BSA (at 1:1000 dilutions) and blot 2 with the rabbit polyclonal antibody for ATP Citrate Lyase in 5% BSA (Cell Signaling #4332) at 1:1000 dilution by incubation overnight at 4 deg C with shaking. After the overnight incubation with primary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 mins and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Actin levels in HDFa and MCF-7 samples.

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The 2 blots were scanned in laser scanner to check for complete stripping. The blots were then activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the antibody for Actin in 5% BSA (Sigma catalog # A5316, clone AC-74) at 1:5000 dilutions for 1 hour at room temperature with shaking. After 1 hour of incubation with primary antibody for Actin, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antimouse; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Western Blot Experiment 2

The cells used in this experiment were SKMEL28, SCC-25, nFib and Heka that were treated or not with coenzyme Q10 at two different concentrations, 50 μM or 100 μM, and harvested after 3, 6 and/or 24 hours of treatment. The samples were processed and run on a 4-12% Bis-Tris Novex NuPAGE gel as described above. The gels were run, transferred and stained essentially as described above.

Levels of IDH1 for the 4 Cell lines

After transfer the blot was dried for 15-20 minutes, activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blot was blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each). This was then probed with the primary antibody for IDH1 (Cell Signaling # 3997) in TBST with 5% BSA (at 1:1000 dilutions) by incubation overnight at 4 deg C with shaking. After the overnight incubation with primary antibody for IDH1, the blot was washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antirabbit; 1:10,000 dilution) for 1 h at room temperature. After 1 h of incubation with secondary antibodies, the blot was washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 mins and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

ATP Citrate Lyase levels in 4 different cell lines.

The Isocitrate dehydrogenase blot was stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blot was scanned in laser scanner to check for complete stripping. The blot was activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blot was blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each). This was then probed with the rabbit polyclonal antibody for ATP Citrate Lyase in 5% BSA (Cell Signaling #4332) at 1:1000 dilution overnight at 4

deg C with shaking. After the overnight incubation with primary antibody for ATP Citrate Lyase, the membrane was washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blot was washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Actin levels in 4 different cell lines.

The ATP Citrate Lyase blot was stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blot was scanned in laser scanner to check for complete stripping. The blot was activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blot was blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the antibody for Actin in 5% BSA (Sigma catalog # A5316, clone AC-74) at 1:5000 dilutions for 1 hour at room temperature with shaking. After 1 hour of incubation with primary antibody for Actin, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antimouse; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Western Blot Experiment 3

The cells used in this experiment were HepG2, HASMC, and PACA2 cells that were treated or not with Coenzyme Q10 at two different concentrations (50 μM and 100 μM) and harvested 48 hours of treatment. In this experiment (western blot experiment 3), and in all of the experiments described below in this Example (*i.e.*, western blot experiments 4 through 9) , the cells were additionally treated with either 5 mM glucose

("5G") or 22 mM glucose ("22G"). The samples derived from the cells were processed and run on a 4-12% Bis-Tris Novex NuPAGE gel as described above. The gels were run, transferred and stained essentially as described above.

IDH1, ATP Citrate Lyase and Actin levels in HASMC vs. PACA2 and HepG2.

The levels of IDH1, ATP citrate lyase and actin levels were determined by probing the blots with primary antibodies for IDH1, ATP citrate lyase and actin, essentially as described above.

Western Blot Experiment 4

The cells used in this experiment were HepG2 cells that were treated or not with Coenzyme Q10 at two different concentrations, 50 or 100 μM, and harvested after 24 or 48 hours of treatment. The samples were processed and run on a 4-12% Bis-Tris Novex NuPAGE gel as described above. The gels were run, transferred and stained essentially as described above.

Lactate Dehydrogenase levels in* HepG2 *cells.

After transfer each blot was dried for 15-20 minutes, activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for Lactate Dehydrogenase (abcam ab2101; polyclonal) in 5% BSA (at 1:1000 dilutions) by incubation overnight at 4 deg C with shaking. After the overnight incubation with primary antibody for Lactate Dehydrogenase, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (rabbit antigoat; 1:10,000 dilution) for 1 h at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 mins and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Pyruvate Kinase Muscle form (PKM2) levels in HepG2 cells.

The lactate dehydrogenase blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The 2 blots were scanned in laser scanner to check for complete stripping. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the rabbit polyclonal antibody for Pyruvate Kinase M2 in 5% BSA (NOVUS BIOLOGICALS catalog # H00005315-D01P) at 1:500 dilution overnight at 4deg C with shaking. After the overnight incubation with primary antibody for Pyruvate Kinase M2, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Pyruvate Dehydrogenase beta levels in HepG2 cells.

The pyruvate kinase blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The 2 blots were scanned in laser scanner to check for complete stripping. After making sure stripping of the antibody and the ECF reagent has worked, the blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots are blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the antibody for Pyruvate Dehydrogenase in 5% BSA (ABNOVA catalog # H00005162-M03) at 1:500 dilutions) overnight at 4deg C with shaking. After the overnight incubation with primary antibody for Pyruvate Dehydrogenase, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each)

and probed with the secondary antibody (antimouse; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Actin levels in HepG2 cells.

The Pyruvate Dehydrogenase blots were stripped and then reprobed for actin, essentially as described above.

Western Blot Experiment 5

The cells used in this experiment were MIAPACA2 (PACA2) cells that were treated or not with Coenzyme Q10 at two different concentrations, 50 or 100 μM, and harvested after 24 or 48 hours of treatment. The PACA2 samples were processed and the gels were run, transferred, stained and scanned essentially as described above.

Lactate Dehydrogenase (LDH) and* Pyruvate Dehydrogenase *(PDH) levels in PaCa2 cells

The levels of LDH and PDH were determined by probing the blots successively with primary antibodies for LDH and PDH, essentially as described above.

Caspase 3 levels in PaCa2 cells.

The blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The 2 blots were scanned in laser scanner to check for complete stripping. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the antibody for Caspase 3 in 5% BSA (Santacruz Biotechnology # sc7272) at 1:200 dilutions) overnight at 4deg C with shaking. After the overnight incubation with primary antibody for Caspase 3, the membranes were washed 3 times with TBS-T (1X-

15'; 2X 5' each) and probed with the secondary antibody (antimouse; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Western Blot Experiment 6

The cells that were used for this Western blot experiment were PC-3, HepG2, MCF-7, HDFa and PACA2 that were treated or not with a Coenzyme Q10 IV formulation and harvested after 24 hours of treatment. The samples were processed and the gels were run, transferred, stained and scanned essentially as described above.

Capase 3 and Actin levels in different cell types.

The levels of Caspase 3 and actin were determined by probing the blots successively with primary antibodies for Caspase 3 and actin, essentially as described above.

Western Blot Experiment 7

The cells used in this experiment were Human Aortic Smooth Muscle (HASMC) cells that were treated or not with Coenzyme Q10 at two different concentrations, 50 µM or 100 µM, and harvested after 24 or 48 hours of treatment. The HASMC samples were processed and the gels were run, transferred, stained and scanned essentially as described above.

Experimental Protocol for Actin:

The levels of actin were determined by probing the blots with a primary antibody for actin, essentially as described above.

Experimental Protocol for Hif 1alpha, Caspase3 and PDHB:

The Actin blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-

T for 30' each. The blots were scanned in laser scanner to check for complete stripping. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for Hif 1 alpha, Caspase 3 or PDHB in 5% BSA (at 1:200 by incubation overnight at 4 deg C with gentle shaking. The primary antibody for Hif 1 alpha (Abcam ab2185; antirabbit) was at 1:500 dilution in 5% BSA. The primary antibody for Caspase 3 (Santacruz sc7272; antirabbit) was at 1:200 dilution in 5% BSA. The primary antibody for Pyruvate Dehydrogenase beta (PDHB) (Novus Biologicals H00005162-M03; antimouse) was at 1:500 dilution in 5% BSA. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (PDHB antimouse; Hif 1a and Caspase 3 antirabbit; 1:10,000 dilution) for 1 h at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Experimental Protocol for PKM2, SDHB and SDHC:

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were scanned in laser scanner to check for complete stripping. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for PKM2, SDHB or SDHC in 5% BSA in TBS-T by incubation overnight at 4 deg C with gentle shaking. The primary antibody for SDHC (ABNOVA H00006391-M02; antimouse) was at 1:500 dilution. The primary antibody for SDHB was from Abcam ab4714-200; antimouse; at 1:1000 dilution. The primary antibody for Pyruvate Kinase M2 (PKM2) was from Novus Biologicals H00005315-D0IP; antirabbit; at 1:500 dilution. After incubation with primary

antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (SDHB & C antimouse; and PKM2 antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Experimental Protocol for LDH and Bik:

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were scanned in laser scanner to check for complete stripping. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for LDH or Bik in 5% BSA in TBS-T by incubation overnight at 4 deg C with gentle shaking. The primary antibody for LDH was from Abcam ab2101; antigoat; at 1:1000 dilution. The primary antibody for Bik was from Cell Signaling #9942; antirabbit; at 1:1000 dilution. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (LDH antigoat; Jackson Laboratories) and Bik antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Western Blot Experiment 9

The cells used were HepG2 cells that were treated or not with Coenzyme Q10 at two different concentrations, 50 μM or 100 μM, and harvested after 24 or 48 hours of treatment. The HepG2 samples processed and the gels were run, transferred, stained and scanned essentially as described above.

Experimental Protocol for Actin:

The levels of actin were determined by probing the blots with a primary antibody for actin, essentially as described above.

Experimental Protocol for Caspase3 and MMP-6:

The Actin blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for Caspase 3 or MMP-6 in 5% BSA by incubation overnight at 4 deg C with gentle shaking. The primary antibody for Caspase 3 (Abcam ab44976-100; antirabbit) was at 1:500 dilution in 5% BSA. The primary antibody for MMP-6 (Santacruz scMM0029-ZB5; antimouse) was at 1:100 dilution in 5% BSA. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (MMP-6 antimouse; Caspase 3 antirabbit; 1:10,000 dilution) for 1 h at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Experimental Protocol for LDH:

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots ere blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for LDH in 5% BSA or 5%

milk by incubation overnight at 4 deg C with gentle shaking. The primary antibody for LDH 080309b1 (Abcam ab2101; antigoat) was at 1:1000 dilution in 5% BSA. The primary antibody for LDH 080309b2 (Abcam ab2101; antigoat) was at 1:1000 dilution in 5% milk. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (Jackson Immuno Research antigoat; 1:10,000 dilution; 305-055-045) for 1 h. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400V and at 500 V.

Experimental Protocol for Transaldolase and Hif1a:

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots are blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for Transaldolase or Hif1a in 5% BSA by incubation overnight at 4 deg C with gentle shaking. The primary antibody for Transaldolase (Abcam ab67467; antimouse) was at 1:500 dilution. The primary antibody for Hif1a (Abcam ab2185; antirabbit) was at 1:500 dilution. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antimouse or antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400 & 500V.

Experimental Protocol for IGFBP3 and TP53:

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots are blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for IGFBP3 or TP53 in 5% BSA by incubation overnight at 4 deg C with gentle shaking. The primary antibody for IGFBP3 (Abcam ab76001; antirabbit) was at 1:100 dilution. The primary antibody for TP53 (Sigma Aldrich AV02055; antirabbit) was at 1:100 dilution. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antirabbit; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3 times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400 & 500V.

Experimental Protocol for Transaldolase and PDHB:

The above blots were stripped by incubating for 30 minutes with methanol, followed by two 10 minute washes with TBS-T, then 30 minutes of incubation with Stripping buffer at 50 deg C, and followed by two washes with 100 ml or more of TBS-T for 30' each. The blots were activated with methanol for 5 seconds, washed with water for 5 minutes, and TBST for 15 minutes. The blots were blocked for 1 hour with 5% blocking reagent in TBS-T at room temperature and then washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the primary antibody for Transaldolase or PDHB in 5% BSA by incubation overnight at 4 deg C with gentle shaking. The primary antibody for Transaldolase (Santacruz sc51440; antigoat) was at 1:200 dilution. The primary antibody for PDHB (Novus Biologicals H00005162-M03; antimouse) was at 1:500 dilution. After incubation with primary antibodies, the membranes were washed 3 times with TBS-T (1X-15'; 2X 5' each) and probed with the secondary antibody (antigoat or antimouse; 1:10,000 dilution) for 1 h on the orbital tilting shaker at room temperature. After 1 h of incubation with secondary antibodies, the blots were washed 3

times with TBS-T (1X-15'; 2X 5' each) and then incubated with ECF reagent for 5 minutes and then each blot scanned with 5100 Fuji Laser scanner at 25 uM resolution, 16 bit, green laser, at 400 & 500V.

RESULTS

Isocitrate Dehydrogenase- 1 (IDH-1)

Isocitrate dehydrogenase is one of the enzymes that is part of the TCA cycle that usually occurs within the mitochondrial matrix. However, IDH1 is the cytosolic form of the enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate and generates carbon dioxide in a two step process. IDH1 is the $NADP^+$ dependent form that is present in the cytosol and peroxisome. IDH1 is inactivated by Ser113 phosphorylation and is expressed in many species including those without a citric acid cycle. IDH1 appears to function normally as a tumor suppressor which upon inactivation contributes to tumorigenesis partly through activation of the HIF-1 pathway (Bayley 2010; Reitman, 2010). Recent studies have implicated an inactivating mutation in IDH1 in the etiology of glioblasotoma (Bleeker, 2009; Bleeker, 2010).

Treatment with Coenzyme Q10 increased expression of IDH1 in cancer cell lines including MCF-7, SKMEL28, HepG2 and PaCa-2 cells. There was a moderate increase in expression in the SCC25 cell lines. In contrast cultures of primary human derived fibroblasts HDFa, nFIB and the human aortic smooth muscle cells HASMC did not demonstrate significant changes in the expression pattern of the IDH1 in resposne to Coenzyme Q10. α-ketoglutarate (α-KG) is a key intermediate in the TCA cycle, biochemically synthesized from isocitrate and is eventually converted to succinyl coA and is a druggable MIM and EpiShifter. The generation of α-KG serves as a critical juncture in the TCA cycle as it can be used by the cell to replenish intermediates of the cycle, resulting in generation of reducing equivalents to increase oxidative phosphorylation. Thus, Coenzyme Q10 mediated increase in IDH1 expression would result in formation of intermediates that can be used by the mitochondrial TCA cycle to augment oxidative phosphorylation in cancer cells. The results are summarized in the tables below.

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Table 29: IDH1 in HDFa and MCF-7

Composition	Average Normalized Intensity
HDF, Media	346
HDF24-50-Coenzyme Q10	519
HDF24-100-Coenzyme Q10	600
MCF, Media	221
MCF24-50-Coenzyme Q10	336
MCF24-100-Coenzyme Q10	649

Table 30: IDH1 in HASMC vs. HepG2 after Treatment

Amount - Composition	Normalized Intensity
HAS5g48-media	20
HAS5g48-50-Coenzyme Q10	948
HAS5g48-100-Coenzyme Q10	1864
HAS22G48-Media	1917
HAS22G48-50-Coenzyme Q10	1370
HAS22G48-100-Coenzyme Q10	1023
Hep5g48-Media	14892
Hep5g48-50-Coenzyme Q10	14106
Hep5g48-100-Coenzyme Q10	15774
Hep22G48-Media	16558
Hep22G48-50-Coenzyme Q10	15537
Hep22G48-100-Coenzyme Q10	27878

Table 31: IDH1 in HASMC vs. PACA2 after Treatment

Amount - Composition	Normalized Intensity
HAS5g48-media	562
HAS5g48-50-Coenzyme Q10	509
HAS5g48-100-Coenzyme Q10	627
HAS22G48-Media	822
HAS22G48-50-Coenzyme Q10	1028

HAS22G48-100-Coenzyme Q10	1015
PACA5g48-Media	1095
PACA5g48-50-Coenzyme Q10	1095
PACA5g48-100-Coenzyme Q10	860
PACA22G48-Media	1103
PACA22G48-50-Coenzyme Q10	1503
PACA22G48-100-Coenzyme Q10	1630

ATP Citrate Lyase (ACL)

ATP citrate Lyase (ACL) is a homotetramer (~126kd) enzyme that catalyzes the formation of acteyl-CoA and oxaloacetate in the cytosol. This reaction is a very important first step for the biosynthesis of fatty acids, cholesterol, and acetylcholine, as well as for glucogenesis (Towle et al., 1997). Nutrients and hormones regulate the expression level and phosphorylation status of this key enzyme. Ser454 phosphorylation of ACL by Akt and PKA has been reported (Berwick., DC MW et al., 2002; Pierce MW et al., 1982).

The data describes the effect of Coenzyme Q10 on ATP citrate Lyase is that in normal and cancer cells. It is consistently observed that in cancer cells there is a dose-dependent decrease in the expression of ACL enzymes. In contrast there appears to be a trend towards increased expression of ACL in normal cells. Cytosolic ACL has been demonstrated to be essential for histone acetylation in cells during growth factor stimulation and during differentiation. The fact that ACL utilizes cytosolic glucose derived citrate to generate Acetyl CoA essential for histone acetylation, a process important in the neoplastic process demonstrates a role of Coenzyme Q10 induced ACL expression in influencing cancer cell function. Acetyl CoA generated from citrate by cytosolic ACL serves as a source for biosynthesis of new lipids and cholesterol during cell division. Thus, Coenzyme Q10 induced changes in ACL expression alters Acetyl CoA availability for synthesis of lipids and cholesterol in normal versus cancer cells. The results are summarized in the tables below.

Table 32: ATPCL in HDFa and MCF-7

Composition	Average Normalized Intensity
HDF-Media	~ 15000
HDF-50-Coenzyme Q10	~ 17500
HDF-100-Coenzyme Q10	~ 25000
MCF-Media	~ 7500
MCF-50-Coenzyme Q10	~7500
MCF-100-Coenzyme Q10	~ 12500

Table 33: ATP Citrate Lysase ~kd band in HASMC vs. HepG2

Amount - Composition	Normalized Intensity
HAS5g48-media	24557
HAS5g48-50-Coenzyme Q10	23341
HAS5g48-100-Coenzyme Q10	25544
HAS22G48-Media	27014
HAS22G48-50-Coenzyme Q10	21439
HAS22G48-100-Coenzyme Q10	19491
Hep5g48-Media	28377
Hep5g48-50-Coenzyme Q10	24106
Hep5g48-100-Coenzyme Q10	22463
Hep22G48-Media	24262
Hep22G48-50-Coenzyme Q10	31235
Hep22G48-100-Coenzyme Q10	50588

Table 34: ATP Citrate Lysase ~kd band in HASMC vs. PACA2

Amount - Composition	Normalized Intensity
HAS5g48-media	11036
HAS5g48-50-Coenzyme Q10	12056
HAS5g48-100-Coenzyme Q10	15265
HAS22G48-Media	18270
HAS22G48-50-Coenzyme Q10	15857
HAS22G48-100-Coenzyme Q10	13892

PACA5g48-Media	11727
PACA5g48-50-Coenzyme Q10	8027
PACA5g48-100-Coenzyme Q10	4942
PACA22G48-Media	8541
PACA22G48-50-Coenzyme Q10	9537
PACA22G48-100-Coenzyme Q10	14901

Table 35: ATP Citrate Lysase in HepG2 and PACA2 as % of CTRL

Amount - Composition	Normalized Intensity
PACA5g48-Media	1.00
PACA5g48-50-Coenzyme Q10	0.68
PACA5g48-100-Coenzyme Q10	0.42
PACA22G48-Media	1.00
PACA22G48-50-Coenzyme Q10	1.12
PACA22G48-100-Coenzyme Q10	1.74
Hep5g48-Media	1.00
Hep5g48-50-Coenzyme Q10	0.85
Hep5g48-100-Coenzyme Q10	0.79
Hep22G48-Media	1.00
Hep22G48-50-Coenzyme Q10	1.29
Hep22G48-100-Coenzyme Q10	2.09

Pyruvate Kinase M2 (PKM2)

Pyruvate Kinase is an enzyme involved in the glycolytic pathway. It is responsible for the transfer of phosphate from phosphoenolpyruvate (PEP) to adenosine diphosphophate (ADP) to generate ATP and pyruvate. PKM2 is an isoenzyme of the glycolytic pyruvate kinase, expression of which is characterized by the metabolic function of the tissue i.e. M2 isoenzyme is expressed in normal rapidly proliferating cells with high energy needs such as embryonic cells and also expressed in few normal differentiated tissues such as lung and pancreatic islet cells that require high rate of nucleic acid synthesis. PKM2 is highly expressed in tumor cells due to their dependence on glycolytic pathway for meeting cellular energetic requirements. The PKM2 isoform

normally thought to be embryonically restricted is re-expressed in cancerous cells. Cells expressing PKM2 favor a stronger aerobic glycolytic phenotype (show a shift in metabolic phenotype) with increased lactate production and decreased oxidative phosphorylation. Thus, decrease in expression of PKM2 in cancer cells would shift or down-regulate energy generation via the glycolytic pathway, a strategy that is useful in the treatment of cancer. Data demonstrates variable expression pattern of PKM2 in normal and cancer cells, with cancer cells demonstrating higher levels of expression compared to normal. Treatment of cells with Coenzyme Q10 altered expression pattern of the PKM2 upper and lower band levels in normal and cancer cells. In cancer cells tested, there was a dose-dependent decrease in the PKM2 expression, and no major changes in normal cells were observed. The results are summarized in the tables below.

Table 36: Pyruvate Kinase Muscle form 2 Upper Band in HepG2

Amount - Composition	Normalized Volume (24 h)	Normalized Intensity (48 h)
5g-Media	28386	413
5g-50-Coenzyme Q10	29269	303
5g-100-Coenzyme Q10	18307	354
22G-Media	25903	659
22G-50-Coenzyme Q10	22294	562
22G-100-Coenzyme Q10	19560	601

Table 37: Pyruvate Kinase Muscle form 2 Lower Band (58 KD) in HepG2

Amount - Composition	Normalized Volume (24 h)	Normalized Volume (48 h)
5g-Media	10483	310
5g-50-Coenzyme Q10	11197	185
5g-100-Coenzyme Q10	7642	122

22G-Media	9150	306
22G-50-Coenzyme Q10	6302	344
22G-100-Coenzyme Q10	6904	465

Table 38: Pyruvate Kinase Muscle form 2 Upper Band in HASMC Cells after Treatment

Amount - Composition	Normalized Intensity
5g48-Media	608
5g48-50-Coenzyme Q10	811
5g48-100-Coenzyme Q10	611
22G48-Media	516
22G48-50-Coenzyme Q10	595
22G48-100-Coenzyme Q10	496
22G24-Media	301
22G24-50-Coenzyme Q10	477
22G24-100-Coenzyme Q10	701

Lactate Dehydrogenase (LDH)

LDH is an enzyme that catalyzes the interconversion of pyruvate and lactate with the simultaneous interconversion of NADH and NAD^+. It has the ability to convert pyruvate to lactate (lactic acid) under low cell oxygen tension for generation of reducing equivalents and ATP generation at the expense of mitochondrial oxidative phosphorylation. Cancer cells typically demonstrate increased expression of LDH to maintain the glycolytic flux to generate ATP and reducing equivalents and reducing mitochondrial OXPHOS. Thus, reducing the expression of the LDH in cancer cells would shift metabolism from generation of lactate to facilitate entry of pyruvate into the TCA cycle. Treatment with Coenzyme Q10 reduced Lactate Dehydrogenase (LDH) expression in cancer with minimal effect on normal cells, supporting a role for Coenzyme Q10 in eliciting a shift in cancer cell bioenergtics for the generation of ATP from glycolytic to mitochondrial OXPHOS sources by minimizing the conversion of cytoplasmic pyruvate to lactic acid. The results are summarized in the tables below.

Table 39: Lactate Dehydrogenase in HepG2

Amount - Composition	Normalized Volume (24 h)	Normalized Volume (48 h)
5g-Media	7981	5997
5g-50-Coenzyme Q10	7900	5188
5g-100-Coenzyme Q10	6616	7319
22G-Media	9171	7527
22G-50-Coenzyme Q10	7550	6173
22G-100-Coenzyme Q10	7124	9141

Table 40: Lactate Dehydrogenase in HepG2 as % Control from 2 Experiments

Amount - Composition	Average Volume as a % of Control
5g24-Media	1.00
5g24-50-Coenzyme Q10	0.64
5g24-100-Coenzyme Q10	1.06
5g48-Media	1.00
5g48-50-Coenzyme Q10	1.12
5g48-100-Coenzyme Q10	1.21
22G24-Media	1.00
22G24-50-Coenzyme Q10	1.21
22G24-100-Coenzyme Q10	1.44
22G48-Media	1.00
22G48-50-Coenzyme Q10	0.95
22G48-100-Coenzyme Q10	0.67

5

Table 41: Lactate Dehydrogenase in PACA2

Amount - Composition	Normalized Volume (24 h)	Normalized Volume (48 h)
5g-Media	2122	2360
5g-50-Coenzyme Q10	5068	2978

5g-100-Coenzyme Q10	3675	2396
22G-Media	4499	2332
22G-50-Coenzyme Q10	10218	2575
22G-100-Coenzyme Q10	7158	3557

Pyruvate Dehydrogenase – B (PDH-E1)

Pyruvate Dehydrogenase beta (PDH-E1) is the first enzyme component that is part of the pyruvate dehydrogenase complex (PDC) that converts pyruvate to acetyl CoA. PDH-E1 requires thiamine as cofactor for its activity, performs the first two biochemical reactions in the PDC complex essential for the conversion of pyruvate to acetyl CoA to enter the TCA cycle in the mitochondria. Thus, concomitant decreases in PKM2 and LDH expression along with increase in expression of PDH-E1 in cancer cells would enhance the rate of entry of pyruvate towards augmenting the mitochondrial OXPHOS for generation of ATP. The data shows that for expression of PDH-E1 in normal and cancer cell lines, the baseline expressions of this enzyme is decreased in cancer compared to normal cells. Treatment with Coenzyme Q10 is associated with progressive increase in the expression of the PDH-E1 proteins in cancer cells with minimal changes in the normal cells. The results are summarized in the tables below.

Table 42: Pyruvate Dehydrogenase Beta in HepG2

Amount - Composition	Normalized Volume (24 h)	Normalized Volume (48 h)
5g-Media	517	100
5g-50-Coenzyme Q10	921	123
5g-100-Coenzyme Q10	433	205
22G-Media	484	181
22G-50-Coenzyme Q10	426	232
22G-100-Coenzyme Q10	340	456

Table 43: Pyruvate Dehydrogenase Beta in PACA2

Amount - Composition	Normalized Volume (24 h)	Normalized Volume (48 h)
5g-Media	323	375
5g-50-Coenzyme Q10	492	339
5g-100-Coenzyme Q10	467	252
22G-Media	572	276
22G-50-Coenzyme Q10	924	279
22G-100-Coenzyme Q10	1201	385

Table 44: Pyruvate Dehydrogenase Beta in HASMC after Treatment

Amount - Composition	Normalized Volume
5g48-Media	140
5g48-50-Coenzyme Q10	147
5g48-100-Coenzyme Q10	147
22G48-Media	174
22G48-50-Coenzyme Q10	149
22G48-100-Coenzyme Q10	123
22G24-Media	140
22G24-50-Coenzyme Q10	145
22G24-100-Coenzyme Q10	150

Caspase 3

Control of the onset of apoptosis is often exerted at the level of the initiator caspases, caspase-2, -9 and -8/10. In the extrinsic pathway of apoptosis, caspase-8, once active, directly cleaves and activates executioner caspases (such as caspase-3). The active caspase-3 cleaves and activates other caspases (6, 7, and 9) as well as relevant targets in the cells (e.g. PARP and DFF). In these studies, the levels of effectors caspase-3 protein were measured in the cancer cell lines and in normal cell lines in response to Coenzyme Q10. It should be noted although control of apoptosis is through initiator caspases, a number of signaling pathways interrupt instead the transmission of the

apoptotic signal through direct inhibition of effectors caspases. For e.g. P38 MAPK phosphorylates caspase-3 and suppresses its activity (Alvarado-Kristensson et al., 2004). Interestingly, activation of protein phosphates (PP2A) in the same study or protein kinase C delta (PKC delta) (Voss et al., 2005) can counteract the effect of p38 MAPK to amplify the caspase-3 activation and bolster the transmission of the apoptotic signal. Therefore, events at the level of caspase-3 activation or after Caspase 3 activation may determine the ultimate fate of the cell in some cases.

Caspase-3 is a cysteine-aspartic acid protease that plays a central role in the execution phase of cell apoptosis. The levels of caspase 3 in the cancer cells were increased with Coenzyme Q10 treatment. In contrast the expression of Caspase-3 in normal cells was moderately decreased in normal cells. The results are summarized in the tables below.

Table 45: Caspase 3 in PACA2

Amount-Composition	Normalized Volume (24 h)	Normalized Volume (48 h)
5g-Media	324	300
5g-50-Coenzyme Q10	325	701
5g-100-Coenzyme Q10	374	291
22G-Media	344	135
22G-50-Coenzyme Q10	675	497
22G-100-Coenzyme Q10	842	559

Table 46: Caspase 3 in HepG2 cells as % Control from 2 Experiments

Amount - Composition	NormalizedVolume as a % of Control
5g24-Media	1..00
5g24-50-Coenzyme Q10	1.08
5g24-100-Coenzyme Q10	1.76
5g48-Media	1.00
5g48-50-Coenzyme Q10	1.44

5g48-100-Coenzyme Q10	0.95
22G24-Media	1.00
22G24-50-Coenzyme Q10	1.39
22G24-100-Coenzyme Q10	1.78
22G48-Media	1.00
22G48-50-Coenzyme Q10	1.50
22G48-100-Coenzyme Q10	1.45

Table 47: Caspase 3 in HASMC after Treatment

Amount - Composition	Normalized Volume
5g48-Media	658
5g48-50-Coenzyme Q10	766
5g48-100-Coenzyme Q10	669
22G48-Media	846
22G48-50-Coenzyme Q10	639
22G48-100-Coenzyme Q10	624
22G24-Media	982
22G24-50-Coenzyme Q10	835
22G24-100-Coenzyme Q10	865

Succinate Dehydrogenase (SDH)

Succinate dehydrogenase, also known as succinate-coenzyme Q reductase is a complex of the inner mitochondrial membrane that is involved in both TCA and electron transport chain. In the TCA, this complex catalyzes the oxidation of succinate to fumarate with the concomitant reduction of ubiquinone to ubiquinol. (Baysal et al., Science 2000; and Tomlinson et al., Nature Genetics 2002). Germline mutations in SDH B, C and D subunits were found to be initiating events of familial paraganglioma or leiomyoma (Baysal et al., Science 2000).

Western blotting analysis was used to characterize expression of SDH Subunit B in mitochondrial preparations of cancer cells treated with Coenzyme Q10. The results suggest that Coenzyme Q10 treatment is associated with increase SDH protein levels in the mitochondrion of the cells. These results suggest one of the mechanisms of action of

Coenzyme Q10 is to shift the metabolism of the cell towards the TCA cycle and the mitochondrion by increasing the levels of mitochondrial enzymes such as SDHB. The results are summarized in the table below.

Table 48: Succinate Dehydrogenase B in NCIE0808 Mitopreps

Composition - Time	Average Normalized Volume
Media	531
50 uM Coezyme Q10, 3h	634
100 uM Coenzyme Q10, 3h	964
50 uM Coenzyme Q10, 6h	1077
100 uM Coenzyme Q10, 6h	934

Hypoxia Induced Factor - 1

Hypoxia inducible factor (Hif) is a transcription factor composed of alpha and beta subunits. Under normoxia, the protein levels of Hif1 alpha are very low owing to its continuous degradation via a sequence of post translational events. The shift between glycolytic and oxidative phosphorylation is generally considered to be controlled by the relative activities of two enzymes PDH and LDH that determine the catabolic fate of pyruvate. Hif controls this crucial bifurgation point by inducing LDH levels and inhibiting PDH activity by stimulating PDK. Due to this ability to divert pyruvate metabolism from mitochondrion to cytosol, Hif is considered a crucial mediator of the bioenergetic switch in cancer cells.

Treatment with Coenzyme Q10 decreased Hif1 alpha protein levels after in mitochondrial preparations of cancer cells. In whole cell lysates of normal cells, the lower band of Hif1a was observed and showed a decrease as well. The results are summarized in the tables below.

Table 49: Hif1 alpha Lower Band in HASMC Cells after Treatment

Amount - Composition	Normalized Volume
5g48-Media	22244
5g48-50-Coenzyme Q10	21664

5g48-100-Coenzyme Q10	19540
22G48-Media	14752
22G48-50-Coenzyme Q10	17496
22G48-100-Coenzyme Q10	23111
22G24-Media	21073
22G24-50-Coenzyme Q10	18486
22G24-100-Coenzyme Q10	17919

Table 50: Hif1 alpha Upper Band in HepG2 after Treatment

Amount - Composition	Normalized Volume
5g24-Media	12186
5g24-50-Coenzyme Q10	8998
5g24-100-Coenzyme Q10	9315
5g48-Media	8868
5g48-50-Coenzyme Q10	8601
5g48-100-Coenzyme Q10	10192
22G24-Media	11748
22G24-50-Coenzyme Q10	14089
22G24-100-Coenzyme Q10	8530
22G48-Media	8695
22G48-50-Coenzyme Q10	9416
22G48-100-Coenzyme Q10	5608

EXAMPLE 21: Analysis of Oxygen Consumption Rates (OCR) and Extracellular Acidification (ECAR) in Normal and Cancer Cells Treated with CoQ10

This example demonstrates that exposure of cells to treatment by a representative MIM / epi-shifter of the invention - CoQ10 - in the absence and/or presence of stressors (e.g., hyperglycemia, hypoxia, lactic acid), is associated with a shift towards glycolysis / lactate biosynthesis and mitochondrial oxidative phosphorylation (as measured by

ECAR and OCR values) representative of values observed in a normal cells under normal physiological conditions.

Applicants have demonstrated in the previous section that treatment with CoQ10 in cancer cells is associated with changes in expression of specific proteins that enhance mitochondrial oxidative phosphorylation, with a concomitant decrease in glycolysis and lactate biosynthesis. This example shows that a direct measure of mitochondrial oxidative phosphorylation can be obtained by measuring the oxygen consumption rates (OCR) in cell lines using the SeaHorse XF analyzer, an instrument that measures dissolved oxygen and extracellular pH levels in an *in vitro* experimental model. (SeaHorse Biosciences Inc, North Billerica, MA).

The pH of the extracellular microenvironment is relatively acidic in tumors compared to the intracellular (cytoplasmic) pH and surrounding normal tissues. This characteristic of tumors serves multiple purposes, including the ability to invade the extracellular matrix (ECM), a hallmark attribute of tumor metastasis that subsequently initiates signaling cascades that further modulate:

- tumor angiogenesis
- increased activation of arrest mechanisms that control cell cycle turn-over
- immuno-modulatory mechanisms that facilitate a cellular evasion system against immunosurveillance
- metabolic control elements that increase dependency on glycolytic flux and lactate utilization
- dysregulation of key apopototic gene families such as Bcl-2, IAP, EndoG, AIF that serve to increase oncogenicity

While not wishing to be bound by any particular theory, the acidic pH of the external microenvironment in the tumor is a consequence of increase in hydrogen ion concentrations extruded from the tumor cells due to the increased lactate production from an altered glycolytic phenotype.

In this experiment, the OCR and extracellular acidification rate (ECAR) in normal cells lines were obtained in the presence and absence of CoQ10 to determine baseline values. It was observed that in its native nutrient environment, the basal OCR

rates in normal cells lines are different, and are usually a function of the physiological roles of the cells in the body.

For example, one set of experiments were conducted using the non-cancerous cell line HDFa, which is a human adult dermal fibroblast cell line. Fibroblasts are cells that primarily synthesize and secrete extracellular matrix (ECM) components and collagen that form the structural framework (stroma) for tissues. In addition, fibroblasts are known to serve as tissue ambassadors of numerous functions such as wound healing and localized immnomodulation. Under normal physiological conditions, energy requirements in normal fibroblasts are met using a combination of glycolysis and oxidative phosphorylation - the glycolysis providing the necessary nutrients for synthesis of ECM.

In contrast to HDFa, the HASMC (human aortic smooth muscle cell) is found in arteries, veins, lymphatic vessels, gastrointestinal tracts, respiratory tract, urinary bladder and other tissues with the ability to undergo regulated excitation-contraction coupling. The ability of smooth muscles such as HASMC cells to undergo contraction requires energy provided by ATP. These tissues transition from low energy modes wherein ATP may be supplied from mitochondria to high energy modes (during exercise/stress) where energy is provided by switching to glycolysis for rapid generation of ATP. Thus, normal smooth muscle cells can use a combination of mitochondrial OXPHOS and glycolysis to meet their energy requirements under normal physiological environment.

The differences in their respective physiological roles (i.e., HDFa and HASMC) were observed in the resting OCR values measured in these cells lines using the SeaHorse XF analyzer. Figures 29 and 30 describe the OCR in HDFa and HASMC cells grown in physiologically normal glucose (about 4.6mM) and high glucose (hyperglycemic) conditions.

The baseline OCR values for HDFa in the absence of any treatments under normal oxygen availability is approximately 40 pmoles/min (Figure 29) in the presence of 5.5 mM glucose. This value was slightly elevated when the cells were maintained at 22 mM glucose. In contrast, in HASMC cells, the OCR values at 5.5 mM glucose is approximately 90 pmoles/min, and the OCR value declined to approximately 40

pmoles/min while at 22 mM glucose. Thus, under hyperglycemic conditions, there is a differential response between HDFa and HASMC, further demonstrating inherent differences in their respective physiological make-up and function.

Treatment with CoQ10 in cells is associated with changes in OCR that is representative of conditions observed at normal (5 mM) glucose conditions. The complexity of physiological response is compounded in the presence of low oxygen tension. Thus, CoQ10 exposure is associated with changes in OCR rates in normal cells towards a physiological state that is native to a particular cell.

Table 51 below describes the ECAR values (mpH/min) in HDFa cells in the presence or absence of CoQ10 under normoxic and hypoxic conditions at 5.5 mM and 22 mM glucose. It can be observed that in normal cells, treatment with CoQ10 had minimal influence on ECAR values, even though it influenced OCR in these cells. In high glucose hypoxic conditions, treatment with CoQ10 was associated with lowering of elevated ECAR to a value that was observed in untreated normoxic conditions.

Table 51: ECAR values in HDFa cells in the absence and presence of CoQ10 under normoxic and hypoxic conditions at 5.5 mM and 22 mM glucose

	Normoxia (5.5mM)		Hypoxia (5.5mM)		Normoxia (22mM)		Hypoxia (22mM)	
Treatment	ECAR	SEM	ECAR	SEM	ECAR	SEM	ECAR	SEM
Untreated	5	1.32	5	0.62	5	0.62	9	0.81
50μM 31510	6	1.11	5	0.78	5	0.78	6	0.70
100μM 31510	6	0.76	5	1.19	5	1.19	8	1.07

In Table 52 the measured baseline ECAR values (mpH/min) in HASMC were higher compared to that of HDFa. Induction of hypoxic conditions caused an increase in ECAR most likely associated with intracellular hypoxia induced acidosis secondary to increased glycolysis.

Table 52: ECAR values in HASMC cells in the absence and presence of CoQ10 under normoxic and hypoxic conditions at 5.5 mM and 22 mM glucose

	Normoxic (5.5mM)		Hypoxic (5.5mM)		Normoxic (22mM)		Hypoxic (22mM)	
Treatment	ECAR	SEM	ECAR	SEM	ECAR	SEM	ECAR	SEM
Untreated	9	2.22	11	2.18	22	2.08	19	1.45
50μM 31510	9	2.13	11	2.54	21	1.72	17	1.60
100μM 31510	9	1.72	13	2.30	22	1.64	17	1.47

Treatment with CoQ10 was observed to be associated with a downward trend of ECAR rates in hyperglycemic HASMC cells in hypoxic conditions towards a value that would be observed in normoxic normal glucose conditions. These data demonstrate the presence of physiological variables that is inherent to the physiological role of a specific type of cell, alterations observed in abnormal conditions (e.g. hyperglycemia) is shifted towards normal when treated with CoQ10.

In contrast, cancer cells (e.g., MCF-7, PaCa-2) are inherently primed to culture at higher levels of glucose compared to normal cells due to their glycolytic phenotype for maintenance in culture. Treatment with CoQ10 caused a consistent reduction in OCR values (Figure 31 and Figure 32).

The effects of CoQ10 on OCR values in MCF-7 and PaCa-2 cells was similar to that of the normal HDFa and HASMC cells, wherein the variable response was suggestive of a therapeutic response based on individual metabolic profile of the cancer cell line.

Table 53: ECAR values in PaCa-2 cells in the absence and presence of CoQ10 under normoxic and hypoxic conditions at 5.5 mM and 22 mM glucose

	Normoxia (17mM)		Hypoxia (17mM)		Normoxia (22mM)		Hypoxia (22mM)	
Treatment	ECAR	SEM	ECAR	SEM	ECAR	SEM	ECAR	SEM

Untreated	21	5.97	16	3.41	24	4.35	36	5.65
50μM 31510	13	3.08	12	1.66	20	5.15	25	4.58
100μM 31510	14	2.14	17	2.59	19	3.38	30	5.62

Table 53 describes the ECAR values in PaCa-2 cells. In contrast to normal cells, cancer cells are phenotypically primed to use high glucose for ATP generation (enhanced glycolysis) resulting in higher ECAR (Table 53, ECAR for untreated normoxia 17mM) at 21 mpH/min. Treatment with CoQ10 produces a significant decrease in ECAR rates under these conditions, most likely associated with a decrease in the glycolysis generated lactic acid. The associated decrease in OCR in these cells was likely associated with increased efficiency of the mitochondrial OXPHOS.

A similar comparison of OCR and ECAR values (data not shown) were determined in numerous other normal and cancer cells lines, including: HAEC (normal human aortic endothelial cells), MCF-7 (breast cancer), HepG2 (liver cancer) and highly metastatic PC-3 (prostate cancer) cell lines. In all of the cell lines tested, exposure to CoQ10 in the absence and/or presence of stressors (e.g., hyperglycemia, hypoxia, lactic acid) was associated with a shift in OCR and ECAR values representative of values observed in a normal cells under normal physiological conditions. Thus, the overall effect of CoQ10 in the treatment of cancer, including cell death, is an downstream effect of its collective influence on proteomic, genomic, metabolomic outcomes in concert with shifting of the cellular bioenergetics from glycolysis to mitochondrial OXPHOS.

EXAMPLE 22: Building Block Molecules for the Biosynthesis of CoQ10

This example demonstrates that certain precursors of CoQ10 biosynthesis, such as those for the biosynthesis of the benzoquinone ring, and those for the biosynthesis of the isoprenoid repeats and their attachment to the benzoquinone ring ("building block components"), can be individually administered or administered in combination to target cells, and effect down-regulation of the apoptosis inhibitor Bcl-2, and/or up-regulation of the apoptosis promoter Caspase-3. Certain precursors or combinations thereof may also inhibit cell proliferation. The data suggests that such CoQ10 precursors may be

used in place of CoQ10 to achieve substantially the same results as CoQ10 administration.

Certain exemplary experimental conditions used in the experiments are listed below.

Skmel-28 melanoma cells were cultured in DMEM/F12 supplemented with 5% Fetal Bovine Serum (FBS) and 1X final concentration of Antibiotics. The cells were grown to 85% confluency and treated with building block components for 3, 6, 12 and 24 hours. The cells were then pelleted and a Western blot analysis was performed.

The test building block components included L-Phenlylalanine, DL-Phenlyalanine, D-Phenlylalanine, L-Tyrosine, DL-Tyrosine, D-Tyrosine, 4-Hydroxy-phenylpyruvate, phenylacetate, 3-methoxy-4-hydroxymandelate (vanillylmandelate or VMA), vanillic acid, 4-hydroxy-benzoate, pyridoxine, panthenol, mevalonic acid, Acetylglycine, Acetyl-CoA, Farnesyl, and 2,3-Dimethoxy-5-methyl-*p*-benzoquinone.

In the Western Blot Analysis, the cells were pelleted in cold PBS, lysed, and the protein levels were quantified using a BCA protein assay. The whole cell lysate was loaded in a 4% loading 12% running Tris-HCl gel. The proteins were then transferred to a nitrocellulose paper then blocked with a 5% milk Tris-buffered solution for 1 hour. The proteins were then exposed to primary antibodies (Bcl-2 and Caspase-3) overnight. The nitrocellulose paper was then exposed to Pico Chemilluminescent for 5 min and the protein expression was recorded. After exposure, actin was quantified using the same method. Using ImageJ the levels of protein expression were quantified. A t-Test was used to analyze for statistical significance.

Illustrative results of the experiments are summarized below.

Western Blot Analysis of Building Block component L-Phenylalanine: Before proceeding to the synthesis pathway for the quinone ring structure, L-Phenylalanine is converted to tyrosine. A western blot analysis was performed to quantify any changes in the expression of the apoptotic proteins in the melanoma cells. The concentrations tested were 5 μM, 25 μM, and 100 μM. Initial studies added L-Phenylalanine to DMEM/F12 medium which contained a concentration of 0.4 M phenylalanine. For the 5 μM, 25 μM, and 100 μM the final concentration of the L-Phenylalanine in the medium was 0.405 M, 0.425 M, and 0.500 M, respectively. These final concentrations were tested on the Skmel-28 cells for incubation periods of 3,6, 12 and 24 hours. The cells

were grown to 80% confluency before adding the treatment medium and harvested using the western blot analysis procedure as described above. A statistically significant decrease in Bcl-2 was observed for the 100 μM L-Phenylalanine after 3 hours and 12 hours incubation. Fr the 5 μM L-phenylalanine, a statistically significant decrease in Bcl-2 was observed after 6 hours of incubation. For the 25 μM L-phenylalanine, a statistically significant decrease in Bcl-2 and a statistically significant increase in Caspase-3 were observed after 12 hours of incubation. A statistically significant decrease in Bcl-2 indicates a change in the apoptotic potential and a statistically significant increase in Caspase-3 confirms the cells are undergoing apoptosis. There was a constant trend for the decrease in Bcl-2 compared to the control even though, due to sample size and standard deviation, these time points were not statistically significant in this experiment.

Western Blot Analysis of Building Block component D-Phenylalanine: D-Phenylalanine, a chemically synthetic form of the bioactive L-Phenylalanine, was tested for comparison to L-phenylalanine. For all three concentrations (5 μM, 25 μM, and 100 μM of D-Phenylalanine, there was a significant reduction in Bcl-2 expression after 6 hours of incubation. In addition, for the 5 μM and 25 μM, there was a significant reduction after 3 hours of incubation. For the 5μM and 100 μM concentrations, a significant increase in Caspase-3 expression was observed after 6 hours of incubation.

Western Blot Analysis of Building Block component DL-Phenylalanine: DL-Phenylalanine was also tested for comparison to L-Phenylalanine. Again, concentrations of 5 μM, 25 μM, and 100 μM were tested on Skmel-28 cells. The incubation periods were 3, 6, 12 and 24 hours. A statistically significant increase in Caspase-3 was observed after 3 hours of incubation. A statistically significant decrease in Bcl-2 was observed after 24 hours of incubation. Although a decreasing Bcl-2 and increasing Caspase-3 trend at all other concentrations and incubation time points, they were not statistically significant in this experiment.

Western Blot Analysis of Building Block component L-Tyrosine: L-Tyrosine is a building block component for the synthesis of quinone ring structure of CoQ10. Initial testing of L-Tyrosine did not result in a high enough protein concentration for western blot analysis. From this study concentrations under 25 μM were tested for Western Blot Analysis. The DMEM/F12 medium used contained L-Tyrosine disodium salt

concentration of 0.398467 M. The initial concentration was increased by 500 nM, 5 μM, and 15 μM. A statistically significant increase in Caspase-3 was observed for the 500 nM concentration after 12 hours of incubation. A statistically significant increase in Caspase-3 was also observed for the 5A statistically significant decrease in Bcl-2 was observed for the 5 μM concentration after 24 hours of incubation. A statistically significant decrease in Bcl-2 was observed for the 500 μM and 5 μM concentrations after 24 hours of incubation.

Western Blot Analysis of Building Block component D-Tyrosine: D-Tyrosine, a synthetic form of L-Tyrosine, was tested for comparison against the L-Tyrosine apoptotic effect on the melanonal cells. Based on initial studies with L-Tyrosine, concentrations below 25 μM were chosen for the western blot analysis. The concentrations tested were 1 μm, 5 μM, and 15 μM. D-Tyrosine showed a reduction in Bcl-2 expression for the 5 μM and 15 μM concentrations for 12 and 24 hour time periods. Caspase-3 was significantly increased for the concentration of 5 μM for 3, 12 and 24 time periods. Also there was an increase in Caspase-3 expression for the 1 μM for 12 and 24 hour time period. In addition there is an increase in Caspase-3 expression for 5 μM for the 12 hour time period.

Western Blot Analysis of Building Block component DL-Tyrosine: DL-Tyrosine, a synthetic form of L-Tyrosine, was also tested for comparison against L-Tyrosine's apoptotic effect on the cells. There is a statistical decrease in Bcl-2 expression seen in the 1 μM and 15 μM concentrations after 12 hours incubation and for the 5 μM after 24 hour of incubation . An increase in Caspase-3 expression was also observed for the 5 μM and 15 μM after 12 hours of incubation.

Western Blot Analysis of Building Block component 4-Hydroxy-phenylpyruvate: 4-Hydroxy-phenylpyruvate is derived from Tyrosine and Phenylalanine amino acids and may play a role in the synthesis of the ring structure. The concentration of 1 μM, 5 μM, and 15 μM were tested for Bcl-2 and Caspase-3 expression. For the 5 μM and 15 μM concentrations there is a significant reduction in Bcl-2 expression after 24 hours of incubation and a significant increase in Caspase-3 expression after 12 hours of incubation.

Western Blot Analysis of Building Block component Phenylacetate: Phenylacetate has the potential to be converted to 4-Hydroxy-benzoate, which plays a

role in the attachment of the side chain to the ring structure. The concentration tested were 1 μM, 5 μM, and 15 μM. For phenylacetate there was a decrease in Bcl-2 expression for the concentration of 5 μM and 15 μM after 12 hours and 24 hours of incubation. An increase in Caspase-3 expression was observed for the concentration of 5 μM and 15 μM after 12 hours and 24 hours of incubation.

Western Blot Analysis of Building Block component 3-methoxy-4-hydroxymandelate (vanillylmandelate or VMA): VMA is an additional component for the synthesis of the CoQ10 quinone ring structure. The concentrations tested were 100 nM, 250 nM, 500 nM, 1 μM, 25 μM, 50 μM, and 100 μM. Though no statistically significant apoptotic effect was observed in this experiment, the data indicated a downward trend of Bcl-2 expression.

Western Blot Analysis of Building Block component Vanillic acid: Vanillic is a precursor for the synthesis of the quinone ring and was tested at a concentration of 500 nm, 5 μM, and 15 μM. A western blot analysis measured Bcl-2 and Caspase-3 expression. Vanillic Acid was shown to significantly reduce Bcl-2 expression for the concentrations of 500 nM and 5 μM at the 24 hour incubation time point. For the 15 μM concentration there is a reduction in Bcl-2 expression after 3 hours of incubation. For the cells incubated with 15 μM for 24 hours there was a significant increase in Caspase-3 expression.

Western Blot Analysis of Building Block component 4-Hydroxybenzoate: 4-Hydroxybenzoate acid plays a role in the attachment of the isoprenoid side chain to the ring structure. The concentrations tested were 500 nM, 1 μM, and 50 μM. There was a significant reduction in Bcl-2 expression for the 15 μM concentration after 24 hours of incubation.

Western Blot Analysis of Building Block component 4-Pyridoxine: Pyridoxine is another precursor building block for the synthesis of the quinone ring structure of CoQ10. The concentrations tested for this compound are 5μM, 25μM, and 100 μM. The cells were assayed for their levels of Bcl-2 and Caspase-3. Pyridoxine showed a significant reduction in Bcl-2 after 24 hours of incubation in melanoma cells.

Western Blot Analysis of Building Block component Panthenol: Panthenol plays a role in the synthesis of the quinone ring structure of CoQ10. The concentrations tested

on melanoma cells were 5 μM, 25 μM, and 100 μM. This compound showed a significant reduction in Bcl-2 expression for the 25 μM concentration.

Western Blot Analysis of Building Block component Mevalonic: Mevalonic Acid is one of the main components for the synthesis of CoQ10. This compound was tested at the concentrations of 500 nM, 1 μM, 25 μm, and 50 μM. There was no significant reduction in Bcl-2 expression or an increase in Caspase-3 expression in this experiment.

Western Blot Analysis of Building Block component Acetylglycine: Another route for the synthesis of CoQ10 is the isoprenoid (side chain) synthesis. The addition of Acetylglycine converts Coenzyme A to Acetyl-CoA which enters the mevalonic pathway for the synthesis of the isoprenoid synthesis. The concentrations tested were 5 μM, 25 μM, and 100 μM. The testing of Acetylglycine showed significant decrease in Bcl-2 expression after 12 hours of incubation for the concentration of 5 μM and 25 μM. A significant decrease in Bcl-2 was recorded for the 100 μM concentration at the 24 hour incubation time point.

Western Blot Analysis of Building Block component Acetyl-CoA: Acetyl-CoA is a precursor for the mevalonic pathway for the synthesis of CoQ10. The concentrations tested were 500 nm, 1 μM, 25 μM, and 50 μM. There was no significant observed reduction in Bcl-2 or increase in Caspase-3 expression for the time points and concentrations tested.

Western Blot Analysis of Building Block component L-Tyrosine in combination with farnesyl: L-Tyrosine is one of the precursors for the synthesis of the quinone ring structure for CoQ10. Previous experiment tested the reaction of L-Tyrosine in medium with L-Phenylalanine and L-Tyrosine. In this study L-Tyrosine was examined in medium without the addition of L-Phenylalanine and L-Tyrosine. In this study the final concentrations of L-Tyrosine tested were 500 nM, 5 μM, and 15 μM. Farnesyl was tested at a concentration of 50 μM. There was no observed significant response for the 3 and 6 hour time points.

Western Blot Analysis of Building Block component L-Phenylalanine in combination with Farnesyl: L-Phenylalanine, a precursor for the synthesis of the quinone ring structure, was examine in combination with farnesyl in medium free of L-Tyrosine and L-Phenylalanine. A western blot analysis was performed to assay the expression of Bcl-2 and Caspase-3. The final concentrations of L-Phenylalanine were: 5

μM, 25 μM, and 100 μM. Farnesyl was added at a concentration of 50 μM. This study showed a decrease in Bcl-2 expression for most of the concentrations and combinations tested as depicted in the table below.

Table 54: L-Phenylalanine and/or Farnesyl

L-Phenylalanine	3 hr		6 hr		12 hr		24 hr	
	Bcl-2	Cas-3	Bcl-2	Cas-3	Bcl-2	Cas-3	Bcl-2	Cas-3
5 μM	X							
5 μM w/ Farnesyl							X	X
25 μM	X		X					
25 μM w/ Farnesyl	X							X
100 μM	X		X				X	
100 μM w/ Farnesyl				X				

Cell Proliferation Assay of the Combination of 4-Hydroxy-Benzoate with Benzoquinone: This set of experiments used a cell proliferation assay to assess the effect of combining different building block molecules on cell proliferation.

The first study examined the effect of combining 4-Hydroxy-Benzoate with Benzoquinone. Cells were incubated for 48 hours, after which a cell count was performed for the live cells. Each test group was compared to the control, and each combination groups were compared to Benzoquinone control. The compounds were statistically analyzed for the addition of Benzoquinone. The following table summarizes the cell count results wherein the X mark indicates a statistical decrease in cell number.

Table 55: 4-Hydroxy-Benzoate and/or Benzoquinone

4-Hydroxy	Compared to Ctrl	Compared to 4-Hydroxy to compound w/o Benzoquinone	Compared to Benzoquinone Control
500 nm	X		
500 nm w/ Benzo (35μM)	X	X	
500 nm w/ Benzo (70μM)	X	X	
1 μm	X		

1 µm w/ Benzo (35µM)	X	X	
1 µm w/ Benzo (70µM)	X	X	
50 µm	X		
50 µm w/ Benzo (35µM)	X		
50 µm w/ Benzo (70µM)	X	X	X

There is a significant decrease in cell number for the cells incubated with 4-Hydroxybenzoic and benzoquinone and in combination. For the combination of 50 µM 4-Hydroxybenzoate in combination with 70 µM Benzoquinone there is significant reduction in cell number compared to the Benzoquinone control. This suggests a synergistic effect for this molar ratio.

Additional studies were performed testing additional molar ratios. For the first test 4-Hydroxybenzoic were tested at concentrations of 500 nM, 1 µM, and 50 µM. These concentrations were tested in combination with 2,3-Dimethoxy-5-methyl-p-benzoquinone (Benzo). The concentration of Benzo tested were 25 µM, 50 µM, and 100 µM. Melanoma cells were grown to 80% confluency and seeded in 6 well plates at a concentration of 40K cells per well. The cells were treated with CoQ10, 4-Hydroxybenzoate, Benzo, and a combination of 4-Hydroxybenzoate/Benzo.

A T-test was performed with $p<0.05$ as statistically significant. An X signifies a statistical decrease in cell number.

Table 56: 4-Hydroxybenzoic and/or 2,3-Dimethoxy-5-methyl-p-benzoquinone (Benzo)

Ctrl vs Benzo 25 µM	X
Ctrl vs Benzo (B) 50 µM	
Ctrl vs Benzo (B) 100 uM	X
Ctrl vs 4-Hydroxybenzoate (HB) 500 nm	X
Ctrl vs HB 1 µM	X
Ctrl vs HB 50 µM	X

500 nM HB vs 500 nM HB w/ 25 B	X
500 nM HB vs 500 nM HB w/ 50 B	X
500 nM HB vs 500 nM HB w/ 100 B	X
1 uM HB vs 1 μM HB w/ 25 B	X
1 uM HB vs 1 μM HB w/ 50 B	X
1 uM HB vs 1 μM HB w/ 100 B	
50 uM HB vs 50 μM HB w/ 25 B	X
50 uM HB vs 50 μM HB w/ 50 B	X
50 uM HB vs 50 μM HB w/ 100 B	
500 nM HB w/ 25 B vs 25 B	X
500 nM HB w/ 50 B vs 50 B	X
500 nM HB w/ 100 B vs 100 B	X
1 μM HB w/ 25 B vs 25 B	X
1 μM HB w/ 50 B vs 50 B	X
1 μM HB w/ 100 B vs 100 B	
50 μM HB w/ 25 B vs 25 B	X
50 μM HB w/ 50 B vs 50 B	X
50 μM HB w/ 100 B vs 100 B	

There is a significant decrease in cell proliferation for the treatment medium containing HB. Moreover the combination of the HB with benzoquinone showed a significant reduction in cell number compare to the cells incubated with the corresponding benzoquinone concentrations.

A cell proliferation assay was also performed on neonatal fibroblast cells. The concentrations of HB tested were 500 nM, 5 μM, and 25 μM. HB was also tested in combination with benzoquinone at a concentrations of 25 μM, 50 μM, and 100 μM. Melanoma cells were seeded at 40k cells per well and were treated for 24 hours. The cells were trypsinized and quantified using a coulter counter.

Statistical analysis did not show a significant reduction in fibroblast cells. This indicates minimal to no toxicity in normal cells.

Cell Proliferation Assay of the Combination of phenylacetate and benzoquinone: Phenyl acetate is a precursor for the synthesis of 4-Hydroxybenzoic acid (facilitates the attachment of the ring structure . A cell proliferation assay was performed to assay the effect of incubating phenylacetate in combination with CoQ10 and Benzoquinone.

Table 57: Phenylacetate and/or Benzoquinone

Ctrl and 25/25 μM Ben	X
Ctrl and 25/50 μM Ben	X
Ctrl and 25/100 μM Ben	X
Ctrl and 25/25 μM Q-10	X
Ctrl and 25/25 μM Q-10	X
Ctrl and 25/50 μM Q-10	X
Ctrl and 25/100 μM Q-10	X
Ctrl and Ben 25	X
Ctrl and Ben 50	X
Ctrl and Ben 100	X
Ctrl and Q-10 25	
Ctrl and Q-10 50	
Ctrl and Q-10 100	X
Ben 25 μM and 500 nM/25 μM Ben	X
Ben 25 μM and 5 nM/25 μM Ben	X
Ben 25 μM and 25 nM/25 μM Ben	X
Ben 50 μM and 500 nM/50 μM Ben	X
Ben 50 μM and 5 nM/50 μM Ben	X
Ben 50 μM and 25 nM/50 μM Ben	X
Ben 100 μM and 500 nM/100 μM Ben	
Ben 100 μM and 5 nM/100 μM Ben	
Ben 100 μM and 25 nM/100 μM Ben	
Q-10 25 μM and 500 nM/25 μM Q-10	X
Q-10 25 μM and 5 nM/25 μM Q-10	X
Q-10 25 μM and 25 nM/25 μM Q-10	X

Q-10 50 μM and 500 nM/50 μM Q-10	X
Q-10 50 μM and 5 nM/50 μM Q-10	X
Q-10 50 μM and 25 nM/50 μM Q-10	X
Q-10 100 μM and 500 nM/100 μM Q-10	X
Q-10 100 μM and 5 nM/100 μM Q-10	X
Q-10 100 μM and 25 nM/100 μM Q-10	X

The data indicates the addition of phenylacetate in combination with benzoquinone significantly decreases the cellular proliferation. The combination with CoQ10 and phenylacetate significantly decrease the cell number compared to incubation with CoQ10 and benzoquinone alone.

Cell Proliferation Assay of the Combination of 4-Hydroxy-Benzoate with Farnesyl: 4-Hydroxy-Benzoate was incubated in combination with Farnesyl. The summary of the results are listed below. 4-Hydroxybenzoate groups were compared to the control and Farnesyl control groups. The X signifies a statistical decrease in cell number.

Table 58: 4-Hydroxy-Benzoate and/or Farnesyl

4-Hydroxy - Benzoate	Compared to Ctrl	Compared to 4-Hydroxy to compound w/o Farnesyl	Compared to Farnesyl Control
500 nm	X		
500 nm w/ Farnesyl (35μM)	X		
500 nm w/ Farnesyl (70μM)	X		
1 μm	Error		
1 μm w/ Farnesyl (35μM)	Error		
1 μm w/ Farnesyl (70μM)	Error		
50 μm	X		
50 μm w/ Farnesyl (35μM)	X		

50 µm w/ Farnesyl (70µM)	X		

Cell Proliferation Assay of the Combination of L-Phenylalanine with Benzoquinone: A cell proliferation assay was performed to test the combination of L-Phenylalanine combined with Benzoquinone. Below is a summary of the results of L-Phenylalanine compared to the control and Benzoquinone control. The X signifies a statistical decrease.

Table 59: L-Phenylalanine and/or Benzoquinone

L-Phenylalanine	Compared to Ctrl	Compared to L-Phenylalanine to compound w/o Benzoquinone	Compared to Benzoquinone Control
5 µM			
5 µm w/ Benzo (50µM)		X	
5 µm w/ Benzo (100µM)		X	
25 µm			
25 µm w/ Benzo (50µM)		X	
25 µm w/ Benzo (100µM)		X	
100 µm			
100 µm w/ Benzo (50 µM)	X	X	X
100 µm w/ Benzo (100 µM)	X	X	X

A similar synergistic role is seen for the L-Phenylalanine combined with Benzoquinone.

Cell Proliferation Assay of the Combination of L-Phenylalanine with Farnesyl: Preliminary results for combination cell proliferation study of L-Phenylalanine incubated in combination with Farnesyl. The L-Phenylalanine were compared to the control and Farnesyl control group. An X signifies a statistical decrease in cell number.

Table 60: L-Phenylalanine and/or Farnesyl

L-Phenylalanine	Compared to Ctrl	Compared to L-Phenylalanine to compound w/o Farnesyl	Compared to Farnesyl Control
5 μM			
5 μm w/ Farnesyl (50μM)			
5 μm w/ Farnesyl (100μM)			
25 μm	X		
25 μm w/ Farnesyl (50μM)	X	X	X
25 μm w/ Farnesyl (100μM)	X	X	X
100 μm	X		
100 μm w/ Farnesyl (50 μM)	X		X
100 μm w/ Farnesyl (100 μM)	X		

Cell Proliferation Assay of the Combination of L-Tyrosine with Benzoquinone: L-Tyrosine was incubated in combination with Benzoquinone after which a cell count was performed. The groups were compared the control groups and Benzoquinone control group.

Table 61: L-Tyrosine and/or Benzoquinone

L-Tyrosine	Compared to Ctrl	Compared to L-Tyrosine to compound w/o Benzoquinone	Compared to Benzoquinone Control
500 nm			
500 nm w/ Benzo (50μM)			
500 nm w/ Benzo (100 μM)			
5 μm	X		
5 μm w/ Benzo (50 μM)	X		

5 µm w/ Benzo (100 µM)	X		
15 µm	X		
15 µm w/ Benzo (50 µM)	X		
15 µm w/ Benzo (100 µM)	x		

The addition of Benzoquinone did not amplify the effect of L-Tyrosine on the cell number.

Cell Proliferation Assay of the Combination of L-Tyrosine with Benzoquinone: This study examined the combination of L-Tyrosine with Farnesyl. The groups were compared to control and Farnesyl control groups.

Table 62: L-Tyrosine and/or Farnesyl

L-Tyrosine	Compared to Ctrl	Compared to L-Tyrosine to compound w/o Farnesyl	Compared to Farnesyl Control
500 nm			
500 nm w/ Farnesyl (50µM)			
500 nm w/ Farnesyl (50µM)			
5 µm	X		
5 µm w/ Farnesyl (50 µM)	X		
5 µm w/ Farnesyl (100 µM)	X		
15 µm	X		
15 µm w/ Farnesyl (50 µM)	X		
15 µm w/ Farnesyl (100 µM)	X		

Combining L-Tyrosine and Farnesyl does not appear to have a synergistic effect on reducing the cell number in this experiment.

The synthesis of the CoQ10 is divided into two main parts, which consist of the synthesis of the ring structure and synthesis of the side chain structure. Here, oncogenic cells were supplemented with compounds which are precursors for the synthesis of the side chain and the ring structure components. These results have focused the study to 3 main components involved in the synthesis of the ring structure and two compounds that play a role in the attachment of the ring structure to the side chain structure. The three compounds that have shown a significant reduction in Bcl-2 and increase in Caspase-3 expression are: 1) L-Phenylalanine, 2) L-Tyrosine and 3) 4-Hydroxyphenylpyruvate. The two compounds involved with the attachment of the side chain to the ring structure are: 1) 4-hydroxy benzoate and 2) Phenylacetate.

These results also showed that exogenous delivery of these compounds in combination with 2,3 Dimethoxy-5-methyl-p-benzoquinone (benzoquinone) significantly inhibits cell proliferation. This indicates a supplementation of the ring structure with compounds for the attachment of the side chain to the benzoquinone ring may supplement an impaired CoQ10 synthesis mechanism. This may also assist in the stabilization of the molecule to maintain the functional properties required by cellular processes. Phenylacetate is a precursor for the synthesis of 4-Hydroxybenzoate, which exogenous delivery in combination with benzoquinone has a similar effect in oncogenic cells.

EXAMPLE 23: Modulation of Gene Expression by Coenzyme Q10 in Cell Model for Diabetes

Coenzyme Q10 is an endogenous molecule with an established role in the maintenance of normal mitochondrial function by directly influencing oxidative phosphorylation. Experimental evidence is presented that demonstrates the ability of Coenzyme Q10 in modulating intracellular targets that serve as key indices of metabolic disorders, such as diabetes, in a manner representative of therapeutic endpoints.

In order to understand how Coenzyme Q10 regulates expression of genes associated with the cause or treatment of diabetes, immortalized primary kidney proximal tubular cell line derived from human kidney (HK-2) and primary cultures of the human aortic smooth muscle cells (HASMC) were used as experimental models.

The HK-2 and HASMC cells are normally maintained in culture at 5.5 mM glucose, which is a concentration that corresponds to a range considered normal in human blood. However, in order to simulate a diabetic environment, both cell lines were subsequently maintained at 22 mM glucose, which corresponds to the range observed in human blood associated with chronic hyperglycemia. The cells were subsequently allowed to propagate over 3 passages so that the intracellular regulation processes were functionally adapted to mimic a diabetic state. The choice of cell line was based on the physiologic influence of diabetes on renal dysfunction and progression to end-stage renal disease (ESRD) in addition to the progressive pathophysiology of a compromised cardiovascular function.

Effect of Coenzyme Q10 on Gene Expression in HK-2 Cells using the Diabetes PCR Array

The Diabetes PCR array (SABiosciences) offers a screen for 84 genes simultaneously. The 4 treatments tested in this study were:

- HK-2;
- HK-2 H maintained 22 mM glucose;
- HK2(H) +50 μM Coenzyme Q10; and
- HK2(H) + 100 μM Coenzyme Q10.

A stringent analysis of the Real time PCR data of the HK-2 samples on the Diabetes Arrays (Cat # PAHS-023E, SABiosciences Frederick MD) was made to exclude all results where gene regulation was not at least a two-fold regulation over HK-2 normal untreated cells with a *p* value of less than 0.05. Genes that were observed to be regulated either by chronic hyperglycemia or by Coenzyme Q10 are listed in Table 63 and their functions and subcellular locations (derived from Ingenuity Pathway Analysis) are listed in Table 64.

Table 63

Genes	HK-2(H) Fold regulation	p value	HK-2(H)-50 μM Coenzyme Q10 Fold regulation	p value	HK-2(H)-100 μM Coenzyme Q10 Fold regulation	p value
CEACAM1	1.26	0.409	3.47	0.067	5.36	0.032
PIK3C2B	1.48	0.131	2.32	0.115	3.31	0.003
INSR	-1.09	0.568	2.51	0.103	2.88	0.024
TNF	2.00	0.005	2.57	0.042	2.81	0.020

ENPP1	-1.50	0.002	1.42	0.238	2.67	0.038
PRKCB	-1.75	0.005	1.82	0.280	2.49	0.042
DUSP4	1.27	0.318	1.24	0.455	2.26	0.060
SELL	-1.58	0.219	1.77	0.042	2.06	0.021
SNAP25	-1.00	0.934	1.46	0.377	1.97	0.059

Table 64

Symbol	Entrez Gene Name	Location	Type(s)
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	Plasma Membrane	transmembrane receptor
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	Cytoplasm	kinase
INSR	insulin receptor	Plasma Membrane	kinase
TNF	tumor necrosis factor (TNF superfamily, member 2)	Extracellular Space	cytokine
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1	Plasma Membrane	enzyme
PRKCB	protein kinase C, beta	Cytoplasm	kinase
DUSP4	dual specificity phosphatase 4	Nucleus	phosphatase
SELL	selectin L	Plasma Membrane	other
SNAP25	synaptosomal-associated protein, 25kDa	Plasma Membrane	transporter

Among the detected RNA transcripts with modulated levels, the Carcino Embryonic Antigen Cell Adhesion Molecule 1 (CEACAM1) was identified as being highly upregulated in HK2(H) cells, particularly with 100 μM Coenzyme Q10 treatment. CEACAM-1, also known as CD66a and BGP-I, is a 115-200 KD type I transmembrane glycoprotein that belongs to the membrane-bound CEA subfamily of the CEA superfamily. On the surface of cells, it forms noncovalent homo- and heterodimers. The extracellular region contains three C2-type Ig-like domains and one N-terminal V-type Ig-like domain. Multiple splice variants involving regions C-terminal to the second C2-type domain (aa 320 and beyond) exist. The lack of intact CEACAM1 expression in mice has been proposed to promote the metabolic syndrome associated with diabetes, while an increase in expression of CEACAM1 is associated with increased insulin internalization, which suggests an increase in insulin sensitivity and glucose utilization

(*e.g.*, movement of glucose from blood into the cells), thus mitigating insulin resistance, a hallmark characteristic of type 2 diabetes mellitus.

As shown in Table 63, insulin receptor (INSR) expression was also altered in diabetic HK-2 cells treated with Coenzyme Q10. Without being bound by theory, the increase in expression of INSR with Coenzyme Q10 treatment should enhance insulin sensitivity (either alone or in addition to expression of CEACAM1) with the potential to reverse a major physiologic/metabolic complication associated with diabetes.

Effect of Coenzyme Q10 on Gene Expression in HK-2 Cells using Mitochondrial Arrays

Differential expression of mitochondrial genes in diabetes was assayed using the mitochondria arrays (Cat# PAHS 087E, SABisociences Frederick MD). Genes that were regulated by chronic hyperglycemia and/or Coenzyme Q10 treatment are listed in Table 65 while their functions and location are included in Table 66.

Table 65

Genes	HK2 (H) untreated	p value	HK-2(H) 50 µM Coenzyme Q10	p value	HK-2(H) 100 µM Coenzyme Q10	p value
GRPEL1	-1.5837	0.151255	-2.6512	0.04704	-1.933	0.139161
SLC25A3	-8.6338	0.071951	-8.2059	0.0425	-1.6984	0.995194
TOMM40	-2.3134	0.140033	-1.1567	0.115407	-1.9509	0.038762
TSPO	-3.6385	0.111056	-6.7583	0.073769	-2.1104	0.167084

Table 66

Symbol	Entrez Gene Name	Location	Type(s)
GRPEL1	GrpE-like 1, mitochondrial (E. coli)	Mitochondria	other
SLC25A3	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	Mitochondrial membrane.	transporter
TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)	Outer membrane of mitochondria.	ion channel
TSPO	translocator protein (18kDa)	Outer membrane of mitochondria.	transmembrane receptor

To date, the role of the four mitochondrial genes identified (Table 65) in diabetic HK-2 cells treated with Coenzyme Q10 in diabetes is uncharacterized.

Study 2: Effect of Coenzyme Q10 on Gene Expression in HASMC Cells using the Diabetes PCR Array

The Diabetes PCR array (SABiosciences) offers a screen for 84 genes simultaneously. The 4 treatments tested in this study were:

- HASMC;
- HASMC H maintained at 22 mM glucose;
- HASMC (H) + 50 μM Coenzyme Q10; and
- HASMC (H) + 100 μM Coenzyme Q10.

A stringent analysis of the Real time PCR data of the HASMC cell samples on the Diabetes Arrays (Cat # PAHS-023E, SABiosciences Frederick MD) was made to exclude all results where gene regulation was not at least a two-fold regulation over HASMC normal untreated cells with a *p* value of less than 0.05. Genes that were observed to be regulated either by chronic hyperglycemia or by Coenzyme Q10 are listed in Table 67.

Table 67

Genes	HASMC-(H)	p value	HASMC-(H)-50 μM Coenzyme Q10	p value	HASMC-(H)-100 μM Coenzyme Q10	p value
AGT	1.3051	0.547507	-1.0169	0.781622	2.3027	0.030195
CCL5	-17.4179	0.013798	-5.3796	0.022489	-4.6913	0.022696
CEACAM1	-5.5629	0.012985	-5.3424	0.014436	-5.8025	0.012948
IL6	2.7085	0.049263	3.8172	0.012685	6.0349	0.000775
INSR	1.4649	0.207788	1.9622	0.081204	2.0801	0.016316
NFKB1	1.482	0.072924	1.3779	0.191191	2.0898	0.027694
PIK3C2B	2.0479	0.218276	1.4331	0.254894	2.6329	0.069422
SELL	-1.9308	0.087513	1.2476	0.393904	4.0371	0.000177
TNF	-1.814	0.108322	-3.2434	0.043526	-1.8489	0.133757

In HASMC cells, treatment of hyperglycemic cells with Coenzyme Q10 resulted in the altered expression of genes involved in regulating vascular function (AGT), insulin sensitivity (CEACAM1, INSR, SELL) and inflammation/immune function (IL-6, TNF, CCL5). Without being bound by theory, an increase in expression of INSR may

be associated with increased insulin sensitivity in HASMC cells, which is a physiological property that would be beneficial in the treatment of diabetes, while IL-6, in addition to its immunoregulatory properties, has been proposed to affect glucose homeostasis and metabolism, both directly and indirectly, by action on skeletal muscle cells, adipocytes, hepatocytes, pancreatic β-cells and neuroendocrine cells. Upon activation, normal T-cell express and secrete RANTES and chemokine(C-Cmotif) ligand (CCL5). CCL5 is expressed by adipocytes, and serum levels of RANTES are increased in obesity and type 2 diabetes. However, as shown in Table 67, treatment of HASMC cells with Coenzyme Q10 causes a significant decrease in the expression of CCL5. Based on the foregoing data, it is expected that administration of Coenzyme Q10 will have a therapeutic benefit in the management of diabetes.

Effect of Coenzyme Q10 on Gene Expression in HASMC Cells using Mitochondrial Arrays

Differential expression of mitochondrial genes in diabetes was assayed using the mitochondria arrays (Cat# PAHS 087E, SABisociences Frederick MD). Genes that were regulated by chronic hyperglycemia and/or Coenzyme Q10 treatment are shown in Table 68.

Table 68

Genes	HASMC-(H)	p value	HASMC-(H) 50uM Coenzyme Q10	p value	HASMC-(H)-100 μM Coenzyme Q10	p value
BCL2L1	-1.6558	0.244494	-2.7863	0.008744	-2.3001	0.014537
MFN1	-1.4992	0.317009	-1.2585	0.021185	-2.2632	0.005961
PMAIP1	-4.7816	0.206848	-6.8132	0.000158	-4.352	0.000286
SLC25A1	-2.2051	0.020868	-1.834	0.00581	-3.0001	0.03285
SLC25A13	-2.0527	0.035987	-1.5	0.029019	-1.5245	0.043712
SLC25A19	-1.0699	0.417217	-1.4257	0.104814	-2.1214	0.007737
SLC25A22	-2.1747	0.007344	-1.9839	0.0013	-10.3747	0.003437
TIMM44	-1.3605	0.414909	-2.3214	0.004118	-1.9931	0.010206
TOMM40	-1.1982	0.428061	-2.0922	0.002195	-2.2684	0.003272
TSPO	-1.402	0.304875	-2.0586	0.061365	-2.3647	0.044656

Treatment of hyperglycemic HASMC cells with Coenzyme Q10 resulted in altered expression of genes that regulate programmed cell death or apoptosis (BCL2L1,

PMIAP1 also known as NOXA), transporter proteins (SLC25A1 [citrate transporter], SLC25A13 [aspartate-glutamate exchanger], SLC25A19 [thiamine pyrophosphate transporter] and SLC25A22 [glutamate-hydrogen cotransporter]) and mitochondrial matrix transport proteins (MFN1, TIMM44 and TOMM40). The activities of these transporters play important role in the regulation of precursors essential for the Kreb's cycle and maintenance of mitochondrial oxidative phosphorylation. These results indicate that exposure of diabetic HASMC cells to Coenzyme Q10 is associated with changes in expression of cytoplasmic and mitochondrial genes, which in turn is consistent with Coenzyme Q10 providing a therapeutic benefit in the treatment of diabetes.

A comparison of the data obtained by treating HASMC cells and HK-2 cells with Coenzyme Q10 or in a hyperglycemic environment reveals that 4 genes were commonly regulated by Coenzyme Q10 in both cell lines (*e.g.*, PIK3C2B and SELL in the gene expression assay and TOMM40 and TSPO in the mitochondrial array assay). These results demonstrate that treatment of cells with Coenzyme Q10 in a diabetic environment is associated with altered expression of genes that are known to be involved in the cause or treatment of diabetes.

Equivalents:

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

2010247750 01 Sep 2016

CLAIMS

1. A method for treating, alleviating symptoms of, or inhibiting progression of obesity in a mammal, the method comprising: administering to the mammal a therapeutically effective amount of a pharmaceutical composition comprising a sole active ingredient consisting of Coenzyme Q10 (CoQ10) in the amount of about 1% to about 25% w/w of the composition, or a building block of CoQ10.

2. A method for selectively augmenting mitochondrial oxidative phosphorylation, in a disease cell of a mammal in need of treatment for obesity, the method comprising: administering to said mammal a therapeutically effective amount of a pharmaceutical composition comprising a sole active ingredient consisting of Coenzyme Q10 (CoQ10) in an amount of about 1% to about 25% w/w of the composition, or a building block of CoQ10, thereby selectively augmenting mitochondrial oxidative phosphorylation in said disease cell of the mammal.

3. A method of reducing lipid levels in a mammal suffering from obesity comprising administering to the mammal an effective amount of a pharmaceutical composition comprising a sole active ingredient consisting of Coenzyme Q10 in an amount of about 1% to about 25% w/w of the composition, or a building block of CoQ10, thereby reducing lipid levels in the mammal.

4. The method of any one of claims 1-3, wherein the CoQ10 or the building block of CoQ10 selectively elicits, in a disease cell of the mammal, a cellular metabolic energy shift towards normalized mitochondrial oxidative phosphorylation.

5. The method of any one of claims 1-3, wherein the CoQ10 or the building block of CoQ10 does not substantially elicit, in normal cells of the mammal, the cellular metabolic energy shift towards mitochondrial oxidative phosphorylation.

6. The method of any one of claims 1-5, wherein the mammal is human.

7. The method of any one of claims 1-5, wherein the mammal is a non-human mammal.

8. The method of any one of claims 1-7, wherein the obesity is responsive or sensitive to treatment by Coenzyme Q10 or a building block of CoQ10.

9. The method of any one of claims 1-7, wherein the obesity is characterized by a dysregulated mitochondrial oxidative phosphorylation function that leads to

altered gene regulation and/or protein-protein interactions which contribute to or causally lead to the obesity.

10. The method of any one of claims 1-9, wherein the building block of CoQ10 comprises:
    (a) benzoquinone or at least one molecule that facilitates the biosynthesis of the benzoquinone ring, and
    (b) at least one molecule that facilitates the synthesis of and/or attachment of isoprenoid units to the benzoquinone ring.

11. The method of claim 10, wherein said at least one molecule that facilitates the biosynthesis of the benzoquinone ring comprises: L-Phenylalanine, DL-Phenylalanine, D-Phenylalanine, L-Tyrosine, DL-Tyrosine, D-Tyrosine, 4-hydroxy-phenylpyruvate, 3-methoxy-4-hydroxymandelate (vanillylmandelate or VMA), vanillic acid, pyridoxine, or panthenol.

12. The method of claim 10, wherein said at least one molecule that facilitates the synthesis of and/or attachment of isoprenoid units to the benzoquinone ring comprises: phenylacetate, 4-hydroxy-benzoate, mevalonic acid, acetylglycine, acetyl-CoA, or farnesyl.

13. The method of any one of claims 1-9, wherein the building block of CoQ10 comprises:
    (a) one or more of L-Phenylalanine, L-Tyrosine, and 4-hydroxyphenylpyruvate; and,
    (b) one or more of 4-hydroxy benzoate, phenylacetate, and benzoquinone.

14. The method of any one of claims 1-9, wherein the CoQ10 or the building block of CoQ10:
    (a) inhibits Bcl-2 expression and/or promotes Caspase-3 expression; and/or,
    (b) inhibits cell proliferation.

15. The method of any one of claims 1-9, wherein the concentration of the CoQ10 or the building block of CoQ10 in the tissues of the mammal being treated is different than that of a control standard of tissue representative of a healthy or normal state.

16. The method of any one of claims 1-9, wherein the form of the CoQ10 or the building block of CoQ10 administered to the mammal is different than the predominant form found in systemic circulation in the mammal.

17. The method of any one of claims 1-16, wherein the amount sufficient to treat the obesity in the mammal up-regulates or down-regulates mitochondrial oxidative phosphorylation.

18. The method of claim 17, wherein the amount sufficient to treat the obesity in the mammal modulates anaerobic use of glucose and/or lactate biosynthesis.

19. The method of any one of claims 1-18, wherein the treatment occurs via an interaction of the CoQ10 or the building block of CoQ10 with HNF4alpha.

20. The method of any one of claims 1-18, wherein the treatment occurs via an interaction of the CoQ10 or the building block of CoQ10 with transaldolase.

21. The method of any one of claims 1-18, wherein the CoQ10 or the building block of CoQ10 affects beta cell oxidation in the mitochondria, decreases in adipocyte size, and/or controls cortisol levels.

22. The method of any one of claims 1-18, wherein said method results in a reduction of waist circumference, visceral fat content, or fasting plasma triglycerides, or an increase of fasting high density lipoprotein level.

23. Use of Coenzyme Q10 (CoQ10) in the manufacture of a medicament for treating, alleviating symptoms of, or inhibiting progression of obesity in a mammal, wherein CoQ10, or a building block thereof, is the sole active ingredient, and is in the amount of 1% to about 25% w/w of the composition.

24. Use of Coenzyme Q10 (CoQ10) in the manufacture of a medicament for selectively augmenting mitochondrial oxidative phosphorylation, in a disease cell of a mammal in need of treatment for obesity, wherein CoQ10, or a building block thereof, is the sole active ingredient, and is in the amount of 1% to about 25% w/w of the composition.

25. Use of Coenzyme Q10 (CoQ10) in the manufacture of a medicament for reducing lipid levels in a mammal suffering from obesity, wherein CoQ10, or a building block thereof, is the sole active ingredient, and is in the amount of 1% to about 25% w/w of the composition.

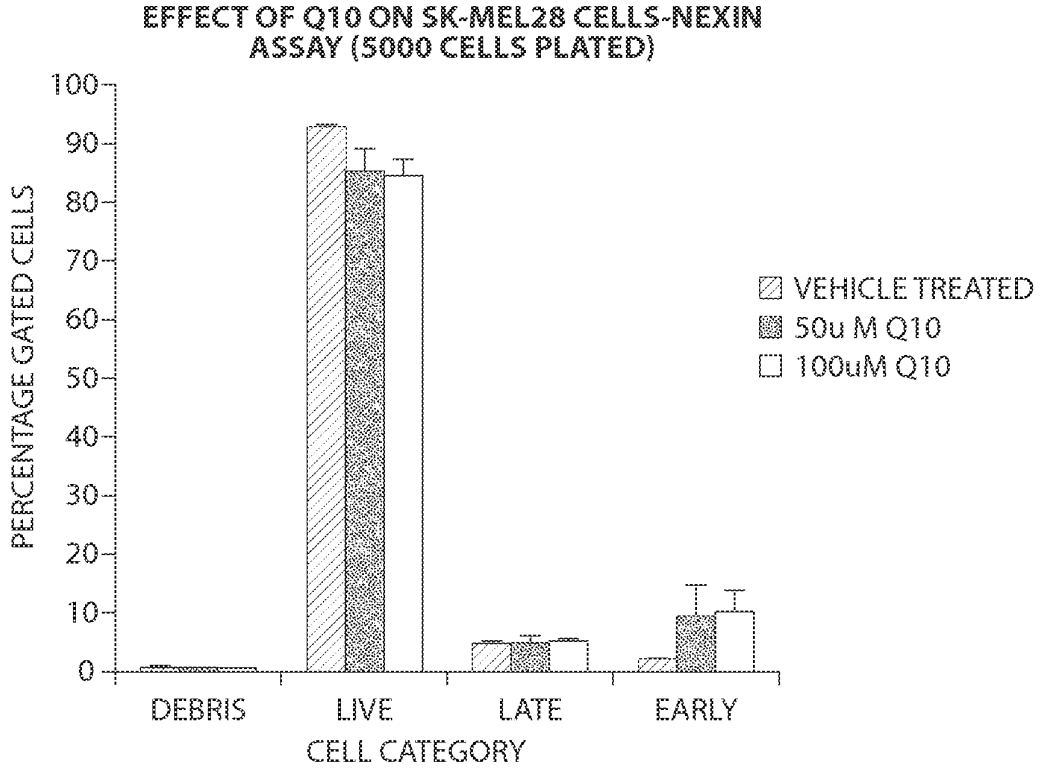
EFFECT OF Q10 ON SK-MEL28 CELLS-NEXIN ASSAY (5000 CELLS PLATED)
PERCENTAGE GATED CELLS
100
90
80
70
60
50
40
30
20
10
0
DEBRIS
LIVE
LATE
EARLY
CELL CATEGORY
VEHICLE TREATED
50u M Q10
100uM Q10

Fig. 1

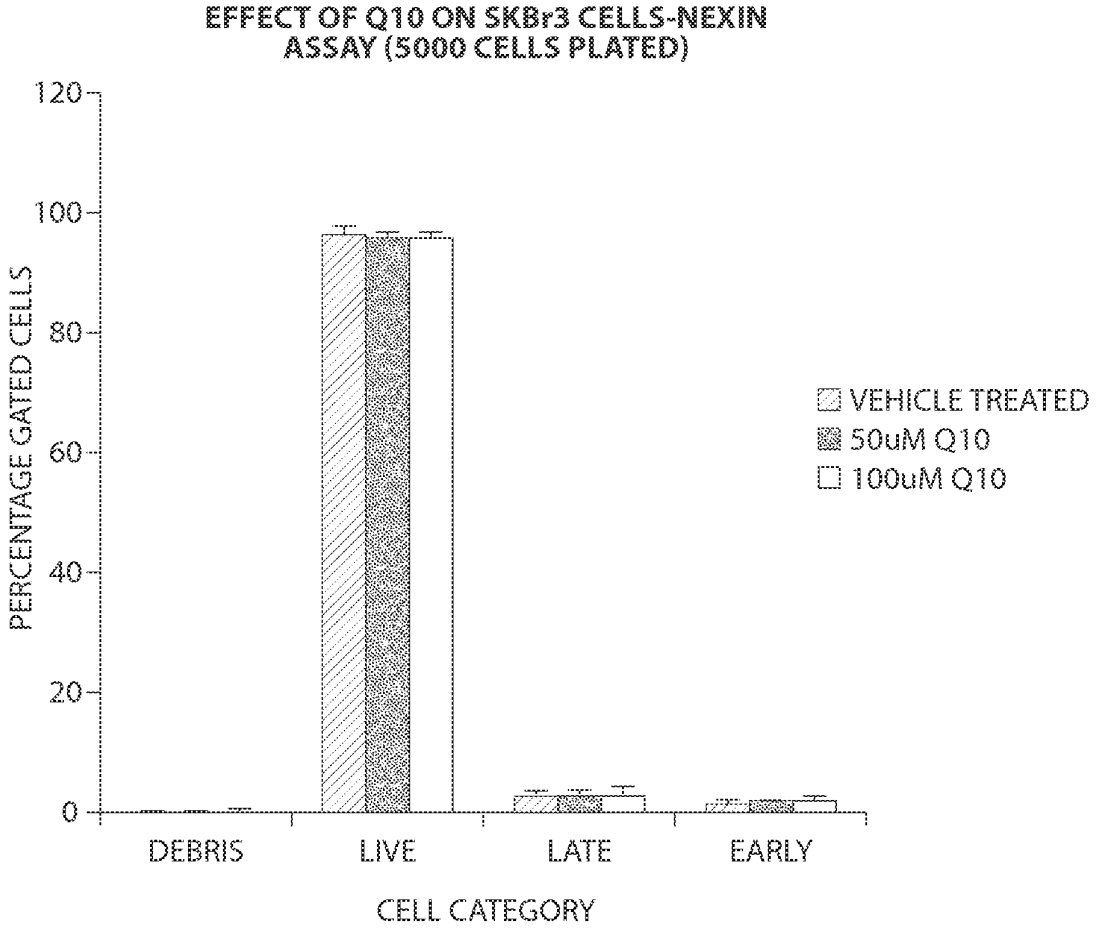
EFFECT OF Q10 ON SKBr3 CELLS-NEXIN ASSAY (5000 CELLS PLATED)
PERCENTAGE GATED CELLS
120
100
80
60
40
20
0
DEBRIS
LIVE
LATE
EARLY
CELL CATEGORY
VEHICLE TREATED
50uM Q10
100uM Q10

Fig. 2

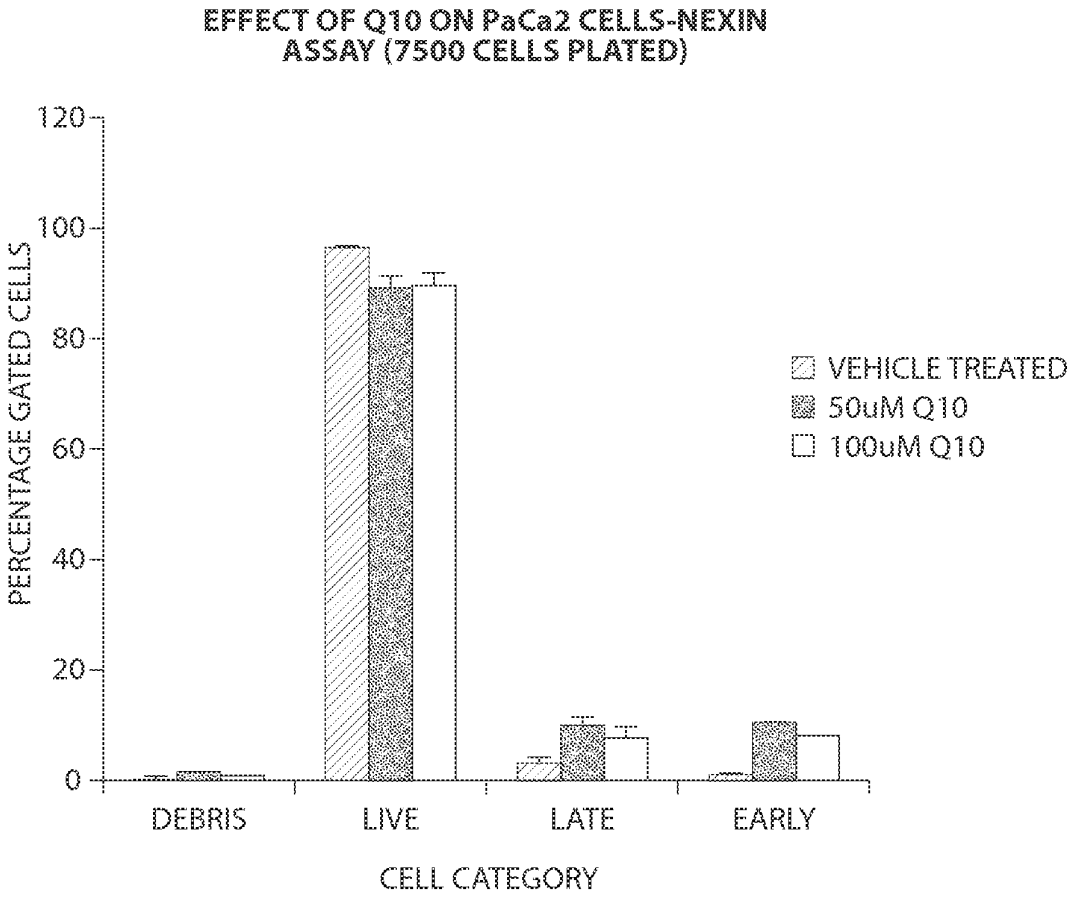
EFFECT OF Q10 ON PaCa2 CELLS-NEXIN ASSAY (7500 CELLS PLATED)
PERCENTAGE GATED CELLS
120
100
80
60
40
20
0
DEBRIS
LIVE
LATE
EARLY
CELL CATEGORY
VEHICLE TREATED
50uM Q10
100uM Q10

Fig. 3

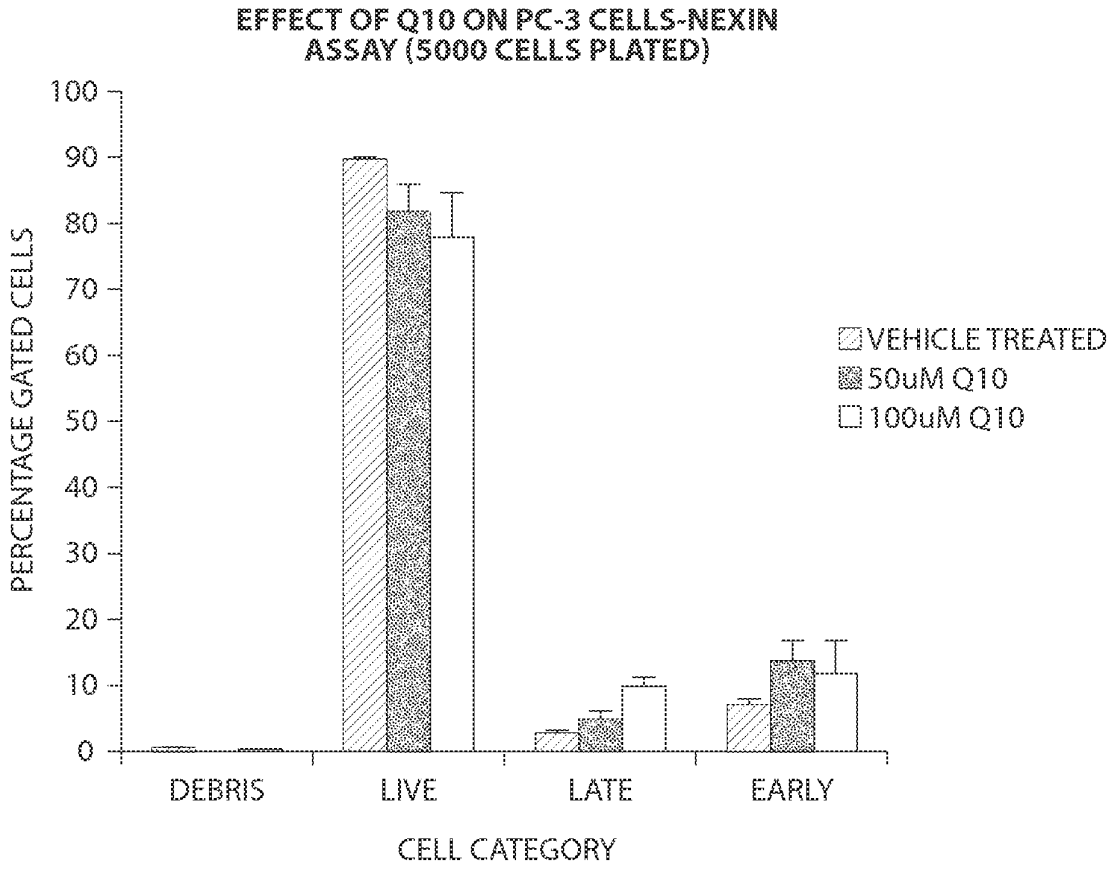
EFFECT OF Q10 ON PC-3 CELLS-NEXIN ASSAY (5000 CELLS PLATED)
PERCENTAGE GATED CELLS
100
90
80
70
60
50
40
30
20
10
0
DEBRIS
LIVE
LATE
EARLY
CELL CATEGORY
VEHICLE TREATED
50uM Q10
100uM Q10

Fig. 4

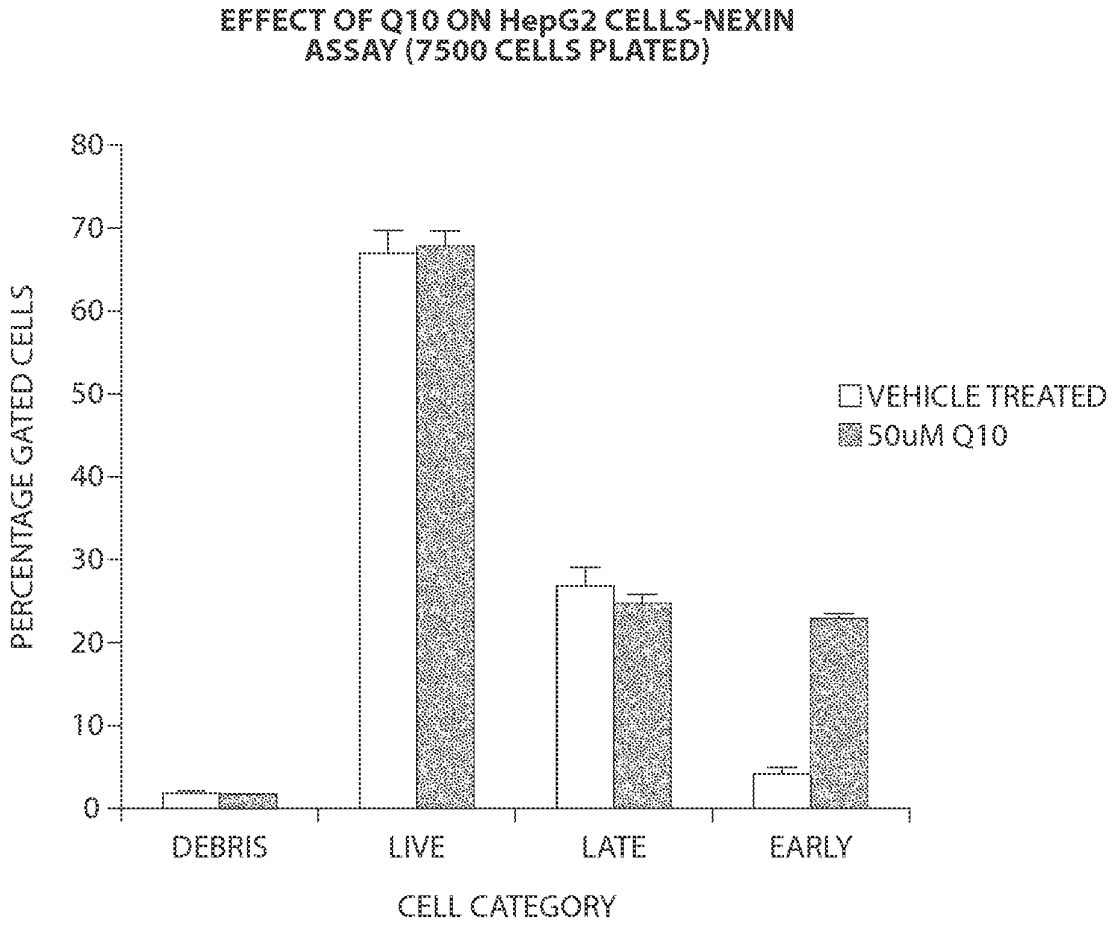
EFFECT OF Q10 ON HepG2 CELLS-NEXIN ASSAY (7500 CELLS PLATED)
PERCENTAGE GATED CELLS
80
70
60
50
40
30
20
10
0
DEBRIS
LIVE
LATE
EARLY
CELL CATEGORY
VEHICLE TREATED
50uM Q10

Fig. 5

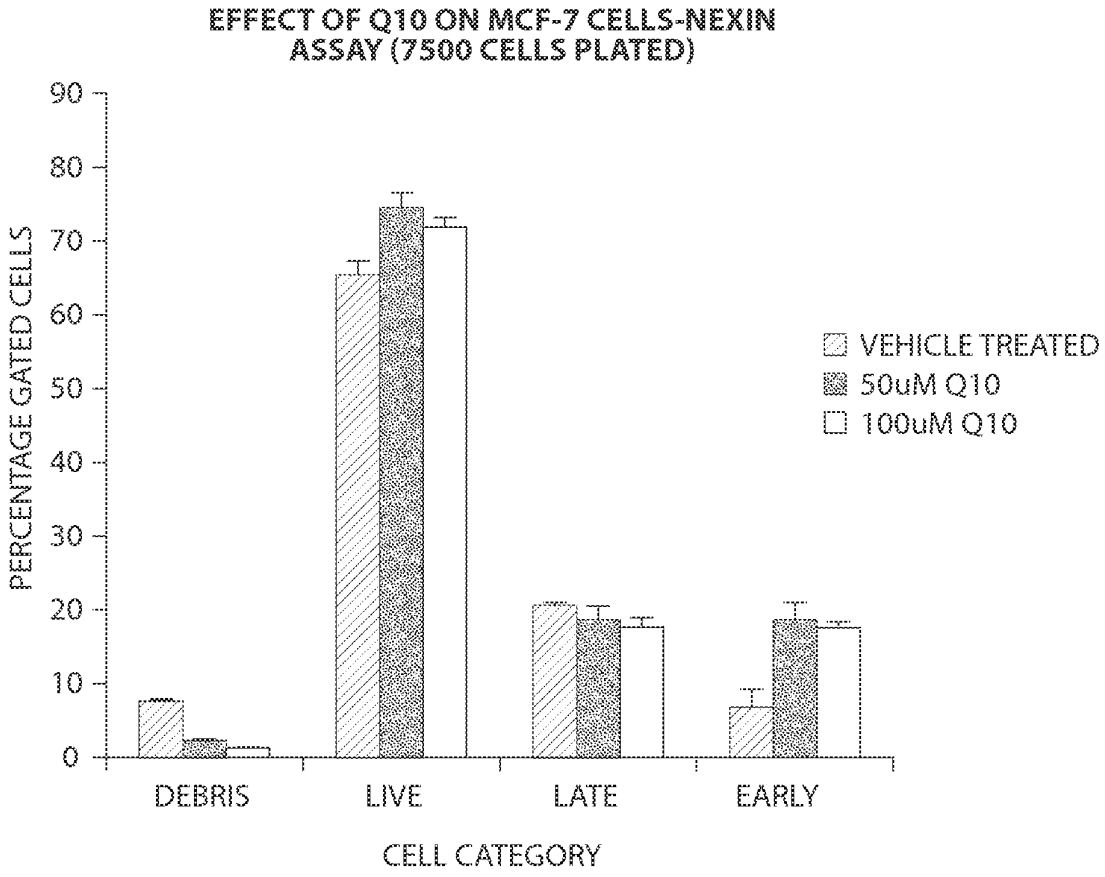
EFFECT OF Q10 ON MCF-7 CELLS-NEXIN ASSAY (7500 CELLS PLATED)
PERCENTAGE GATED CELLS
90
80
70
60
50
40
30
20
10
0
DEBRIS
LIVE
LATE
EARLY
CELL CATEGORY
VEHICLE TREATED
50uM Q10
100uM Q10

Fig. 6

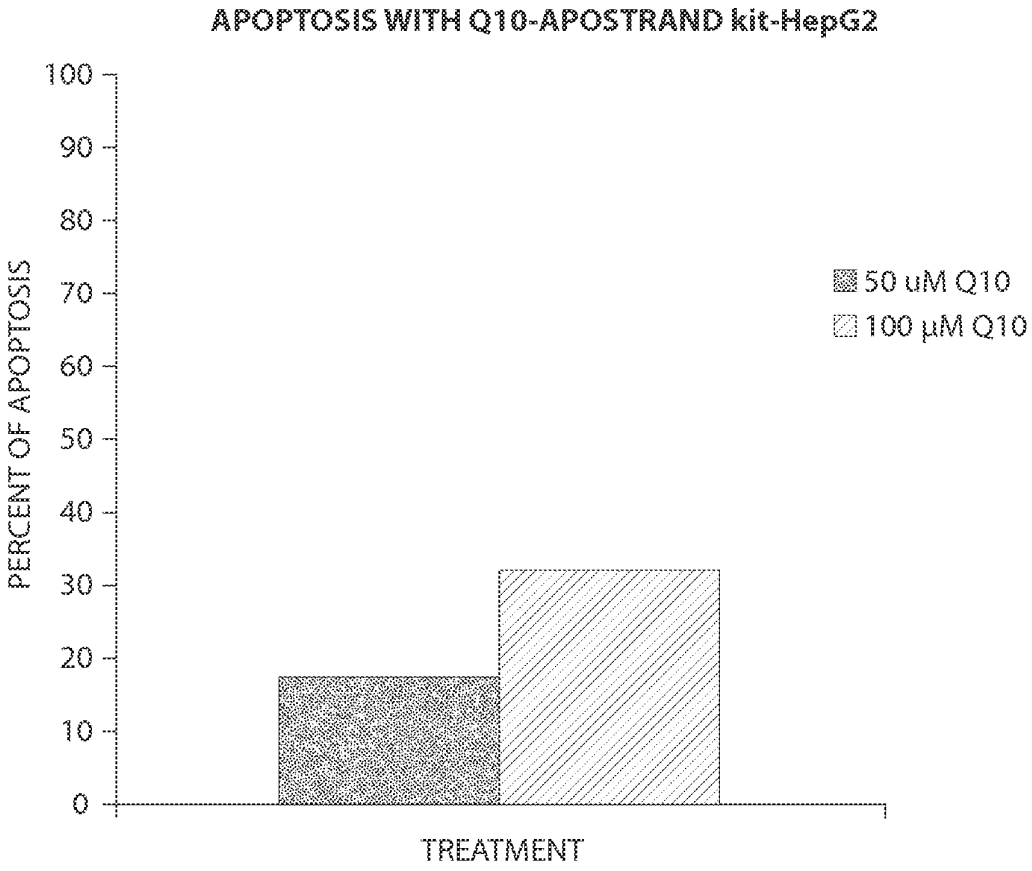
APOPTOSIS WITH Q10-APOSTRAND kit-HepG2
PERCENT OF APOPTOSIS
100
90
80
70
60
50
40
30
20
10
0
50 uM Q10
100 µM Q10
TREATMENT

Fig. 7

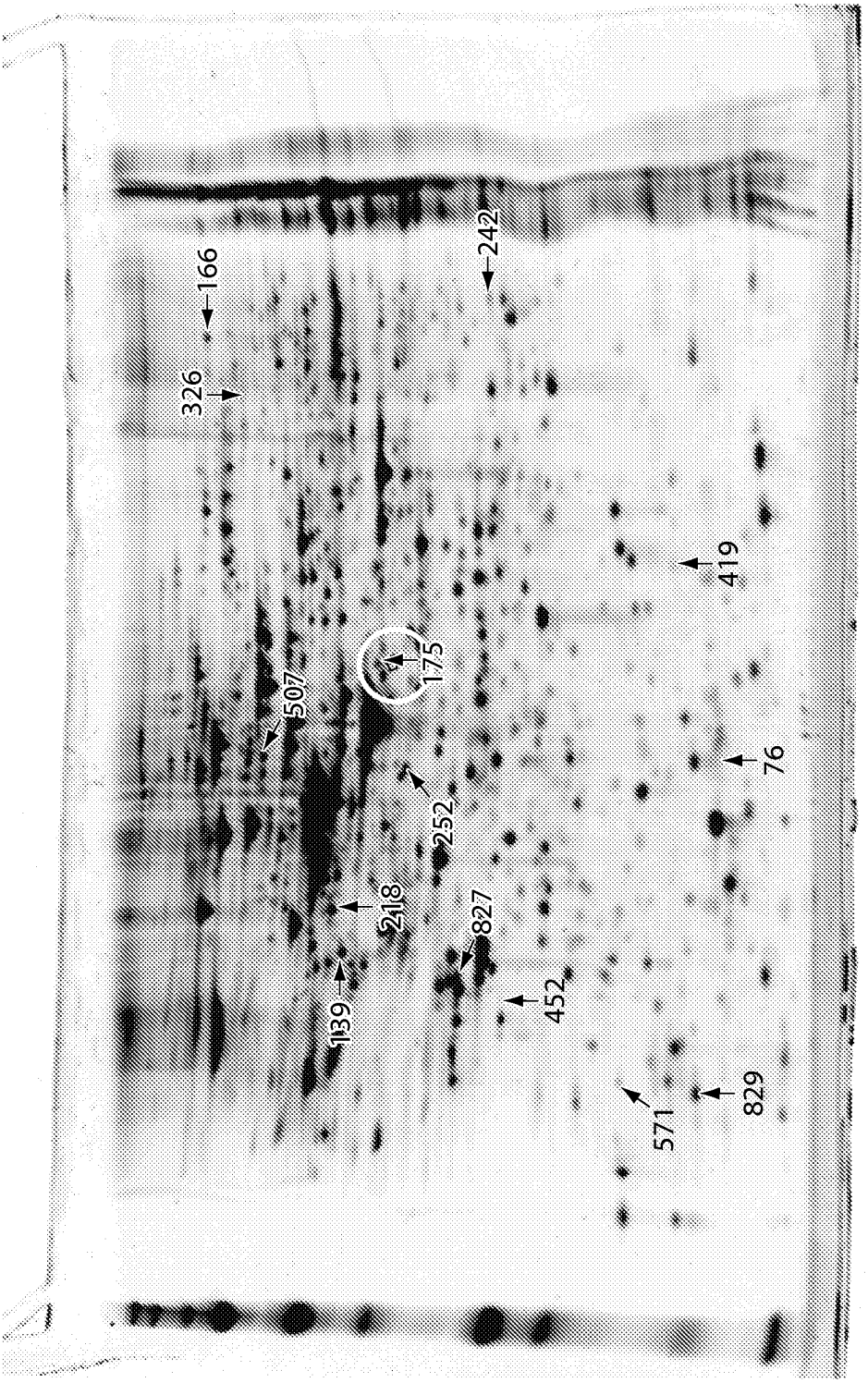

Fig. 8

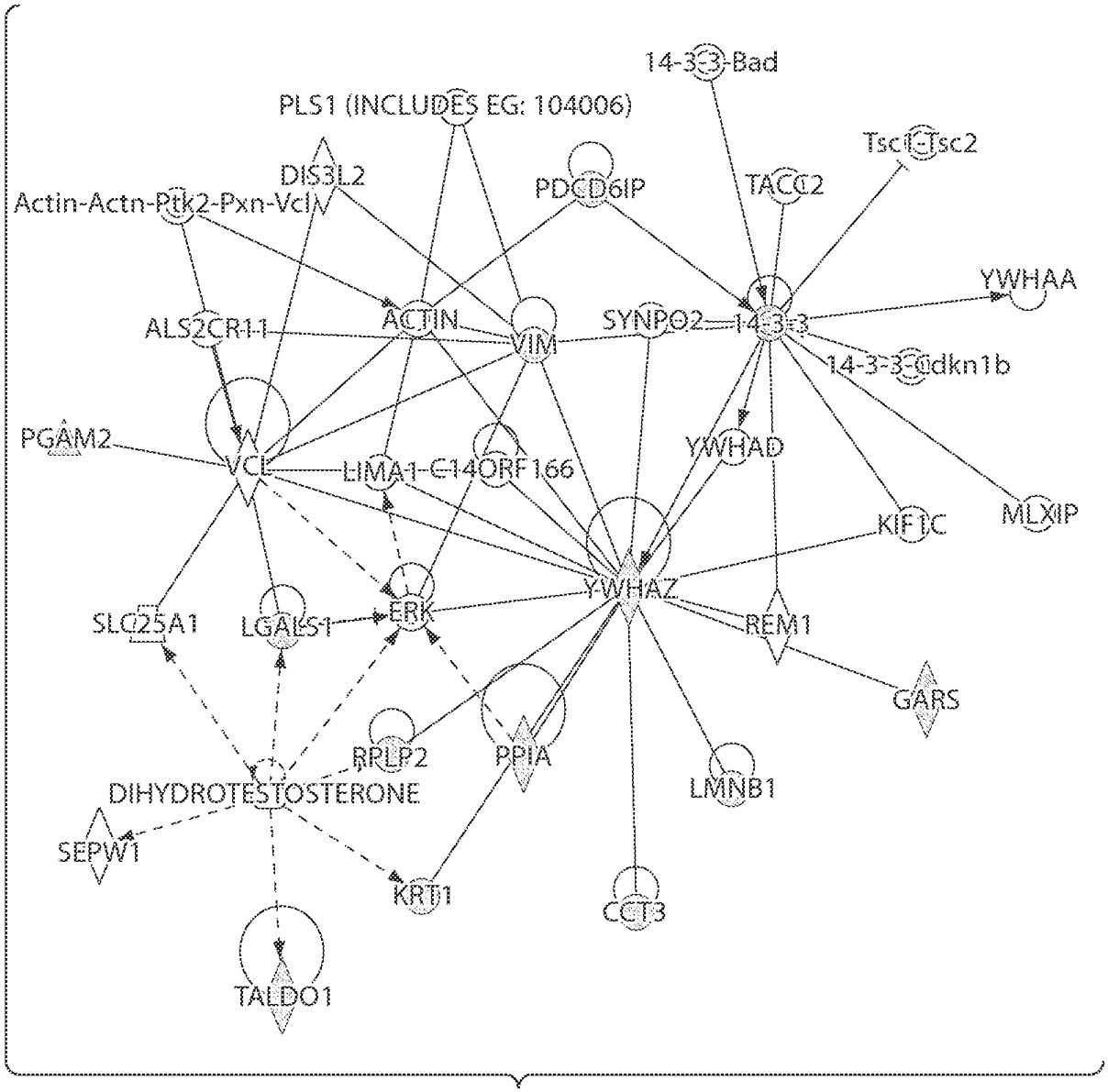
14-3-3-Bad
PLS1 (INCLUDES EG: 104006)
Tsc1-Tsc2
DIS3L2
PDCD6IP
TACC2
Actin-Actn-Ptk2-Pxn-Vcl
YWHAA
ALS2CR11
ACTIN
VIM
SYNPO2
14-3-3
14-3-3-@dkn1b
PGAM2
VCL
LIMA1-C14ORF166
YWHAD
KIF1C
MLXIP
YWHAZ
SLC25A1
LGALS1
ERK
REM1
GARS
RRLP2
PPIA
LMNB1
DIHYDROTESTOSTERONE
SEPW1
KRT1
CCT3
TALDO1

Fig. 9

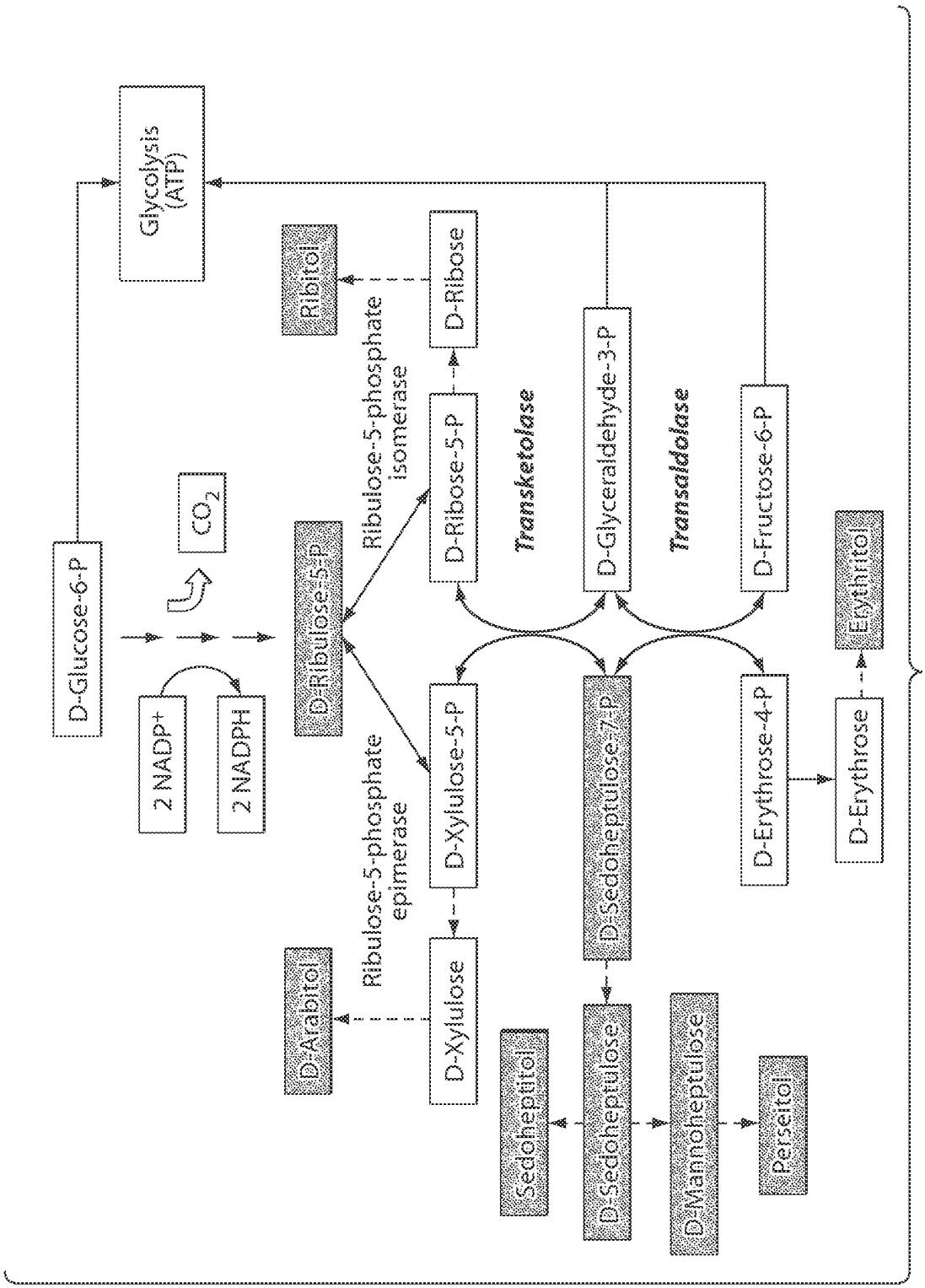
Glycolysis (ATP)
D-Glucose-6-P
2 NADP+
2 NADPH
CO2
D-Ribulose-5-P
Ribulose-5-phosphate isomerase
Ribulose-5-phosphate epimerase
Ribitol
D-Ribose
D-Ribose-5-P
Transketolase
D-Glyceraldehyde-3-P
Transaldolase
D-Fructose-6-P
Erythritol
D-Xylulose-5-P
D-Sedoheptulose-7-P
D-Erythrose-4-P
D-Erythrose
D-Arabitol
D-Xylulose
Sedoheptitol
D-Sedoheptulose
D-Mannoheptulose
Perseitol

Fig. 10

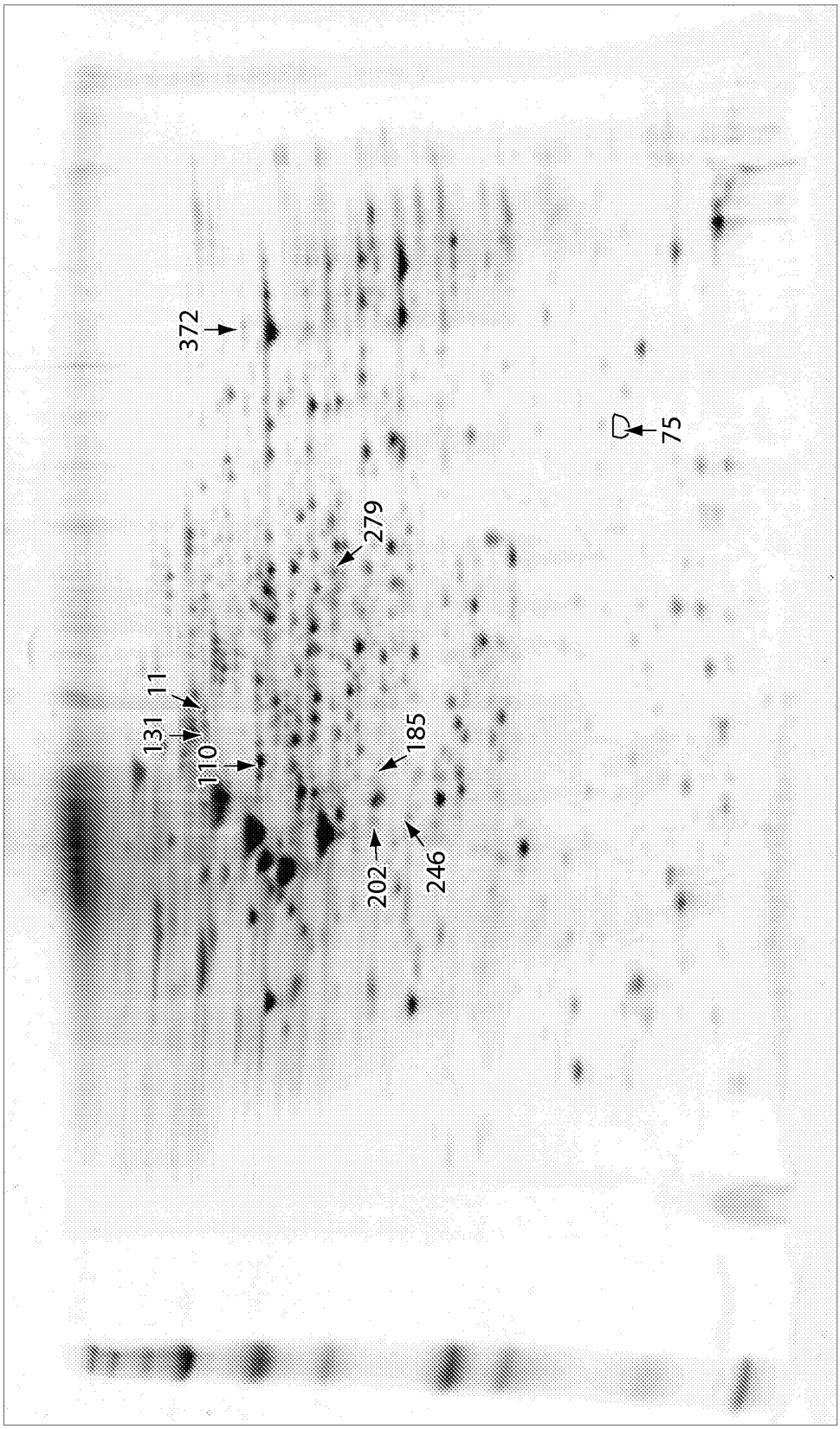

Fig. 11

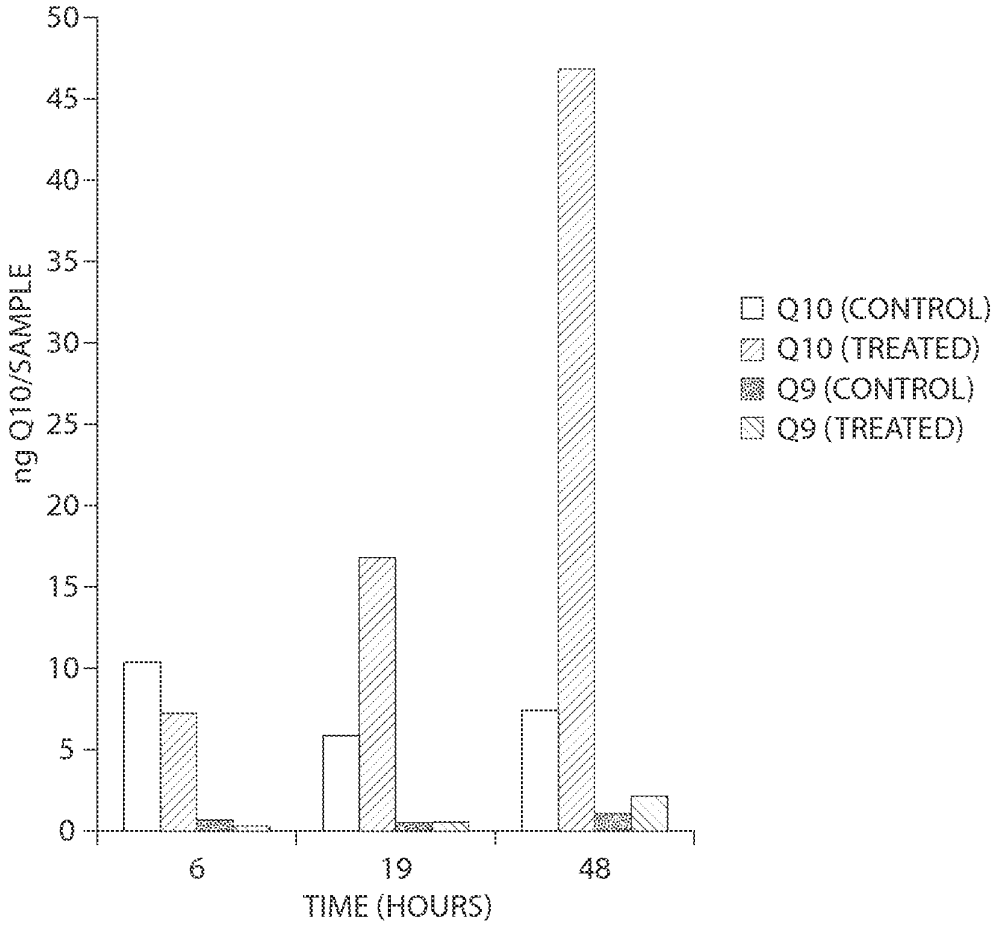
50
45
40
35
30
25
20
15
10
5
0
ng Q10/SAMPLE
6
19
48
TIME (HOURS)
Q10 (CONTROL)
Q10 (TREATED)
Q9 (CONTROL)
Q9 (TREATED)

Fig. 12

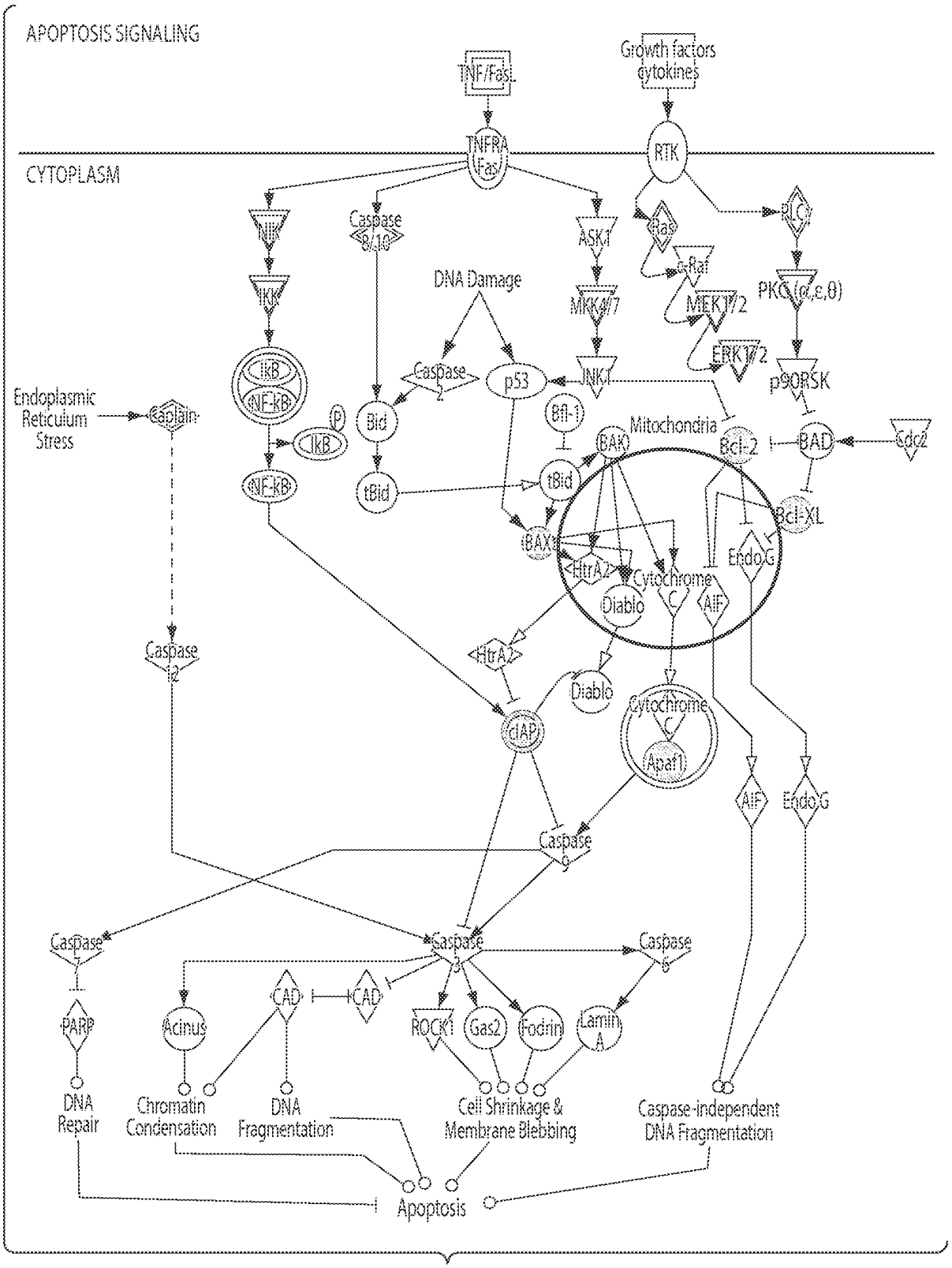
APOPTOSIS SIGNALING
CYTOPLASM
TNF/FasL
Growth factors cytokines
TNFRA (Fas)
RTK
NIK
IKK
IkB
NF-kB
P
IkB
NF-kB
Caspase 8/10
DNA Damage
Caspase 2
Bid
tBid
p53
ASK1
MKK4/7
JNK
Ras
Raf
MEK1/2
ERK1/2
PKC (α,ε,θ)
p90RSK
Bfl-1
BAK
Mitochondria
Bcl-2
BAD
Sdc2
Bcl-XL
tBid
tBAX
HtrA2
Cytochrome C
Diablo
AIF
EndoG
Endoplasmic Reticulum Stress
Calpain
Caspase 12
HtrA2
Diablo
cIAP
Cytochrome C
Apaf1
AIF
EndoG
Caspase 9
Caspase 7
Caspase 3
Caspase 6
PARP
Acinus
CAD
CAD
ROCK1
Gas2
Fodrin
Lamin A
DNA Repair
Chromatin Condensation
DNA Fragmentation
Cell Shrinkage & Membrane Blebbing
Caspase-independent DNA Fragmentation
Apoptosis

Fig. 13A

14/25

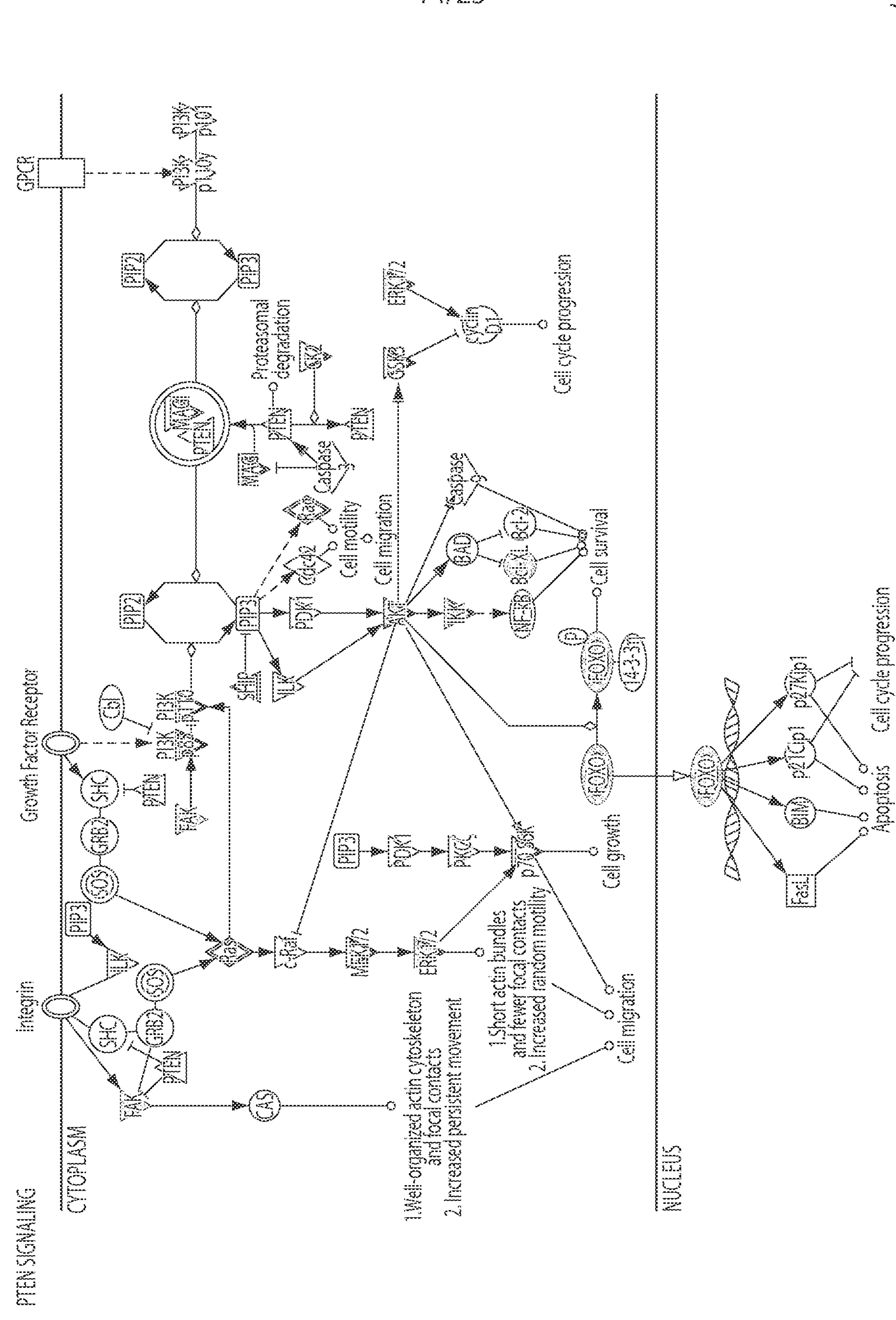
PTEN SIGNALING
CYTOPLASM
NUCLEUS
integrin
Growth Factor Receptor
GPCR
SHC
GRB2
SOS
PIP3
ILK
FAK
PTEN
CAS
Ras
c-Raf
MEK1/2
ERK1/2
PIP2
PI3K
Cbl
PDK1
PKCζ
p70 S6K
SHIP
AKT
IKK
NF-κB
BAD
Bcl-XL
Bcl-2
Caspase
Cdc42
Rac
MAGI
CK2
GSK3
Cyclin D1
FOXO
14-3-3
BIM
FasL
p21Cip1
p27Kip1
Proteasomal degradation
Cell motility
Cell migration
Cell survival
Cell growth
Cell cycle progression
Apoptosis
1.Well-organized actin cytoskeleton and focal contacts
2. Increased persistent movement
1.Short actin bundles and fewer focal contacts
2. Increased random motility

Fig. 13B

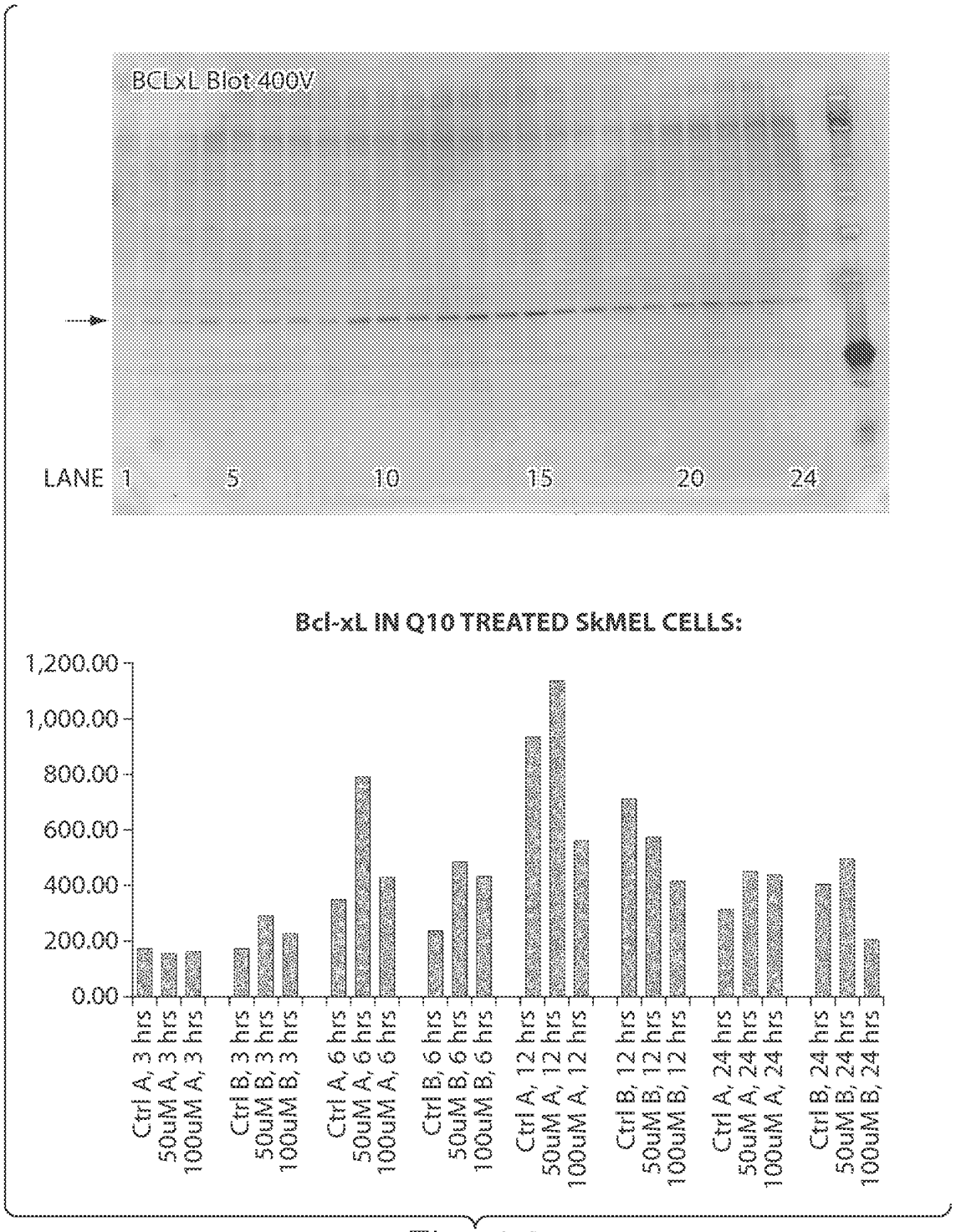
BCLxL Blot 400V
LANE 1 5 10 15 20 24
Bcl-xL IN Q10 TREATED SkMEL CELLS:
1,200.00
1,000.00
800.00
600.00
400.00
200.00
0.00
Ctrl A, 3 hrs
50uM A, 3 hrs
100uM A, 3 hrs
Ctrl B, 3 hrs
50uM B, 3 hrs
100uM B, 3 hrs
Ctrl A, 6 hrs
50uM A, 6 hrs
100uM A, 6 hrs
Ctrl B, 6 hrs
50uM B, 6 hrs
100uM B, 6 hrs
Ctrl A, 12 hrs
50uM A, 12 hrs
100uM A, 12 hrs
Ctrl B, 12 hrs
50uM B, 12 hrs
100uM B, 12 hrs
Ctrl A, 24 hrs
50uM A, 24 hrs
100uM A, 24 hrs
Ctrl B, 24 hrs
50uM B, 24 hrs
100uM B, 24 hrs

Fig. 14

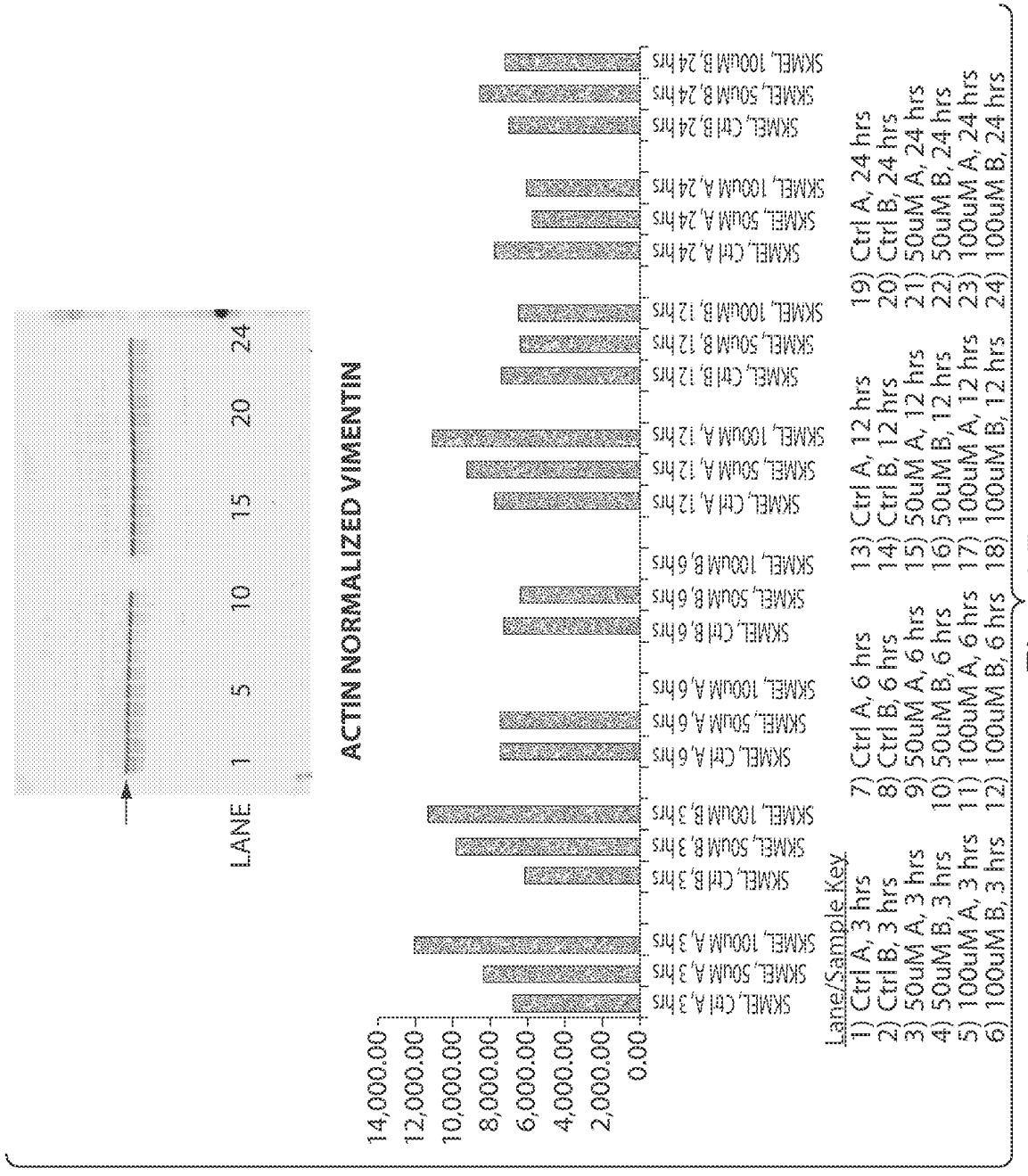
ACTIN NORMALIZED VIMENTIN
14,000.00
12,000.00
10,000.00
8,000.00
6,000.00
4,000.00
2,000.00
0.00
SKMEL, Ctrl A, 3 hrs
SKMEL, 50uM A, 3 hrs
SKMEL, 100uM A, 3 hrs
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl A, 6 hrs
SKMEL, 50uM A, 6 hrs
SKMEL, 100uM A, 6 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl A, 12 hrs
SKMEL, 50uM A, 12 hrs
SKMEL, 100uM A, 12 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl A, 24 hrs
SKMEL, 50uM A, 24 hrs
SKMEL, 100uM A, 24 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
Lane/Sample Key
1) Ctrl A, 3 hrs
2) Ctrl B, 3 hrs
3) 50uM A, 3 hrs
4) 50uM B, 3 hrs
5) 100uM A, 3 hrs
6) 100uM B, 3 hrs
7) Ctrl A, 6 hrs
8) Ctrl B, 6 hrs
9) 50uM A, 6 hrs
10) 50uM B, 6 hrs
11) 100uM A, 6 hrs
12) 100uM B, 6 hrs
13) Ctrl A, 12 hrs
14) Ctrl B, 12 hrs
15) 50uM A, 12 hrs
16) 50uM B, 12 hrs
17) 100uM A, 12 hrs
18) 100uM B, 12 hrs
19) Ctrl A, 24 hrs
20) Ctrl B, 24 hrs
21) 50uM A, 24 hrs
22) 50uM B, 24 hrs
23) 100uM A, 24 hrs
24) 100uM B, 24 hrs
LANE
1
5
10
15
20
24

Fig. 15

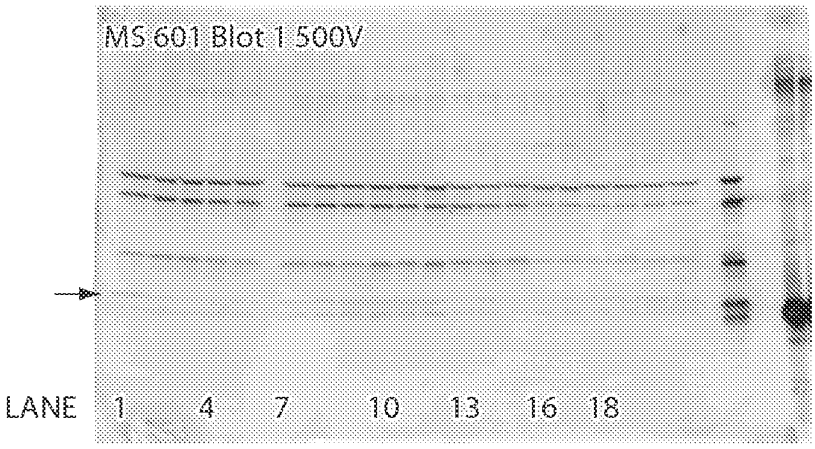
MS 601 Blot 1 500V
LANE 1 4 7 10 13 16 18

Fig. 16

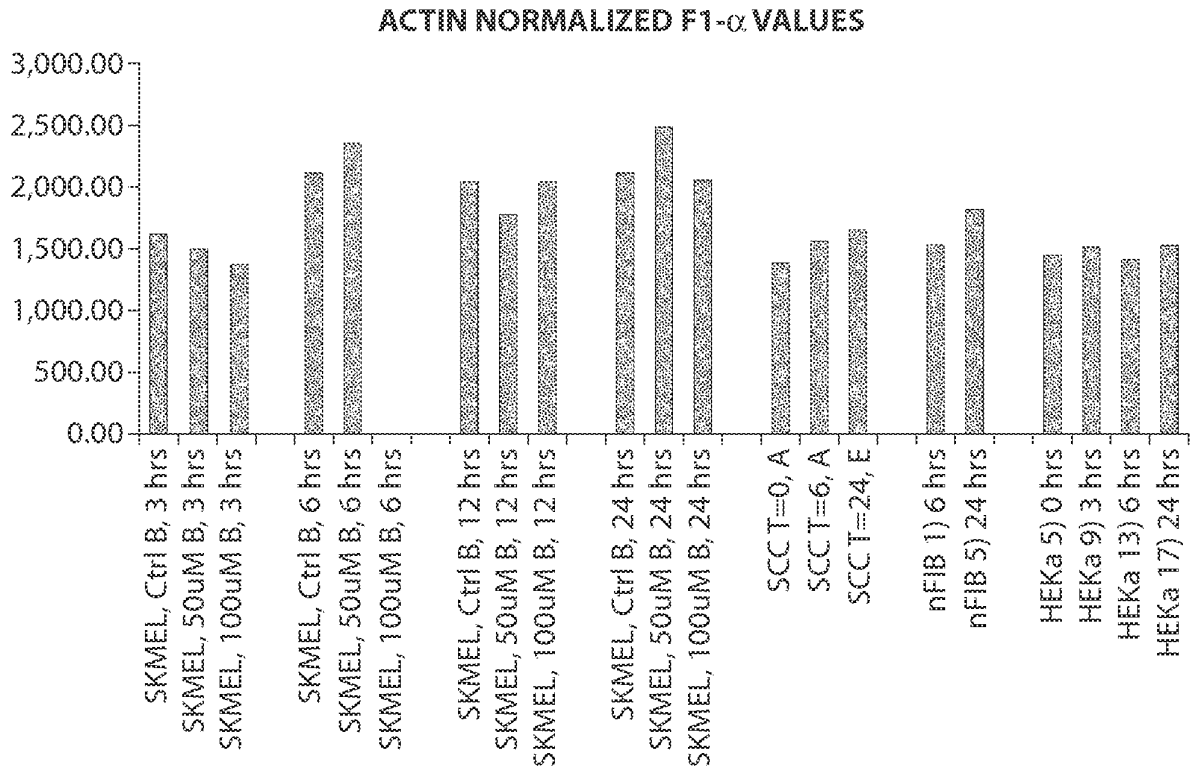
ACTIN NORMALIZED F1-α VALUES
3,000.00
2,500.00
2,000.00
1,500.00
1,000.00
500.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 17

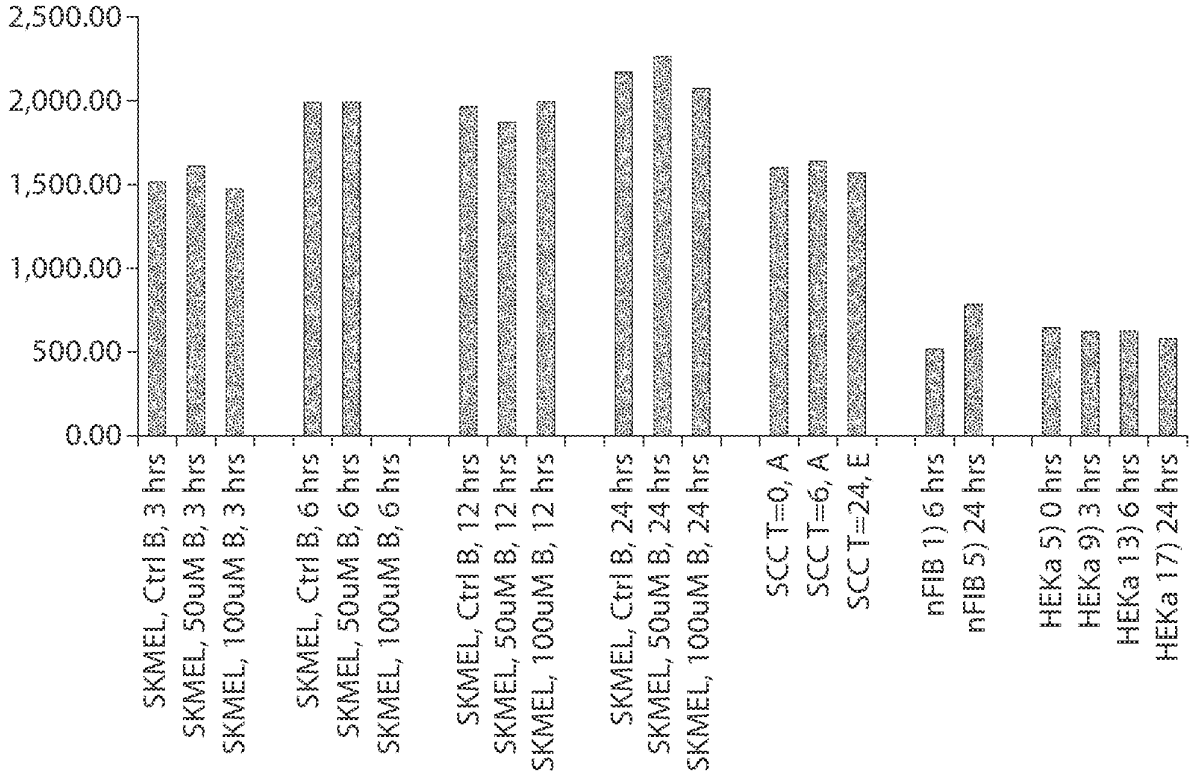
ACTIN NORMALIZED C-III-CORE 2 VALUES
2,500.00
2,000.00
1,500.00
1,000.00
500.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 18

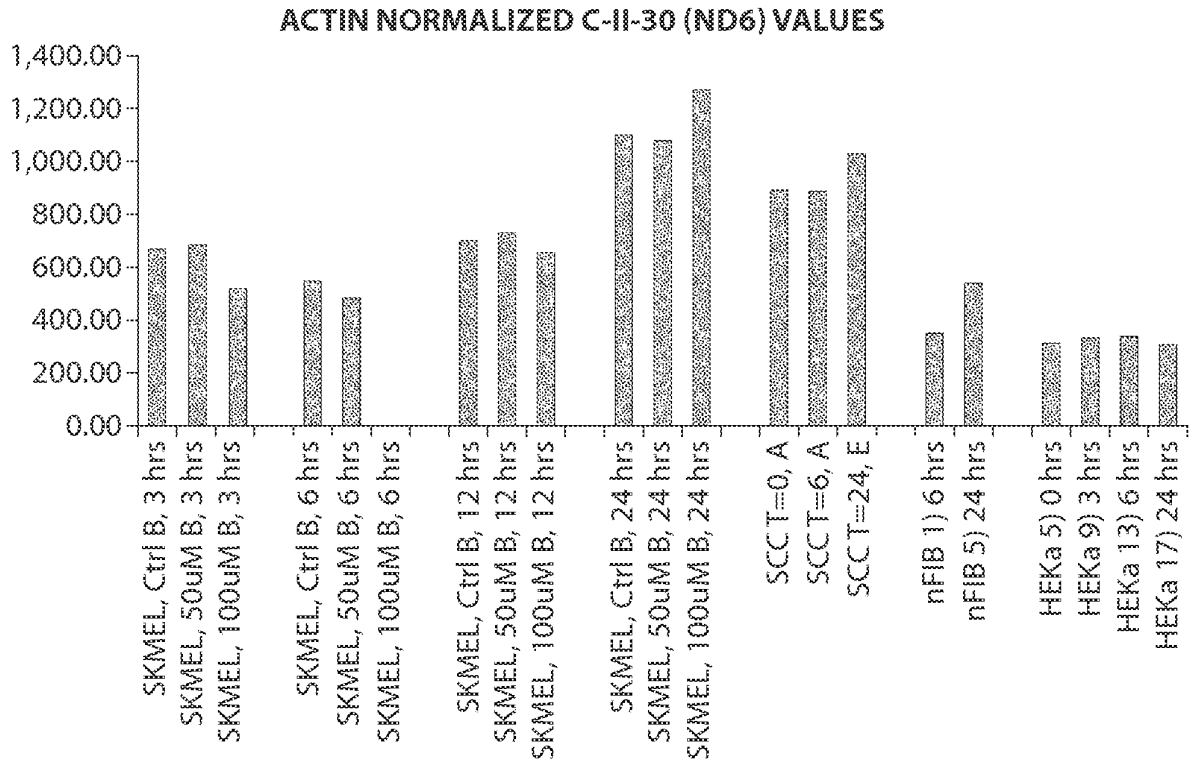
ACTIN NORMALIZED C-II-30 (ND6) VALUES
1,400.00
1,200.00
1,000.00
800.00
600.00
400.00
200.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 19

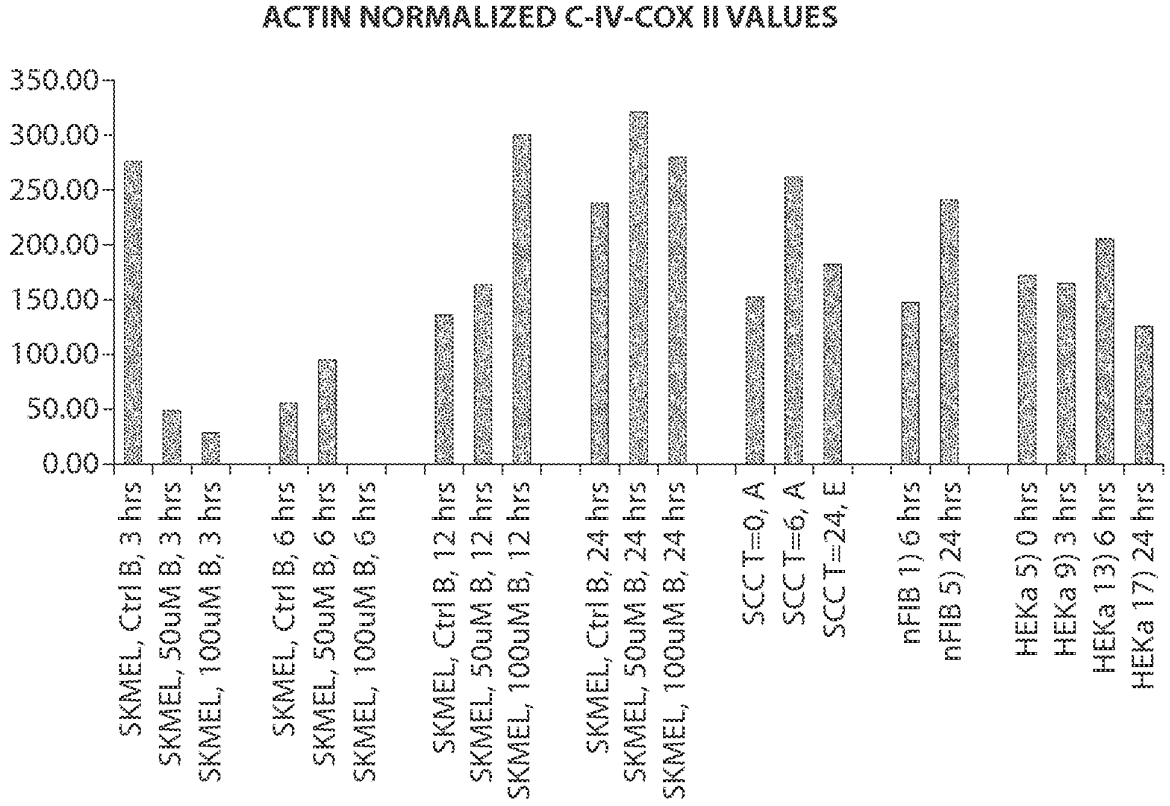
ACTIN NORMALIZED C-IV-COX II VALUES
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 20

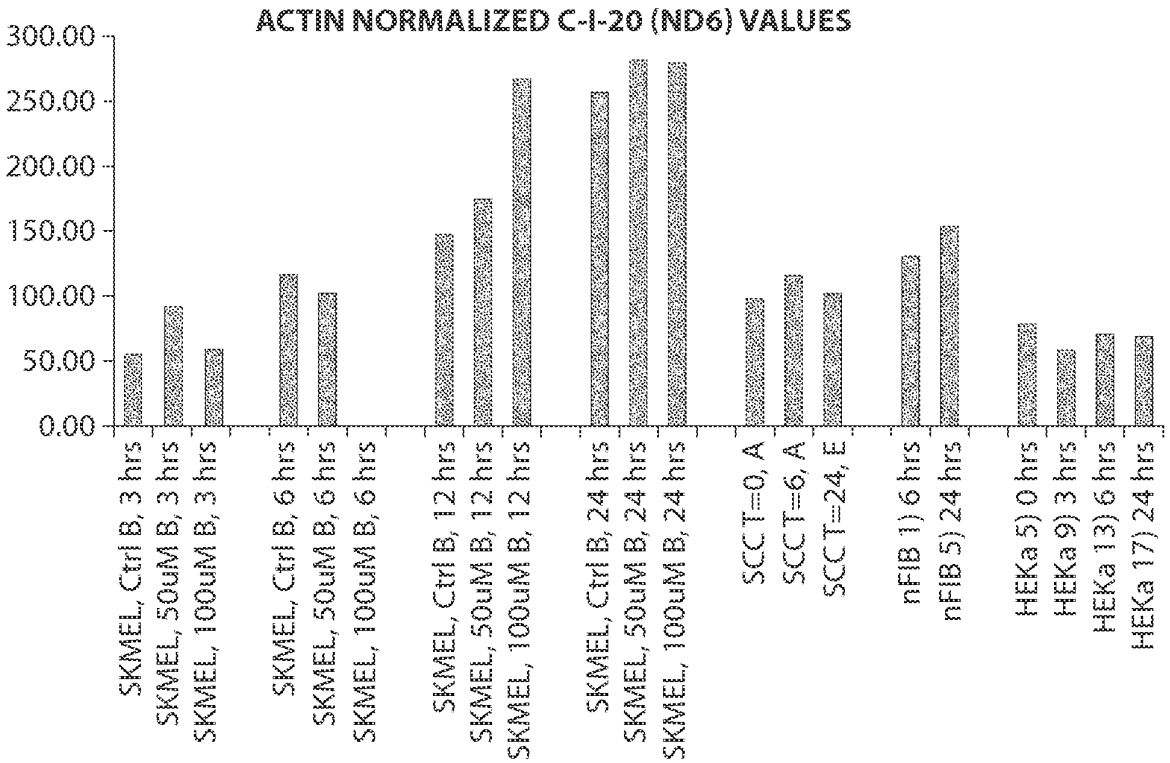
ACTIN NORMALIZED C-I-20 (ND6) VALUES
300.00
250.00
200.00
150.00
100.00
50.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 21

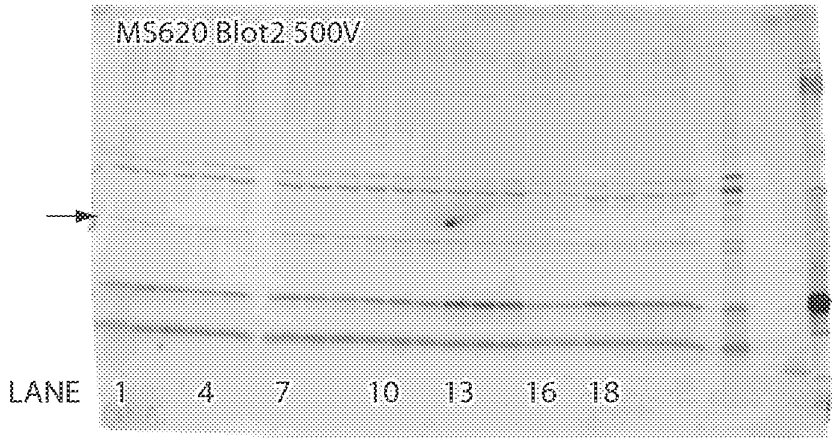
MS620 Blot2 500V
LANE 1 4 7 10 13 16 18

Fig. 22

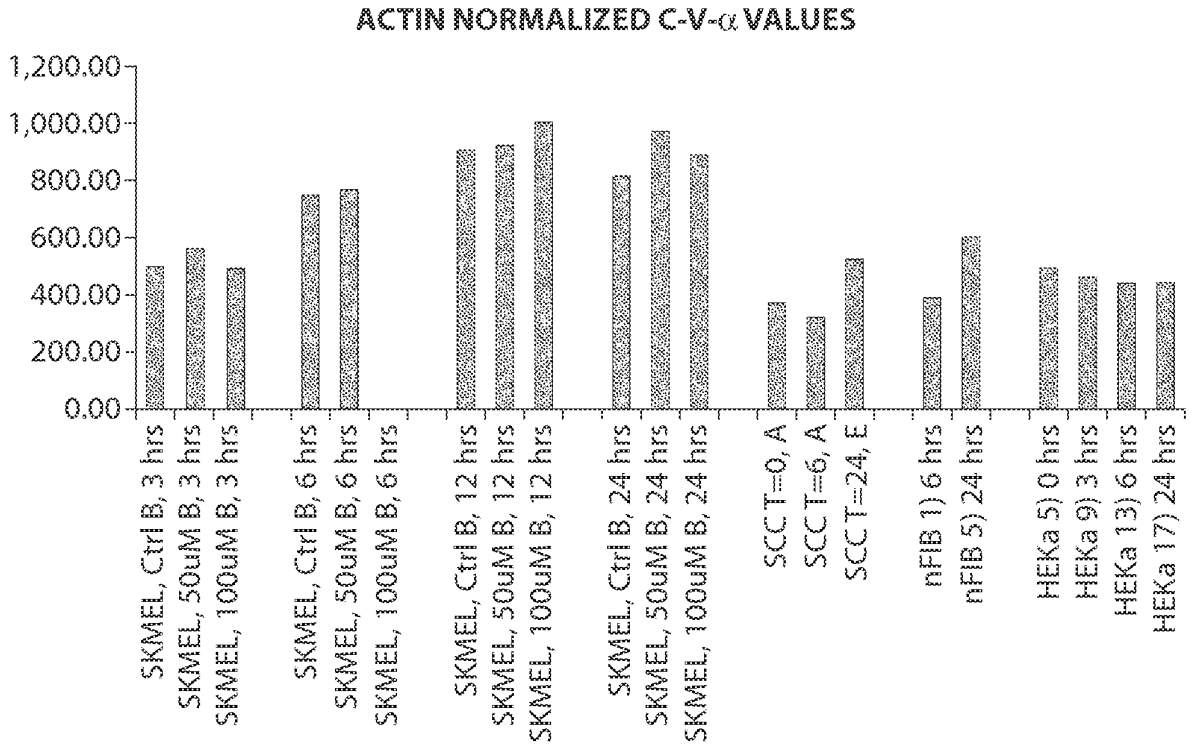
ACTIN NORMALIZED C-V-α VALUES
1,200.00
1,000.00
800.00
600.00
400.00
200.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 23

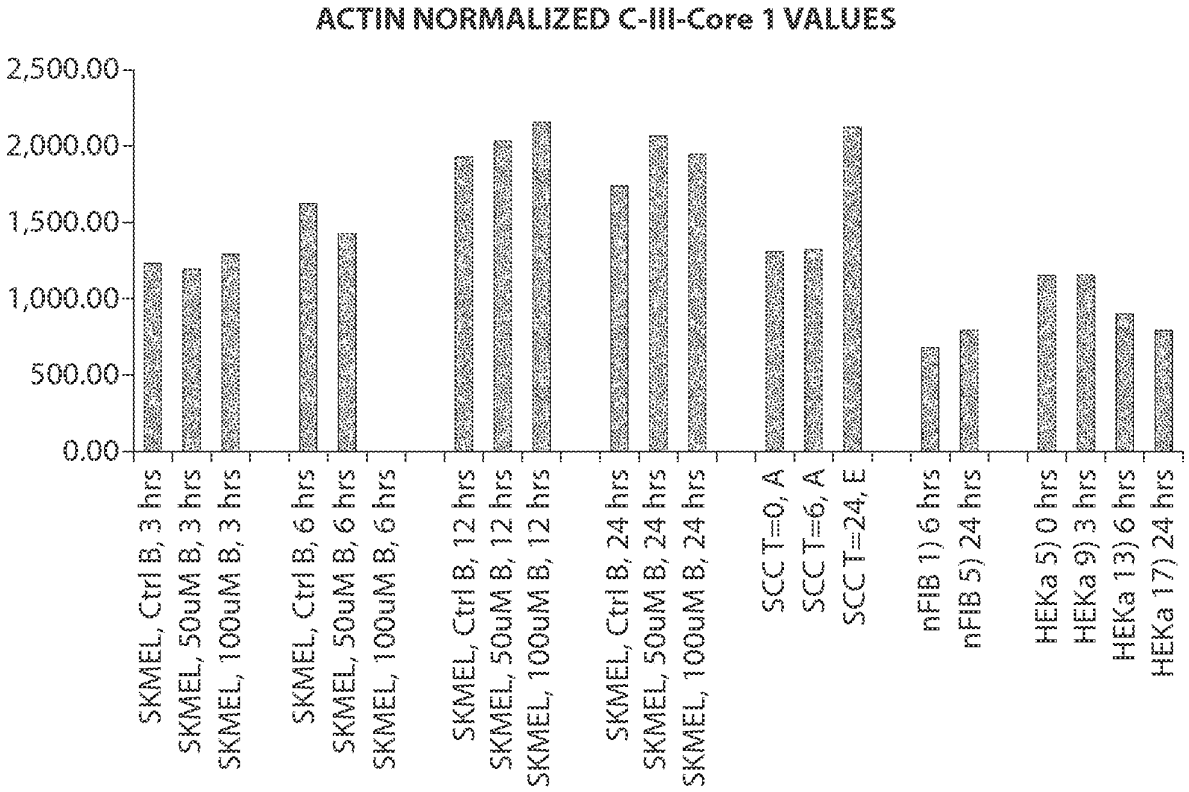
ACTIN NORMALIZED C-III-Core 1 VALUES
2,500.00
2,000.00
1,500.00
1,000.00
500.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 24

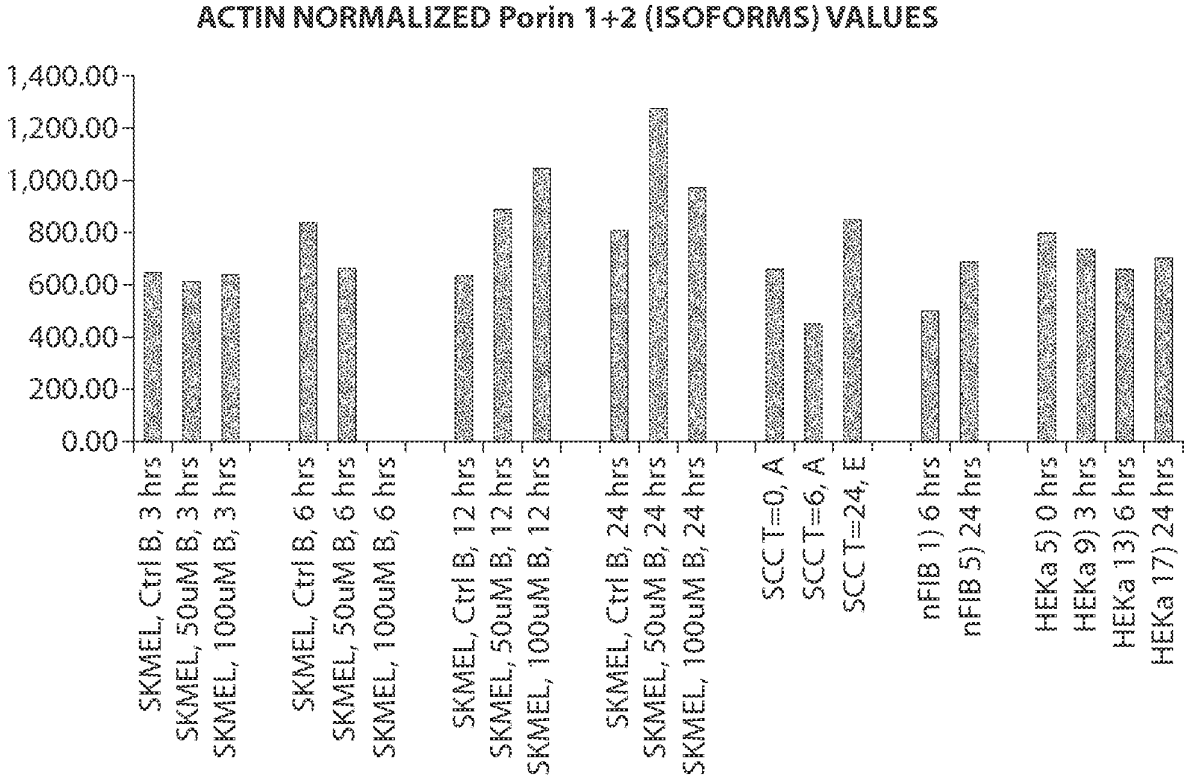
ACTIN NORMALIZED Porin 1+2 (ISOFORMS) VALUES
1,400.00
1,200.00
1,000.00
800.00
600.00
400.00
200.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 25

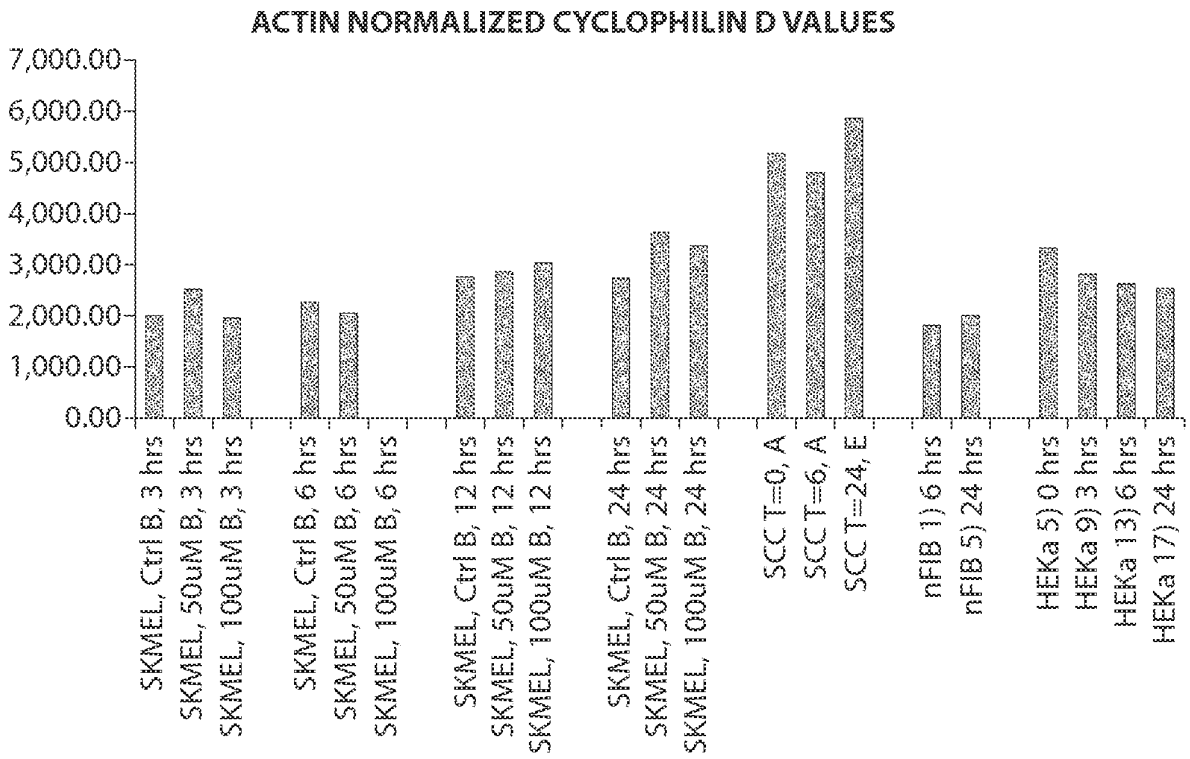
ACTIN NORMALIZED CYCLOPHILIN D VALUES
7,000.00
6,000.00
5,000.00
4,000.00
3,000.00
2,000.00
1,000.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 26

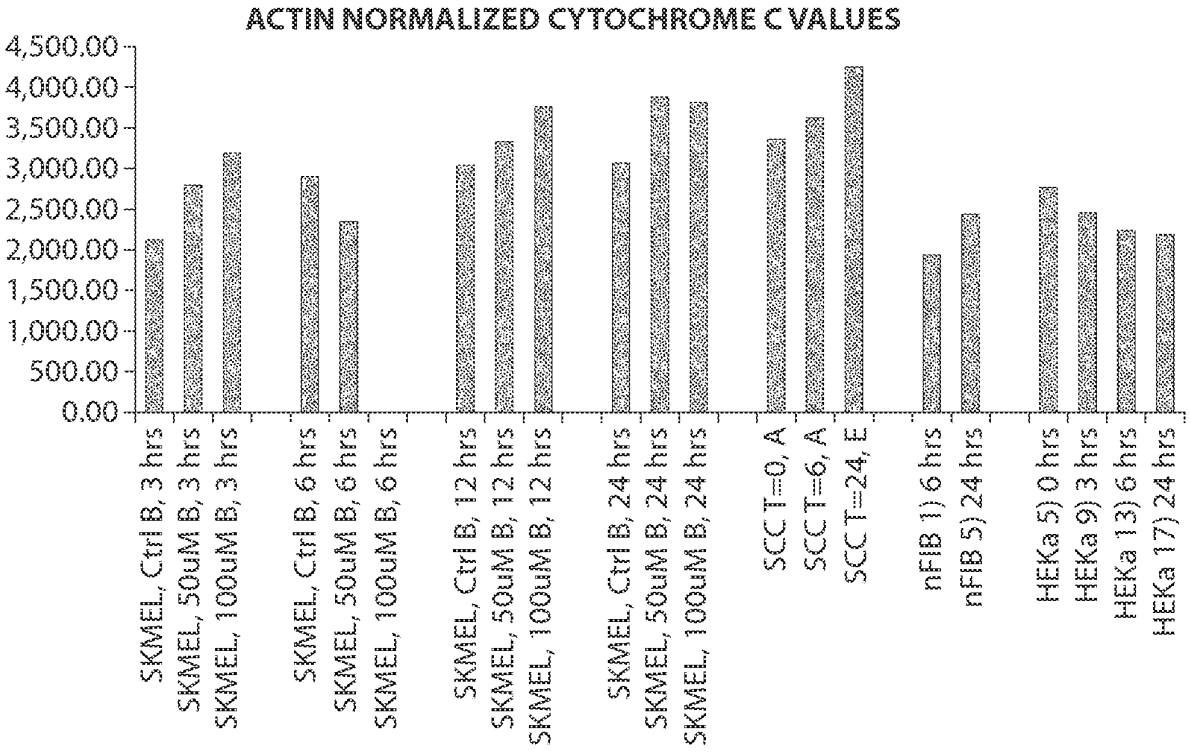
ACTIN NORMALIZED CYTOCHROME C VALUES
4,500.00
4,000.00
3,500.00
3,000.00
2,500.00
2,000.00
1,500.00
1,000.00
500.00
0.00
SKMEL, Ctrl B, 3 hrs
SKMEL, 50uM B, 3 hrs
SKMEL, 100uM B, 3 hrs
SKMEL, Ctrl B, 6 hrs
SKMEL, 50uM B, 6 hrs
SKMEL, 100uM B, 6 hrs
SKMEL, Ctrl B, 12 hrs
SKMEL, 50uM B, 12 hrs
SKMEL, 100uM B, 12 hrs
SKMEL, Ctrl B, 24 hrs
SKMEL, 50uM B, 24 hrs
SKMEL, 100uM B, 24 hrs
SCC T=0, A
SCC T=6, A
SCC T=24, E
nFIB 1) 6 hrs
nFIB 5) 24 hrs
HEKa 5) 0 hrs
HEKa 9) 3 hrs
HEKa 13) 6 hrs
HEKa 17) 24 hrs

Fig. 27

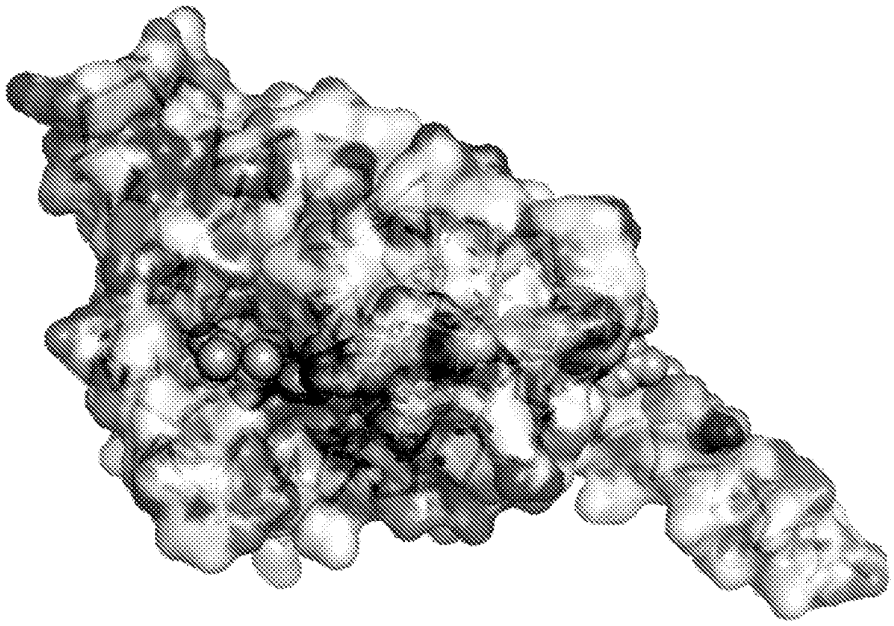

Fig. 28

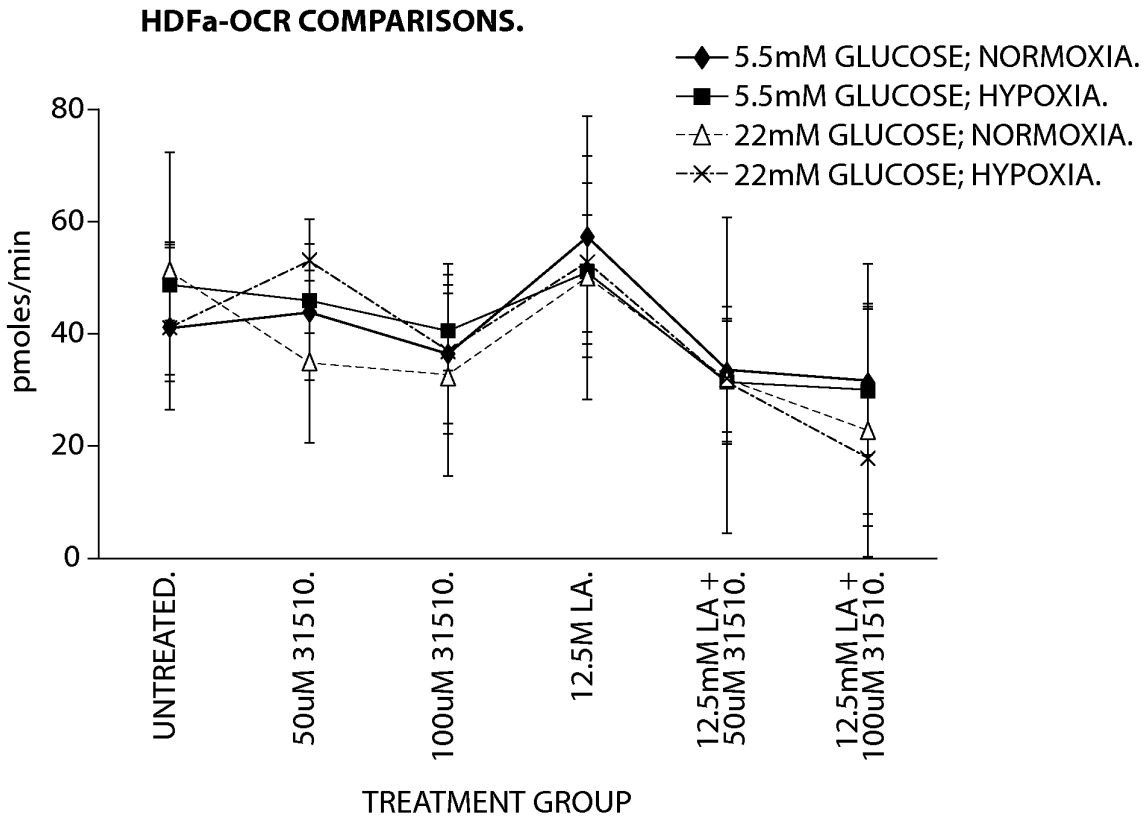
HDFa-OCR COMPARISONS.
5.5mM GLUCOSE; NORMOXIA.
5.5mM GLUCOSE; HYPOXIA.
22mM GLUCOSE; NORMOXIA.
22mM GLUCOSE; HYPOXIA.
pmoles/min
0
20
40
60
80
UNTREATED.
50uM 31510.
100uM 31510.
12.5M LA.
12.5mM LA + 50uM 31510.
12.5mM LA + 100uM 31510.
TREATMENT GROUP

Fig. 29

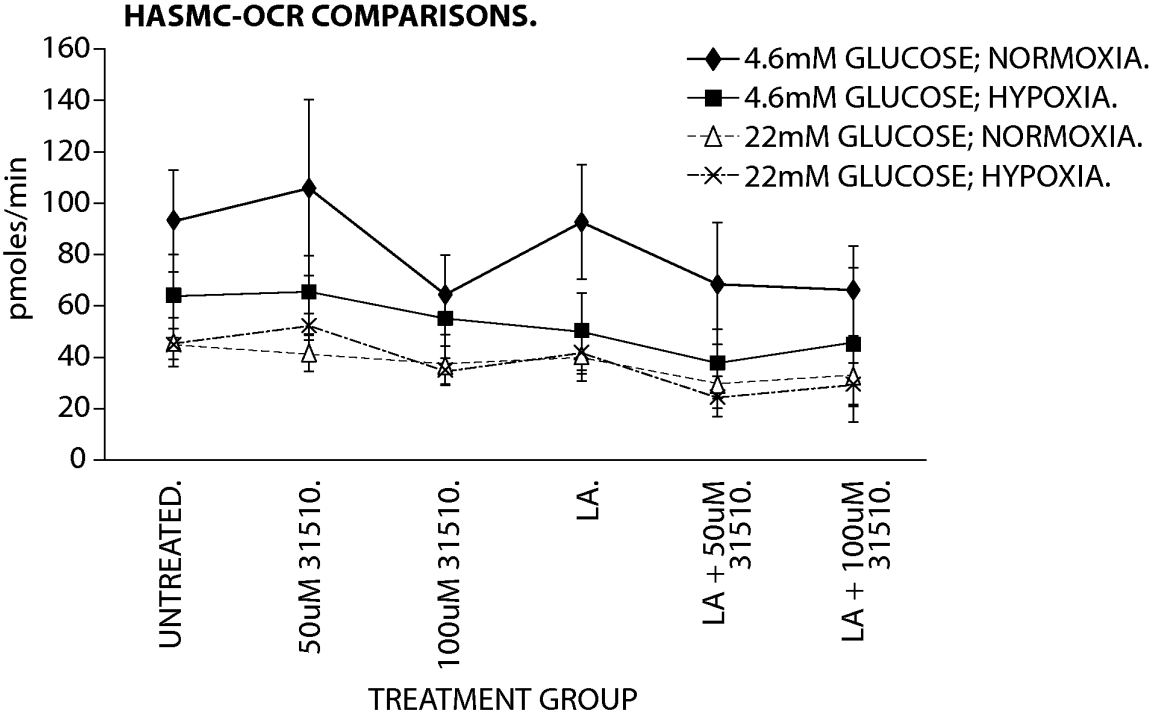
HASMC-OCR COMPARISONS.
4.6mM GLUCOSE; NORMOXIA.
4.6mM GLUCOSE; HYPOXIA.
22mM GLUCOSE; NORMOXIA.
22mM GLUCOSE; HYPOXIA.
pmoles/min
0
20
40
60
80
100
120
140
160
UNTREATED.
50uM 31510.
100uM 31510.
LA.
LA + 50uM 31510.
LA + 100uM 31510.
TREATMENT GROUP

Fig. 30

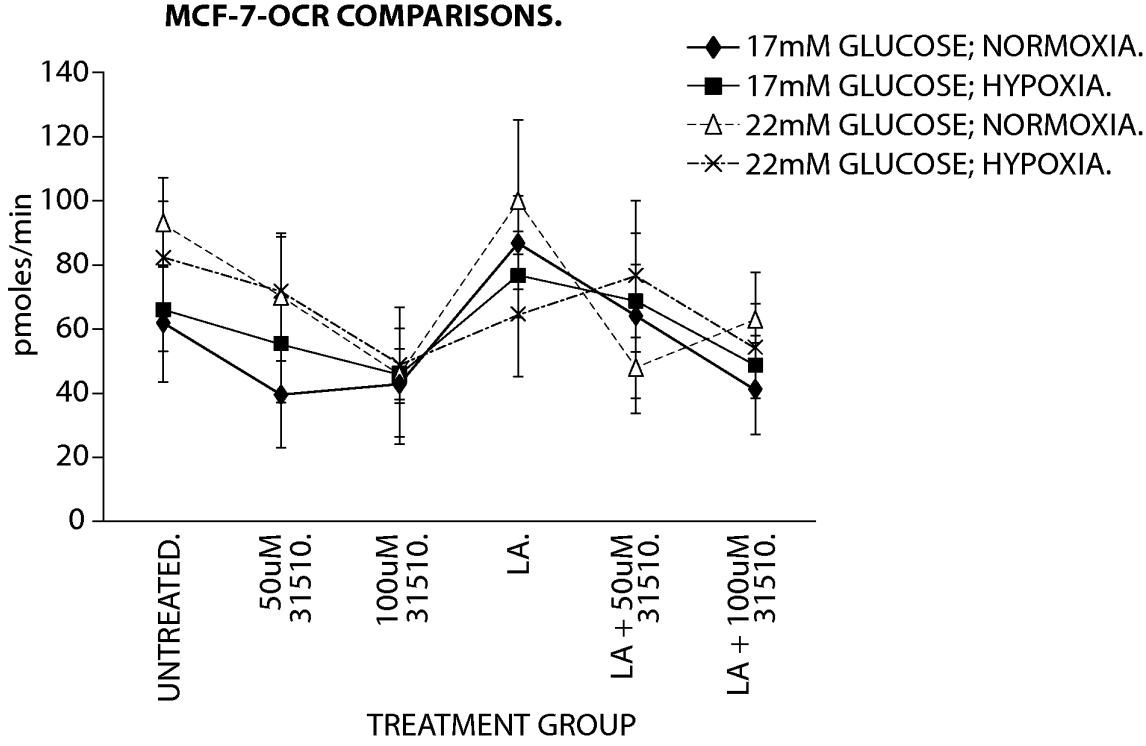
MCF-7-OCR COMPARISONS.
17mM GLUCOSE; NORMOXIA.
17mM GLUCOSE; HYPOXIA.
22mM GLUCOSE; NORMOXIA.
22mM GLUCOSE; HYPOXIA.
pmoles/min
140
120
100
80
60
40
20
0
UNTREATED.
50uM 31510.
100uM 31510.
LA.
LA + 50uM 31510.
LA + 100uM 31510.
TREATMENT GROUP

Fig. 31

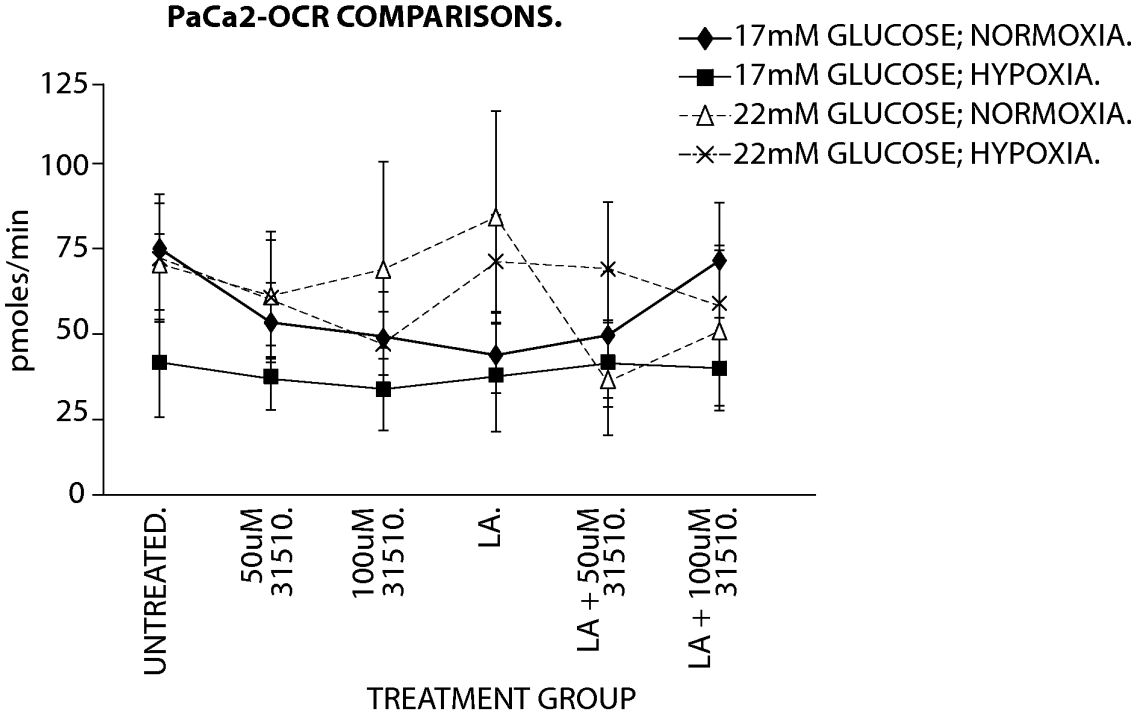
PaCa2-OCR COMPARISONS.
17mM GLUCOSE; NORMOXIA.
17mM GLUCOSE; HYPOXIA.
22mM GLUCOSE; NORMOXIA.
22mM GLUCOSE; HYPOXIA.
pmoles/min
125
100
75
50
25
0
UNTREATED.
50uM 31510.
100uM 31510.
LA.
LA + 50uM 31510.
LA + 100uM 31510.
TREATMENT GROUP

Fig. 32