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INVENTOR(S)

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☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

COMPOUNDS AND METHODS FOR TREATING CONDITIONS ASSOCIATED WITH HYPOXIA
INDUCIBLE FACTOR

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☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE

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COMPOUNDS AND METHODS FOR TREATING CONDITIONS ASSOCIATED WITH HYPOXIA INDUCIBLE FACTOR

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FIELD OF THE INVENTION

The present invention relates to compounds that stabilize the alpha subunit of hypoxia inducible factor and to the use of the compounds in preventing or treating conditions associated with HIF, including ischemic conditions such as ischemic stroke, myocardial infarction, spinal cord injury, and the like, and pulmonary disorders such as pulmonary embolism, pneumonitis, chronic obstructive pulmonary disease, and the like.

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BACKGROUND OF THE INVENTION

Ischemia, a reduction or cessation of blood flow, reduces or blocks the supply of oxygen and nutrients to tissues and organs. Ischemia can be caused by atherosclerosis, or obstruction of an artery or vein by formation of a blood clot (thrombus) or by any foreign circulating matter (embolus). Ischemia can be acute, having a sudden onset and short duration, or chronic, having a long duration or frequent recurrence. Acute ischemia is often associated with an infarct, and chronic ischemia is usually associated with transient hypoxic injury. However, if the decrease in perfusion is prolonged or severe, chronic ischemia can also be associated with an infarct. Infarctions commonly occur in the spleen, kidney, lungs, brain, and heart and can lead to disorders including intestinal infarction, pulmonary infarction, ischemic stroke, and myocardial infarction.

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Occlusive arterial disease includes both coronary artery disease, which can lead to myocardial infarction, and peripheral arterial disease, which can affect the abdominal aorta, its major branches, and arteries of the legs. Peripheral arterial diseases include Buerger's disease, Raynaud's disease, and acrocyanosis. Although peripheral arterial disease is commonly caused by atherosclerosis, other major causes include, e.g., diabetes, etc. Complications associated with peripheral arterial disease include severe leg cramps, angina, abnormal heart rhythms, heart failure, heart attack, stroke, and kidney failure.

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Hypoperfusion or infarction in one organ often affects other organs. Ischemia of the lung, caused by, for example, a pulmonary embolism, pneumonitis, chronic obstructive pulmonary disease (COPD), or congestive heart failure, not only affects the lung, but also puts the heart and other organs, such as the brain, under hypoxic stress. Myocardial infarction, which often involves coronary artery blockage due to arteriosclerosis, arterial wall vasospasms, or viral infection of the heart, can lead to congestive heart failure and systemic hypotension. Secondary complications such as global ischemic encephalopathy can

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develop if the cardiac arrest is prolonged with continued hypoperfusion. Cerebral ischemia, most commonly caused by vascular occlusion due to atherosclerosis, can range in severity from transient ischemic attacks (TIAs) to cerebral infarction or stroke. While the symptoms of TIAs are temporary and reversible, TIAs tend to recur and are often followed by a stroke.

Pathologic changes in ischemic disorders depend on the duration and severity of ischemia, and on the length of patient survival. Necrosis can be seen within the infarct in the first 24 hours, and an acute inflammatory response develops in the viable tissue adjacent to the infarct with leukocytes migrating into the area of dead tissue. Over succeeding days, there is a gradual breakdown and removal of the infarcted cells by phagocytosis, and replacement with a collagenous or glial scar.

Cellular Response to Hypoxia

An early response to tissue hypoxia is induction of hypoxia inducible factor (HIF), a basic helix-loop-helix (bHLH) PAS (Per/Arnt/Sim) transcriptional activator that mediates changes in gene expression in response to changes in cellular oxygen concentration. HIF is a heterodimer consisting of an oxygen-regulated alpha subunit (HIF α) and a constitutively expressed beta subunit (HIF β), also known as aryl hydrocarbon receptor nuclear transporter (ARNT). In oxygenated (normoxic) cells, HIF α subunits are rapidly degraded by a mechanism that involves ubiquitination by the von Hippel-Lindau tumor suppressor (pVHL) E3 ligase complex. Under hypoxic conditions, HIF α is not degraded, and an active HIF complex accumulates in the nucleus and activates the expression of several genes including glycolytic enzymes, glucose transporter (GLUT)-1, erythropoietin (EPO), and vascular endothelial growth factor (VEGF). (Jiang et al. (1996) J Biol Chem 271:17771-17778; Iliopoulos et al. (1996) Proc Natl Acad Sci USA 93:10595-10599; Maxwell et al. (1999) Nature 399:271-275; Sutter et al. (2000) Proc Natl Acad Sci USA 97:4748-4753; Cockman et al. (2000) J Biol Chem 275:25733-25741; and Tanimoto et al. (2000) EMBO J 19:4298-4309.)

Levels of HIF α protein are elevated in most cells in response to hypoxia and are induced *in vivo* when animals are subjected to anemia or hypoxia. HIF α levels rise within a few hours after the onset of hypoxia and then return to baseline under continued hypoxic conditions. HIF has been implicated in numerous cellular and developmental processes including apoptosis, cell proliferation, and angiogenesis. HIF α has also been associated with myocardial acute ischemia and early infarction, pulmonary hypertension, and inflammation. Although HIF α has been associated with tumor growth and metastasis, there is little indication that HIF is directly involved in tumorigenesis. Hypoxic preconditioning, in which a target organ is subjected to brief periods of hypoxia, has been shown to protect both myocardium and

brain against hypoxic-ischemic injury. (Wang and Semenza (1993) *Proc Natl Acad Sci USA* 90:4304-4308; Stroka et al. (2001) *FASEB J* 15:2445-2453; Semenza et al. (1997) *Kidney Int* 51:553-555; Carmeliet et al. (1998) *Nature* 394:485-490; Zhong et al. (1999) *Cancer Res* 59:5830-5835; Lee et al. (2000) *N Engl J Med* 343:148-149; Sharp et al. (2000) *J Cereb Blood Flow Metab* 20:1011-1032; Semenza et al. (2000) *Adv Exp Med Biol* 475:123-130; Thornton et al. (2000) *Biochem J* 350:307-312; Deindl and Schaper (1998) 186:43-51; Bergeron et al. (2000) 48:285-296.)

Several investigators have studied the mechanism of interaction between HIF α and pVHL. An oxygen-dependent degradation domain (ODD) within HIF-1 α from residue 401 to 603 was originally identified as sufficient to confer oxygen-dependent instability to chimeric protein constructs. A smaller domain within the ODD, from residue 526 to 652, was found to be required for pVHL-dependent degradation. Further, mutation of P₅₆₄YI to aspartic acids or mutation of K₅₃₂ to arginine within a region conserved among HIF α homologs (residue 556 to 574 in HIF-1 α) rendered the full-length HIF α protein stable under normoxic conditions and resistant to pVHL-mediated degradation. (Huang et al. (1998) *Proc Natl Acad Sci USA* 95:7987-7992; and Tanimoto et al. (2000) *EMBO J* 19:4298-4309.)

HIF α levels are increased by a number of factors that mimic hypoxia, including iron chelators such as desferrioxamine (DFO) and divalent metals such as CoCl₂. In vascular smooth muscle cells, angiotensin II, thrombin, and platelet-derived growth factor stimulate HIF α stabilization under normoxic conditions using a mechanism involving reactive oxygen species. Reports have also suggested HIF α is regulated by phosphorylation through pathways involving nitric oxide-activated phosphatidylinositol 3'-kinase (PI3K), hepatocyte growth factor, or mitogen-activated protein kinase. Glycogen-synthase kinase, which is a downstream target of PI3K, directly phosphorylates the HIF α ODD domain. (Richard et al. (2000) *J Biol Chem* 275:26765-26771; Sandau et al. (2000) *Biochem Biophys Res Commun* 278:263-267; Tacchini et al. (2001) *Carcinogenesis* 22:1363-1371; and Sodhi et al. (2001) *Biochem Biophys Res Commun* 287:292-300.)

Ischemic disorders are a major cause of morbidity and mortality. Cardiovascular diseases cause at least 15 million deaths every year and are responsible for 30% of deaths worldwide. Among the various cardiovascular diseases, ischemic heart disease and cerebrovascular diseases cause approximately 17% of deaths. Annually, 1.3 million cases of nonfatal acute myocardial infarction are reported, making the prevalence approximately 600 per 100,000 people. Further, an estimated five million Americans suffer from venous thrombosis every year, and approximately 600,000 of these cases result in pulmonary

embolism. About one-third of the pulmonary embolisms end in death, making pulmonary embolism the third most common cause of death in the United States.

Currently, treatment of ischemic disorders is focused on relief of symptoms and treatment of causative disorders. For example, treatments for myocardial infarction include nitroglycerin and analgesics to control pain and relieve the workload of the heart. Other medications, including digoxin, diuretics, amrinone, β -blockers, lipid-lowering agents and angiotensin-converting enzyme inhibitors, are used to stabilize the condition, but none of these therapies directly address ischemia and the resulting tissue damage.

Due to deficiencies in current treatments, there remains a need for compounds that are effective in the treatment of ischemic conditions such as occlusive arterial disease, angina pectoris, intestinal infarctions, pulmonary infarctions, cerebral ischemia, and myocardial infarction. There is also a need for compounds that are effective in the prevention of tissue damage caused by ischemia that occurs due to, e.g., atherosclerosis, diabetes, and pulmonary disorders such as COPD and the like. There also remains a need for compounds and methods for preventing and treating disorders or conditions related to HIF. In summary, there is a need in the art for compounds that stabilize HIF α and for methods of using the compounds to treat and prevent HIF-associated disorders including conditions involving ischemia and hypoxia.

SUMMARY OF THE INVENTION

Described herein are compounds that stabilize the alpha subunit of hypoxia inducible factor (HIF α) and methods of using the compounds to prevent, pre-treat, or treat conditions, diseases, or disorders associated with HIF. Disorders associated with HIF include, e.g., ischemic conditions such as atherosclerosis, diabetes, angina pectoris, occlusive arterial disease, deep vein thrombosis, pulmonary infarction, intestinal infarction, stroke, myocardial infarction, and the like.

In one aspect, a compound of the present invention stabilizes HIF α by specifically inhibiting hydroxylation of amino acid residues in the HIF α protein. In one embodiment, the agent inhibits hydroxylation of HIF α proline residues. In yet another embodiment, the agent inhibits hydroxylation of the HIF α P₅₆₄ residue.

In another aspect, a compound of the invention can be used to treat HIF-associated disorders including, but not limited to, ischemic and pulmonary disorders, the method comprising administering a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient to a patient in need. In one embodiment, the compound is administered immediately following the diagnosis of an acute ischemic disorder such as pulmonary, intestinal, cerebral, and/or myocardial infarction. In another embodiment, the compound is administered to a patient in need during the course of a chronic ischemic condition such as occlusive arterial disease, liver cirrhosis, or congestive heart failure, etc. In yet another embodiment, the ischemia is due to a transient or acute trauma, insult, or injury such as, e.g., a spinal cord injury. In still yet another embodiment, the compound is administered to a patient in need following diagnosis of a pulmonary disorder such as COPD and the like.

In another aspect, a compound of the invention can be used to prevent the tissue damage caused by HIF-associated disorders including, but not limited to, ischemic and pulmonary disorders, the method comprising administering a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient to a patient in need. In one embodiment, the compound can be administered based on pre-disposing conditions, e.g. hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, and systemic sclerosis.

In yet another aspect, a compound of the invention can be used as a pre-treatment to decrease or prevent the tissue damage caused by HIF-associated disorders including, but not limited to, ischemic and pulmonary disorders. In one embodiment, the compound is administered to a patient who has a history of recurring episodes of an ischemic condition, e.g., myocardial infarction or transient ischemic attacks, or has symptoms of impending ischemia, e.g. angina pectoris. In another embodiment, the compound can be administered based on physical parameters implicating possible ischemia or hypoxia, such as is the case with, e.g., individuals placed under general anesthesia or temporarily working at high altitudes. In yet another embodiment, the compounds may be used in the context of organ transplants to pre-treat organ donors and to maintain organs removed from the body prior to implantation in a recipient.

In some embodiments, compounds used in the methods of the invention are selected from the group consisting of phenanthroline derivatives and heterocyclic carbonyl glycine derivatives. In one embodiment, the compound is a phenanthroline derivative selected from the group consisting of 3-carboxy-4-oxo-3,4-dihydro-1,10-phenanthroline; 3-carboxy-5-hydroxy-4-oxo-3,4-dihydro-1,10-

phenanthroline; 3-carboxy-5-methoxy-4-oxo-3,4-dihydro-1,10-phenanthroline; and 3-carboxy-8-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline. In another embodiment, the phenanthroline derivative is 3-carboxy-4-oxo-3,4-dihydro-1,10-phenanthroline.

In another embodiment, the compound of the present invention is a heterocyclic carbonyl glycine selected from the group consisting of substituted quinoline-2-carboxamides, isoquinoline-3-carboxamides, 3-methoxy pyridine carbonyl glycines, 3-methoxy pyridine carbonyl glycine esters, 3-hydroxy pyridine carbonyl glycines, 3-hydroxypyridine carbonyl glycine esters, 5-sulfonamidocarbonyl pyridine carboxylates, and 5-sulfonamidocarbonyl-pyridine-carbonyl-glycine esters. In one embodiment, the compound is a substituted quinoline-2-carboxamide selected from the group consisting of N-((6-(1-butyloxy)-3-hydroxyquinolin-2-yl)-carbonyl)-glycine; N-((6-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine; N-((3-hydroxy-6-(2-propyloxy)-quinolin-2-yl)-carbonyl)-glycine; and N-((7-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine. In another embodiment, the compound is an substituted isoquinoline-3-carboxamide selected from the group consisting of N-((1-chloro-4-hydroxy-7-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxy-6-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxy-7-methoxyisoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxy-6-methoxy-isoquinolin-3-yl)-carbonyl)-glycine; N-((7-butyloxy)-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((7-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((6-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((8-chloro-4-hydroxy-isoquinolin-3-yl)-carbonyl)-glycine; and 1-Chloro-4-hydroxy-7-(2-propyloxy)-isoquinoline-3-carboxylic acid N-(2-hydroxyethyl)-amide. In yet another embodiment, the compound is a 3-methoxy pyridine carbonyl glycine or an ester thereof selected from the group consisting of [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid; 3-methoxypyridine-2-carboxylic acid N-(((hexadecyloxy)-carbonyl)-methyl)-amide hydrochloride; 3-methoxypyridine-2-carboxylic acid N-(((1-octyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((hexyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((nonyloxy)-carbonyl)-methyl)-amide racemate; 3-methoxypyridine-2-carboxylic acid N-(((heptyloxy)-carbonyl)-methyl)-amide; 3-benzyloxypyridine-2-carboxylic acid N-(((octyloxy)-carbonyl)-methyl)-amide; 3-benzyloxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide; 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxy carbonyl)-methyl)-amide; 5-(((3-(1-Butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((1-butyloxy)-carbonyl)-methyl)-amide; and 5-(((3-Lauryloxy)-propyl)amino)-carbonyl)-3-

methoxypyridine-2-carboxylic acid N-(((benzyloxy)-carbonyl)-methyl)-amide. In a specific embodiment, the compound is N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine.

In one aspect, a compound of the invention inhibits one or more 2-oxoglutarate dioxygenase enzymes. In one embodiment, the compound inhibits at least two 2-oxoglutarate dioxygenase family members, e.g., HIF prolyl hydroxylase and procollagen prolyl 4-hydroxylase, with either the same specificity or with differential specificity. In another embodiment, the compound is specific for one 2-oxoglutarate dioxygenase, e.g., HIF prolyl hydroxylase, and shows little to no specificity for other family members.

The compounds can be administered singly or in combination with various other therapeutic approaches. In one embodiment, the compound is administered with another 2-oxoglutarate dioxygenase inhibitor, wherein the two compounds have differential specificity for individual 2-oxoglutarate dioxygenase family members. The two compounds may be administered at the same time as a ratio of one relative to the other, e.g., 10:1, 1:1, or 1:10; or may be administered consecutively during a treatment time course, e.g., following myocardial infarction. In one specific embodiment, one compound specifically inhibits HIF prolyl hydroxylase activity, and a second compound specifically inhibits procollagen prolyl 4-hydroxylase activity. In another embodiment, the compound is administered with another therapeutic agent having a different mode of action, e.g., an ACE inhibitor (ACEI), angiotensin-II receptor blocker (ARB), diuretic, and/or digoxin. In yet another embodiment, the compound is administered with carnitine.

Preferred embodiments of the invention comprise methods using oral and transdermal delivery mechanisms. Thus, the present invention also provides an oral formulation comprising a compound of the invention. In another preferred embodiment, the present methods involve transdermal administration of a compound of the invention. Thus, the present invention also provides a transdermal patch or pad comprising a compound of the invention.

These and other embodiments of the subject invention will readily occur to those of skill in the art in light of the disclosure herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a dose response for HIF-1 α stabilization and accumulation in human cells treated with the heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine. Cell lines

shown in the figure are transformed fetal kidney cells (293A), cervical adenocarcinoma cells (HeLa), hepatocarcinoma cells (Hep3B), and foreskin fibroblasts (HFF).

Figure 2 shows HIF-1 α stabilization and accumulation in human cells treated with compounds of the invention. Compounds shown are the phenanthroline derivative 3-carboxy-4-oxo-3,4-dihydro-1,10-phenanthroline (compound A), and the heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B). Cells shown in the figure are cardiomyocytes, transformed fetal kidney cells (293A), and fibroblasts (HFF).

Figure 3 shows levels of vascular endothelial growth factor (VEGF), a HIF-responsive gene, in human cell culture media following treatment with the heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B). Cell lines shown in the figure are foreskin fibroblasts (HFF) and hepatocarcinoma cells (Hep3B).

Figure 4 shows levels of VEGF in mouse plasma at 2, 5, and 20 hours following final treatment with the heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B) relative to an untreated control group.

Figures 5A and 5B show oxygen consumption and cell viability in human cells treated with the heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B). Figure 5A shows a dose response curve for oxygen consumption in cervical adenocarcinoma (HeLa) and transformed fetal kidney (293A) cells treated with compound B. Figure 5B shows cell proliferation and viability as measured by cleavage of WST-1 tetrazolium salt (Roche Diagnostics Corp. (Roche), Indianapolis IN) in HeLa and 293A cells treated with varying doses of compound B.

Figures 6A and B show improvements to heart architecture following myocardial infarction in animals treated with the heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine. Figure 6A shows statistically significant improvement ($p < 0.05$) in fractional shortening in treated animals relative to untreated controls one week after induced myocardial infarction. Figure 6B shows statistically significant improvement in left ventricle end-diastolic diameter (LVEDD; $p < 0.005$) and left ventricular end-systolic diameter (LVESD; $p < 0.001$) in treated animals relative to untreated controls one week after induced myocardial infarction.

DESCRIPTION OF THE INVENTION

Before the present compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, cell lines, assays, and reagents described, as these may vary. It is also to be understood that the terminology used herein is intended to describe particular
 5 embodiments of the present invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural references unless context clearly dictates otherwise. Thus, for example, a reference to “a
 10 fragment” includes a plurality of such fragments, a reference to an “antibody” is a reference to one or more antibodies and to equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any
 15 methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications cited herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing the methodologies, reagents, and tools reported in the publications that might be used in connection with the invention. Nothing herein is to be construed as an admission that the
 20 invention is not entitled to antedate such disclosure by virtue of prior invention.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, cell biology, genetics, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Gennaro, A.R.,
 25 ed. (1990) Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co.; Hardman, J.G., Limbird, L.E., and Gilman, A.G., eds. (2001) The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill Co.; Colowick, S. et al., eds., Methods In Enzymology, Academic Press, Inc.; Weir, D.M., and Blackwell, C.C., eds. (1986) Handbook of Experimental Immunology, Vols. I-IV, Blackwell Scientific Publications; Maniatis, T. et al., eds. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Vols. I-III, Cold
 30 Spring Harbor Laboratory Press; Ausubel, F.M. et al., eds. (1999) Short Protocols in Molecular Biology, 4th edition, John Wiley & Sons; Ream et al., eds. (1998) Molecular Biology Techniques: An Intensive Laboratory Course, Academic Press; Newton, C.R., and Graham, A., eds. (1997) PCR (Introduction to Biotechniques Series), 2nd ed., Springer Verlag.

DEFINITIONS

The term “ischemia” refers to a reduction in blood flow and, consequently, a reduction in nutrients, including oxygen, delivered to tissues. Ischemia may arise due to conditions such as atherosclerosis, formation of a thrombus in an artery or vein, or blockage of an artery or vein by an embolus, etc. Such conditions may reduce blood flow, producing a state of hypoperfusion to an organ or tissue, or block blood flow completely. Other conditions that can produce ischemia include tissue damage due to trauma or injury, such as, e.g., spinal cord injury; and viral infection, e.g., leading to congestive heart failure. The terms “ischemic conditions” and “ischemic disorders” refer to acute ischemic conditions including myocardial infarction, ischemic stroke, pulmonary embolism, perinatal hypoxia, neonatal respiratory distress syndrome, hemorrhagic shock, mountain sickness, and acute respiratory failure; and chronic ischemic conditions including atherosclerosis, chronic venous insufficiency, chronic heart failure, cardiac cirrhosis, diabetes, macular degeneration, chronic obstructive lung disease, sleep apnea, Raynaud’s disease, systemic sclerosis, nonbacterial thrombotic endocarditis, occlusive artery disease, angina pectoris, TIAs, and chronic alcoholic liver disease. Ischemic conditions may also result when individuals are placed under general anesthesia, and in organs prepared for transplant.

The terms “hypoxia” and “hypoxic” refer to an environment with levels of oxygen below normal. Oxygen levels that define hypoxia in cell culture are preferably about 0.1 to 2%. Hypoxia may be induced in cells by culturing the cells in a reduced oxygen environment, or cells may be treated with compounds that mimic hypoxia. Compounds that mimic hypoxia-induced stabilization of HIF α include iron chelators, cobalt (II), nickel (II), or manganese (II), all of which may be used at a concentration of about 20 to 500 μ M, e.g., 100 μ M. Oxygen levels that define hypoxia in animals include exposure to an environment composed of less than about 12% oxygen, e.g., 8% oxygen.

The terms “hypoxic conditions” and “hypoxic disorders” include, but are not limited to, ischemic disorders (ischemic hypoxia) such as those listed above, wherein hypoxia results from reduced circulation; pulmonary disorders (hypoxic hypoxia) such as COPD, severe pneumonia, pulmonary edema, pulmonary hypertension, hyaline membrane disease, and the like, wherein hypoxia results from reduced oxygenation of the blood in the lungs; anemic disorders (anemic hypoxia) such as gastric or duodenal ulcers, liver or renal disease, thrombocytopenia or blood coagulation disorders, cancer or other chronic illness, and the like, wherein hypoxia results from a decreased concentration of hemoglobin or red blood cells; and altitude sickness.

The terms “disorders” and “diseases” are used inclusively and refer to any condition deviating from normal. The terms “ischemic conditions” and “ischemic disorders” refer to any condition, disease, or disorder that is associated with ischemia. The terms “hypoxic conditions” and “hypoxic disorders” refer to any condition, disease, or disorder that is associated with hypoxia. Such ischemic and hypoxic disorders include those disorders described above.

The term “normoxic” refers to an environment with normal levels of oxygen. Oxygen levels that define normoxic in cell culture are preferably about 10 to 30% oxygen. A normal environment for cell culture would be composed of about 10 to 30% oxygen, about 1 to 10% carbon dioxide, and a balance of nitrogen or another relatively inert and non-toxic gas; a preferable environment being about, e.g., 20% O₂, 5% CO₂, and balance N₂. A normal environment for animal maintenance is about, e.g., 21% O₂, 0.04% CO₂, and balance N₂. Normoxia may also be induced or enhanced in cells, for example, by culturing the cells in the presence of hydrogen peroxide at about 50 to 200 mM.

The term “HIF α ” refers to the alpha subunit of hypoxia inducible factor protein. HIF α may be any human or other mammalian protein, or fragment thereof, including human HIF-1 α (Genbank Accession No. Q16665), HIF-2 α (Genbank Accession No. AAB41495), and HIF-3 α (Genbank Accession No. AAD22668); murine HIF-1 α (Genbank Accession No. Q61221), HIF-2 α (Genbank Accession No. BAA20130 and AAB41496), and HIF-3 α (Genbank Accession No. AAC72734); rat HIF-1 α (Genbank Accession No. CAA70701), HIF-2 α (Genbank Accession No. CAB96612), and HIF-3 α (Genbank Accession No. CAB96611); and cow HIF-1 α (Genbank Accession No. BAA78675). HIF α may also be any non-mammalian protein or fragment thereof, including *Xenopus laevis* HIF-1 α (Genbank Accession No. CAB96628), *Drosophila melanogaster* HIF-1 α (Genbank Accession No. JC4851), and chicken HIF-1 α (Genbank Accession No. BAA34234). HIF α gene sequences may also be obtained by routine cloning techniques, for example by using all or part of a HIF α gene sequence described above as a probe to recover and determine the sequence of a HIF α gene in another species.

Fragments of HIF α include the regions defined by human HIF-1 α from amino acid 401 to 603 (Huang et al., *supra*), amino acid 531 to 575 (Jiang et al. (1997) J Biol Chem 272:19253-19260), amino acid 556 to 575 (Tanimoto et al., *supra*), amino acid 557 to 571 (Srinivas et al. (1999) Biochem Biophys Res Commun 260:557-561), and amino acid 556 to 575 (Ivan and Kaelin (2001) Science 292:464-468). Further, a fragment of HIF α includes any fragment containing at least one occurrence of the motif LXXLAP, e.g., as occurs in the HIF α native sequence at L₃₉₇TLLAP and L₅₅₉EMLAP. Additionally, a

fragment of HIF α includes any fragment retaining at least one functional or structural characteristic of HIF α .

The terms “amino acid sequence” or “polypeptide” as used herein, e.g., to refer to HIF α and fragments thereof, refer to an oligopeptide, peptide, or protein sequence, or to a fragment of any of these, and to naturally occurring or synthetic molecules. “Fragments” can refer to any portion of a sequence that retains at least one structural or functional characteristic of the protein. Immunogenic fragments or antigenic fragments refer to fragments of polypeptides, preferably, fragments of about five to fifteen amino acids in length, that retain at least one biological or immunological activity. Where “amino acid sequence” is recited to refer to the polypeptide sequence of a naturally occurring protein molecule, “amino acid sequence” and like terms are not meant to limit the amino acid sequence to the complete native sequence associated with the recited protein molecule.

The term “related proteins” as used herein, for example, to refer to proteins related to HIF α prolyl hydroxylase, encompasses other 2-oxoglutarate dioxygenase enzymes, especially those family members that similarly require Fe²⁺, 2-oxoglutarate, and oxygen to maintain hydroxylase activity. Such enzymes may include, e.g., procollagen lysyl hydroxylase and procollagen prolyl 4-hydroxylase.

The term “agonist” refers to a molecule that increases or prolongs the duration of the effect of a particular molecule. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that bind to and modulate the effects of the target molecule.

The term “antagonist” refers to a molecule which, when bound to a particular molecule, decreases the extent or duration of the effect of the biological or immunological activity. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules that decrease the effect of the target molecule.

The term “antibody” refers to intact molecules as well as to fragments thereof, such as Fab, F(ab')₂, and Fv fragments, capable of binding the epitopic determinant, and includes polyclonal or monoclonal antibodies. Antibodies that bind HIF α , proline hydroxylated-HIF α , or fragments thereof can be prepared using intact polypeptides or using fragments containing small peptides of interest as the immunizing antigen. The polypeptide or oligopeptide used to immunize an animal (e.g., a mouse, rat, rabbit, chicken, turkey, goat, etc.) can be derived from the translation of RNA or synthesized chemically, can be modified by, e.g., incubation in a cell lysate such as rabbit reticulocyte lysate, and can be conjugated to a carrier

protein if desired. Commonly used carriers chemically coupled to peptides include, for example, bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin (KLH).

The term “antigenicity” relates to the ability of a substance to, when introduced into the body, stimulate the immune response and the production of an antibody. An agent displaying the property of antigenicity is referred to as being antigenic. Antigenic agents can include, but are not limited to, a variety of macromolecules such as, for example, proteins, lipoproteins, polysaccharides, nucleic acids, bacteria and bacterial components, and viruses and viral components. Antigenic fragments refer to fragments of HIF α or proline hydroxylated-HIF α polypeptide, preferably, fragments of about five to fifteen amino acids in length, that retain at least one biological or immunological aspect of HIF α or polypeptide activity.

The term “immunogenicity” relates to the ability to evoke an immune response within an organism. An agent displaying the property of immunogenicity is referred to as being immunogenic. Agents can include, but are not limited to, a variety of macromolecules such as, for example, proteins, lipoproteins, polysaccharides, nucleic acids, bacteria and bacterial components, and viruses and viral components. Immunogenic agents often have a fairly high molecular weight (usually greater than 10 kDa). Immunogenic fragments refer to fragments of HIF α or proline hydroxylated-HIF α polypeptide, preferably, fragments of about five to fifteen amino acids in length, that retain at least one biological or immunological aspect of HIF α polypeptide activity.

The terms “nucleic acid” or “polynucleotide” refer to oligonucleotides, nucleotide sequences, or polynucleotides, or any fragments thereof, and to DNA or RNA of natural or synthetic origin which may be single- or double-stranded and may represent the sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin. Polynucleotide fragments include any portion of a polynucleotide sequence that retains at least one structural or functional characteristic of the polynucleotide. Polynucleotide fragments can be of variable length, for example, greater than 60 nucleotides in length, at least 100 nucleotides in length, at least 1000 nucleotides in length, or at least 10,000 nucleotides in length.

“Altered” polynucleotides include those with deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally equivalent polypeptide. Included within this definition are sequences displaying polymorphisms that may or may not be readily detectable using particular oligonucleotide probes or through deletion of improper or unexpected hybridization to alleles, with a locus other than the normal chromosomal locus for the subject polynucleotide sequence.

“Altered” polypeptides may contain deletions, insertions, or substitutions of amino acid residues that produce a silent change and result in a functionally equivalent polypeptide. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological or immunological activity of the encoded polypeptide is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid; positively charged amino acids may include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values may include leucine, isoleucine, and valine, glycine and alanine, asparagine and glutamine, serine and threonine, and phenylalanine and tyrosine.

A polypeptide or amino acid “variant” is an amino acid sequence that is altered by one or more amino acids from a particular amino acid sequence. A polypeptide variant may have conservative changes, wherein a substituted amino acid has similar structural or chemical properties to the amino acid replaced, e.g., replacement of leucine with isoleucine. A variant may also have non-conservative changes, in which the substituted amino acid has physical properties different from those of the replaced amino acid, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Preferably, amino acid variants retain certain structural or functional characteristics of a particular polypeptide. Guidance in determining which amino acid residues may be substituted, inserted, or deleted may be found, for example, using computer programs well known in the art, such as LASERGENE software (DNASTAR Inc., Madison, WI).

A polynucleotide variant is a variant of a particular polynucleotide sequence that preferably has at least about 80%, 85%, 90%, 95%, or 98% polynucleotide sequence similarity to the particular polynucleotide sequence. It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of variant polynucleotide sequences encoding a particular protein, some bearing minimal homology to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard codon triplet genetic code, and all such variations are to be considered as being specifically disclosed.

A “deletion” is a change in an amino acid or nucleotide sequence that results in the absence of one or more amino acid residues or nucleotides, respectively.

An “insertion” or “addition” is a change in an amino acid or nucleotide sequence that results in the addition of one or more amino acid residues or nucleotides, respectively.

5 The term “functional equivalent” as it is used herein refers to a polypeptide or polynucleotide that possesses at least one functional and/or structural characteristic of a particular polypeptide or polynucleotide. A functional equivalent may contain modifications that enable the performance of a specific function. The term “functional equivalent” is intended to include fragments, mutants, hybrids, variants, analogs, or chemical derivatives of a molecule.

10 The term “microarray” refers to any arrangement of nucleic acids, amino acids, antibodies, etc., on a substrate. The substrate can be any suitable support, e.g., beads, glass, paper, nitrocellulose, nylon, or any appropriate membrane, etc. A substrate can be any rigid or semi-rigid support including, but not limited to, membranes, filters, wafers, chips, slides, fibers, beads, including magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles, capillaries, etc. The substrate can provide a surface for coating and/or can have a variety of surface forms, such as wells, pins, trenches, channels, and pores, to which the nucleic acids, amino acids, etc., may be bound.

15 The term “sample” is used herein in its broadest sense. Samples may be derived from any source, for example, from bodily fluids, secretions, tissues, cells, or cells in culture including, but not limited to, saliva, blood, urine, serum, plasma, vitreous, synovial fluid, cerebral spinal fluid, amniotic fluid, and organ tissue (e.g., biopsied tissue); from chromosomes, organelles, or other membranes isolated from a cell; from genomic DNA, cDNA, RNA, mRNA, etc.; and from cleared cells or tissues, or blots or imprints from such cells or tissues. Samples may be derived from any source, such as, for example, a human subject, or a non-human mammalian subject, etc. Also contemplated are samples derived from any animal model of disease. A sample can be in solution or can be, for example, fixed or bound to a substrate. A sample can refer to any material suitable for testing for the presence of HIF α or of fragments of HIF α or suitable for screening for molecules that bind to HIF α or to fragments thereof. Methods for obtaining such samples are within the level of skill in the art.

INVENTION

30 The present invention relates to the discovery that stabilization of the alpha subunit of hypoxia inducible factor (HIF α) is an effective therapeutic approach with unexpected benefits when applied to treatment or prevention of conditions that are associated with hypoxia and/or ischemia, e.g., myocardial infarction,

stroke, occlusive arterial disease, angina pectoris, cardiac cirrhosis, atherosclerosis, etc. Further, the present invention provides compounds that stabilize HIF α and methods of using the compounds to treat disorders associated with HIF including, but not limited to, hypoxic and/or ischemic disorders such as those described above.

Presented herein is the discovery that stabilization of HIF α is modulated by proline hydroxylation and that HIF α stabilization is effective for treating or preventing the development or persistence of ischemic conditions such as DVT, angina pectoris, pulmonary embolism, stroke, and myocardial infarction. Specifically, it has been shown that HIF α and a HIF α peptide corresponding to residues 556 to 575 [HIF(556-575)] preincubated with rabbit reticulocyte lysate (RRL) binds specifically to the von Hippel Lindau protein (pVHL), and that such binding leads to the ubiquitination and degradation of HIF α . It has also been shown that mutation of the highly conserved colinear sequence M₅₆₁LAPYIPM within HIF(556-575) to eight consecutive alanines stabilized HIF(556-575) under normoxic conditions. (Srinivas et al., *supra*.) An alanine scan of the region showed that mutation of P₅₆₄ to alanine in the context of full-length HIF-1 α or a glutathione *S*-transferase (GST)-HIF α oxygen degradation domain (ODD) fusion protein (Gal4-ODD) abrogated pVHL-binding activity. The modification of P₅₆₄ was identified as an hydroxylation by electrospray ion trap tandem mass spectrometry (MS/MS), and by thin layer chromatography of Gal4-HIF(555-575) that was *in vitro* translated using RRL in the presence of [³H]proline. The functional significance of the proline hydroxylation was demonstrated by showing that P₅₆₄-hydroxylated HIF α bound pVHL but a HIF-1 α mutant containing a single point mutation of P₅₆₄ to alanine was stable in COS7 cells and was insensitive to the hypoxia mimetic desferrioxamine. (See Ivan and Kaelin, *supra*; Jaakkola et al. (2001) Science 292:468-472.)

In principle, drugs that stabilize HIF will augment angiogenesis, the response to acute hypoxia, and adaptation to chronic hypoxia. As tissue ischemia is a major cause of morbidity and mortality, the identification of compounds that stabilize HIF α would be beneficial in the treatment of hypoxic conditions. As HIF α is modified by proline hydroxylation and the hydroxylation reaction requires oxygen and Fe²⁺, the present invention contemplates that the enzyme responsible for HIF α hydroxylation is a member of the 2-oxoglutarate dioxygenase family. All of the enzymes in the 2-oxoglutarate dioxygenase family, including procollagen lysyl hydroxylase, procollagen prolyl 3-hydroxylase, procollagen prolyl 4-hydroxylase, thymine 7-hydroxylase, aspartyl (asparaginyl) β -hydroxylase, ϵ -N-trimethyllysine hydroxylase, and γ -butyrobetaine hydroxylase, etc., require oxygen, Fe²⁺, 2-oxoglutarate, and ascorbic acid for their hydroxylase activity. (See, e.g., Majamaa et al. (1985) Biochem J 229:127-133; Myllyharju and Kivirikko (1997) EMBO J

16:1173-1180; Thornburg et al. (1993) 32:14023-14033; and Jia et al. (1994) Proc Natl Acad Sci USA 91:7227-7231.)

Several small molecule inhibitors of prolyl 4-hydroxylase have been identified. (See, e.g., Majamaa et al., *supra*; Kivirikko and Myllyharju (1998) Matrix Biol 16:357-368; Bickel et al. (1998) Hepatology 28:404-411; Friedman et al. (2000) Proc Natl Acad Sci USA 97:4736-4741; and Franklin et al. (2001) Biochem J 353:333-338.) The present invention contemplates the use of these compounds in ischemic disorders.

Further, phenanthrolines are specifically encompassed by the present invention. U.S. Patent Nos. 5,916,898 and 6,200,974, and PCT Publication WO 9921860 describe phenanthroline derivatives. All compounds listed in the foregoing patents and publication, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are 3-carboxy-4-oxo-3,4-dihydro-1,10-phenanthroline (see, e.g., Seki et al. (1974) Chem Abstracts 81:424, No. 21), 3-carboxy-5-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline, 3-carboxy-5-methoxy-4-oxo-3,4-dihydro-1,10-phenanthroline, and 3-carboxy-8-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline.

Still further, heterocyclic carbonyl glycines are encompassed by the present invention. U.S. Patent Nos. 5,719,164 and 5,726,305 describe substituted quinoline-2-carboxamides. All compounds listed in the foregoing patents, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are N-((6-(1-butyloxy)-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, N-((6-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, N-((3-hydroxy-6-(2-propyloxy)-quinolin-2-yl)-carbonyl)-glycine, and N-((7-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine.

Additionally, U.S. Patent No. 6,093,730 describes substituted isoquinoline-3-carboxamides. All compounds listed in U.S. Patent No. 6,093,730, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are N-((1-chloro-4-hydroxy-7-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-7-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-butyloxy)-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((6-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((1-

chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((8-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, and 1-Chloro-4-hydroxy-7-(2-propyloxy)-isoquinoline-3-carboxylic acid N-(2-hydroxyethyl)-amide.

5 Additionally, U.S. Patent No. 5,658,933 describes 3-methoxy pyridine carbonyl glycine esters. All compounds listed in U.S. Patent No. 5,658,933, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are 3-methoxypyridine-2-carboxylic acid N-(((hexadecyloxy)-carbonyl)-methyl)-amide hydrochloride, 3-methoxypyridine-2-carboxylic acid N-(((1-octyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((hexyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((nonyloxy)-carbonyl)-methyl)-amide racemate, 3-methoxypyridine-2-carboxylic acid N-(((heptyloxy)-carbonyl)-methyl)-amide, 3-benzyloxypyridine-2-carboxylic acid N-(((octyloxy)-carbonyl)-methyl)-amide, 3-benzyloxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide, 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxycarbonyl)-methyl)-amide, 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((1-butyloxy)-carbonyl)-methyl)-amide, and 5-(((3-lauryloxy)-propyl)amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxy)-carbonyl)-methyl)-amide.

10 Additionally, European Patent No. EP0650961 describes 3-methoxy pyridine carbonyl glycines. All compounds listed in European Patent No. EP0650961, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. An exemplary compound contained therein is [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid.

20 Additional compounds contemplated by the present invention are 3-hydroxypyridine carbonyl glycines described in U.S. Patent No. 5,620,995; 3-hydroxypyridine carbonyl glycine esters described in U.S. Patent No. 6,020,350; 5-sulfonamidocarbonyl pyridine carboxylates described in U.S. Patent No. 5,607,954; and 5-sulfonamidocarbonyl-pyridine-carbonyl-glycine esters described in U.S. Patent Nos. 5,610,172 and 5,620,996. All compounds listed in these patents, in particular, those compounds listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein.

Based on the common mechanism of action of the 2-oxoglutarate dioxygenase family members, such as dependence on Fe^{2+} and 2-oxoglutarate for activity, the invention contemplates the use of the above-referenced compounds to inhibit HIF α hydroxylation and thus stabilize HIF α in an oxygen-independent manner. Further, the examples and figures of the present invention demonstrate that application of such compounds stabilize HIF α and subsequently induce HIF-regulated gene products *in vitro* and *in vivo*, thus producing a specific benefit in the prevention and treatment of ischemic conditions.

Figures 1 and 2 show that compounds of the present invention stabilize HIF α in a dose-dependent manner in cells grown in a normoxic environment. Although the different cell types show different levels of HIF α in the presence of the compound, all of the cell lines tested showed some level of HIF α stabilization. The level of HIF α in the untreated cells is low to undetectable. Stabilization of HIF α leads to the expression of glycolytic enzymes, the angiogenic factor VEGF, and the hematopoietic stem cell factor erythropoietin. Figure 3 shows that stabilization of HIF α by a compound of the invention leads to HIF-dependent gene expression *in vitro* in a dose-dependent manner. Further, Figure 4 shows that HIF-dependent gene expression *in vivo* persists up to 20 hours after treatment with a compound of the invention. Additionally, Figure 5 shows a dose-dependent decrease in oxygen consumption in cells treated with a compound of the invention without any affect on cell viability. As stable HIF complexes activate transcription of glycolytic enzymes, the reduction in oxygen consumption is potentially due to a shift in cellular metabolism from aerobic to anaerobic energy production.

The ability to generate energy under low oxygen conditions would be beneficial in ischemic conditions such as, for example, peripheral arterial disease, DVT, angina pectoris, pulmonary embolism, stroke, and myocardial infarction. An ability to increase glycolysis within cells of the body may also be generally applicable to the treatment of other conditions, e.g., diabetes. Further, the ability to stabilize HIF α would also have potential benefits in the induction of angiogenesis and in conditions associated with anemia due to low red blood cell count. Additionally, angiotensin II has been shown to induce expression and stabilization of HIF α in an oxygen-independent manner (Richard et al., *supra*). Use of ACEIs or ARBs, therefore, may be expected to reduce levels of HIF α within, e.g., sites of injury. The present invention contemplates a dual therapy approach to maintaining HIF α levels during inhibition of angiotensin II signaling that involves use of the compounds of the invention in conjunction with ACEI or ARB therapy.

Further, compounds of the invention produce significant therapeutic benefit when administered following ischemia. Figure 6A shows statistically significant improvement in fractional shortening in animals pre-treated with a compound of the invention and then subjected to myocardial infarction. Experiments were

conducted as described in Example 6. One week after surgery, fractional shortening was reduced from 51% in the sham operated group to 29% in the myocardial infarction (control) group. Fractional shortening rose to 41% in a group treated with a compound of the invention, a statistically significant improvement when compared to the control untreated group ($p < 0.05$; one-way ANOVA/Tukey's test).

- 5 Figure 6B shows statistically significant improvement in left ventricular end diastolic (LVEDD) and systolic (LVESD) diameters in a group treated with a compound of the invention relative to an untreated control group. Myocardial infarction increased LVEDD and LVESD by 65% and 15%, respectively, when compared to a sham operated control group. In animals treated with a compound of the invention, end diastolic dilation was reduced to 18% over sham operated group and end systolic dilation was the
- 10 same as the sham operated group.

Previous studies have shown that certain compounds of the invention are effective inhibitors of procollagen prolyl 4-hydroxylase. While it is recognized that recovery from the initial infarct requires connective tissue deposition within the necrotic region, the present invention demonstrates no adverse affects of treatment with respect to scar formation.

Therefore, the invention provides methods for treating ischemic conditions, in particular, using the compounds described herein. Further, the invention provides methods for treating a patient having a high likelihood of developing an ischemic condition, e.g., individuals at high risk for atherosclerosis, etc., using the compounds described herein. Risk factors for atherosclerosis include, e.g., hyperlipidemia, cigarette smoking, hypertension, diabetes mellitus, hyperinsulinemia, and abdominal obesity. In a specific embodiment, the present invention provides compounds that stabilize HIF α before, during, or immediately after ischemia, particularly ischemia due to myocardial infarction and/or stroke.

- 25 Based on the benefits provided by certain compounds of the invention on treatment and prevention of hypoxic tissue damage and fibrosis, the present invention also contemplates a "dual-therapy" approach to treatment or prevention of conditions involving ischemia, including ischemia associated with subsequent reactive fibrosis, e.g., myocardial infarction and resultant congestive heart failure. The method may use one compound that inhibits more than one 2-oxoglutarate dioxygenase enzyme, e.g., HIF prolyl
- 30 hydroxylase and procollagen prolyl 4-hydroxylase, with either the same specificity or with different specificities. Alternatively, the method may use a combination of compounds wherein each compound specifically inhibits only one 2-oxoglutarate dioxygenase enzyme, e.g., one compound specifically inhibits HIF prolyl hydroxylase and a second compound specifically inhibits procollagen prolyl 4-hydroxylase.

Methods of Using the Compounds to Prevent and Treat Ischemic Conditions

The present invention provides compounds that inhibit HIF α proline hydroxylation and methods of using the compounds to treat disorders or conditions associated with HIF. Disorders associated with HIF include, but are not limited to, ischemic conditions that arise due to, e.g., myocardial infarction, stroke, occlusive arterial disease, DVT, angina pectoris, liver cirrhosis, and atherosclerosis.

In one aspect, the present invention provides methods of using the compounds to treat ischemia, the method comprising administering a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient to a patient in need. In one embodiment, the compound is administered immediately following a condition producing acute ischemia, e.g., myocardial infarction, pulmonary embolism, intestinal infarction, and ischemic stroke. In another embodiment, the compound is administered to a patient diagnosed with a condition associated with the development of chronic ischemia, e.g., cardiac cirrhosis, macular degeneration, chronic obstructive lung disease, acute respiratory failure, neonatal respiratory distress syndrome, and congestive heart failure. In yet another embodiment, the compound is administered immediately after a trauma or injury.

In another aspect, the present invention provides methods of using the compounds to prevent ischemic tissue injury, the method comprising administering a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient to a patient in need. In one embodiment, the compound can be administered based on pre-disposing conditions, e.g. hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, and systemic sclerosis.

In another aspect, the invention provides methods of using the compounds to pre-treat a subject to decrease or prevent the development of ischemia, the method comprising administering a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient to a patient with a history of ischemic disorders, e.g., myocardial infarctions, or having symptoms of impending ischemia, e.g. angina pectoris. In another embodiment, the compound can be administered based on physical parameters implicating possible ischemia, e.g., individuals placed under general anesthesia or temporarily working at high altitudes. In yet another embodiment, the compounds may be used in organ transplants to pre-treat organ donors and to maintain organs removed from the body prior to implantation in the recipient.

In one aspect, a compound of the invention inhibits one or more 2-oxoglutarate dioxygenase enzymes. In one embodiment, the compound inhibits at least two 2-oxoglutarate dioxygenase family members, e.g., HIF prolyl hydroxylase and procollagen prolyl 4-hydroxylase, with either the same specificity or with differential specificity. In another embodiment, the compound is specific for one 2-oxoglutarate dioxygenase, e.g., HIF prolyl hydroxylase, and shows little to no specificity for other family members.

The compounds can be administered in combination with various other therapeutic approaches. In one embodiment, the compound is administered with another 2-oxoglutarate dioxygenase inhibitor, wherein the two compounds have differential specificity for individual 2-oxoglutarate dioxygenase family members. The two compounds may be administered at the same time as a ratio of one relative to the other, e.g., 10:1, 1:1, or 1:10; or may be administered consecutively during a treatment time course, e.g., following myocardial infarction. In one specific embodiment, one compound specifically inhibits HIF prolyl hydroxylase activity, and a second compound specifically inhibits procollagen prolyl 4-hydroxylase activity. In another embodiment, the compound is administered with another therapeutic agent having a different mode of action, e.g., an ACE inhibitor (ACEI), angiotensin-II receptor blocker (ARB), diuretic, and/or digoxin. In yet another embodiment, the compound is administered with carnitine. Preferred routes of administration include oral and transdermal delivery mechanisms.

Pharmaceutical Formulations And Routes Of Administration

The compositions of the present invention can be delivered directly or in pharmaceutical compositions along with suitable carriers or excipients, as is well known in the art. Present methods of treatment can comprise administration of an effective amount of a compound of the invention to a subject having or at risk for an ischemic condition, e.g., congestive heart failure, atherosclerosis, etc. In a preferred embodiment, the subject is a mammalian subject, and in a most preferred embodiment, the subject is a human subject.

An effective amount of such agents can readily be determined by routine experimentation, as can the most effective and convenient route of administration and the most appropriate formulation. Various formulations and drug delivery systems are available in the art. (See, e.g., Gennaro, ed. (1995) Remington's Pharmaceutical Sciences, *supra*; and Hardman, Limbird, and Gilman, eds. (2001) The Pharmacological Basis of Therapeutics, *supra*.)

Suitable routes of administration may, for example, include oral, rectal, transmucosal, nasal, or intestinal administration and parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. The agent or composition thereof may be administered in a local rather than a systemic manner. For example, a suitable agent can be delivered via injection or in a targeted drug delivery system, such as a depot or sustained release formulation.

The pharmaceutical compositions of the present invention may be manufactured by any of the methods well-known in the art, such as by conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. As noted above, the compositions of the present invention can include one or more physiologically acceptable carriers such as excipients and auxiliaries that facilitate processing of active molecules into preparations for pharmaceutical use.

Proper formulation is dependent upon the route of administration chosen. For injection, for example, the composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal or nasal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject. 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.

Pharmaceutical preparations for oral use can be obtained as solid excipients, 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 carboxymethylcellulose, and/or polyvinylpyrrolidone (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 coatings. 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 for oral administration 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.

In one embodiment, the compounds of the present invention can be administered transdermally, such as through a skin patch, or topically. In one aspect, the transdermal or topical formulations of the present invention can additionally comprise one or multiple penetration enhancers or other effectors, including agents that enhance migration of the delivered compound. Transdermal or topical administration could be preferred, for example, in situations in which location specific delivery is desired.

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 any other suitable gas. In the case of a pressurized aerosol, the appropriate dosage unit may be determined by providing a valve to deliver a metered amount.

Capsules and cartridges of, for example, gelatin, for use in an inhaler or insufflator may be formulated. These typically contain a powder mix of the compound and a suitable powder base such as lactose or starch.

Compositions formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion, can 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. Formulations for parenteral administration include aqueous solutions or other compositions in water-soluble form.

Suspensions of the active compounds may also be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil and synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain
5 substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

10 As mentioned above, the compositions of the present invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneous or intramuscular) or by intramuscular injection. Thus, for example, the present 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
15 soluble salt.

Suitable carriers for the hydrophobic molecules of the invention are well-known in the art and include co-solvent systems comprising, for example, benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The co-solvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W)
20 consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system is effective in dissolving hydrophobic compounds and produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied. For example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80, the fraction size of polyethylene glycol may be varied, other biocompatible polymers may replace polyethylene glycol,
25 e.g. polyvinyl pyrrolidone, and other sugars or polysaccharides may substitute for dextrose.

30 Alternatively, other delivery systems for hydrophobic molecules may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Liposomal delivery systems are discussed above in the context of gene-delivery systems. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity.

Additionally, the compounds may be delivered using sustained-release systems, such as semi-permeable matrices of solid hydrophobic polymers containing the effective amount of the composition to be administered. Various sustained-release materials are established and available to those of skill in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

For any composition used in the present methods of treatment, a therapeutically effective dose can be estimated initially using a variety of techniques well-known in the art. For example, in a cell culture assay, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Dosage ranges appropriate for human subjects can be determined, for example, using data obtained from cell culture assays and other animal studies.

A therapeutically effective dose of an agent refers to that amount of the agent that results in amelioration of symptoms or a prolongation of survival in a subject. Toxicity and therapeutic efficacy of such molecules can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by 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 of toxic to therapeutic effects is the therapeutic index, which can be expressed as the ratio LD_{50}/ED_{50} . Agents that exhibit high therapeutic indices are preferred.

Dosages preferably fall within a range of circulating concentrations that includes the ED_{50} with little or no toxicity. Dosages may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage should be chosen, according to methods known in the art, in view of the specifics of a subject's condition.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety, which are sufficient to modulate HIF α stabilization and HIF-regulated gene induction, as desired, i.e., minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from, for example, *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Agents or compositions thereof should be administered using a regimen which maintains plasma levels above the MEC for about 10-90% of the duration of treatment, preferably about 30-90% of the duration of treatment, and most preferably between 50-90%. In cases of

local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of agent or composition administered will, of course, be dependent on a variety of factors, including the sex, age, and weight of the subject being treated, the severity of the affliction, the manner of administration, and the judgment of the prescribing physician.

The present compositions may, if desired, be presented in a pack or dispenser device containing one or more unit dosage forms containing the active ingredient. Such a pack or device 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. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of disorders or diseases in which congestive heart failure is a major indication.

Compound Screening and Identification

The present invention further provides methods of screening for and identifying additional compounds that inhibit HIF α proline hydroxylation.

Various assays and screening techniques, including those described below, can be used to identify small molecules that modulate (e.g., increase or decrease) the level or activity of HIF α . Assays will typically provide for detectable signals associated with the consumption of a reaction substrate or production of a reaction product. Detection can involve, for example, fluorophores, radioactive isotopes, enzyme conjugates, and other detectable labels well known in the art. The results may be qualitative or quantitative. Isolation of the reaction product may be facilitated by a label, such as biotin or a histidine tag, that allows purification from other reaction components via precipitation or affinity chromatography.

Assays for HIF α hydroxylation may involve measuring hydroxylated proline or lysine residues in HIF α or a fragment thereof (see, e.g., Palmerini et al. (1985) J Chromatogr 339:285-292), or measuring formation of succinate from 2-oxoglutarate in the presence of enzyme and HIF α or a fragment thereof (see, e.g., Cunliffe et al. (1986) Biochem J 240:617-619). An exemplary procedure that measures HIF α hydroxylation is described in Ivan et al. (*supra*). An exemplary procedure that measures production of succinate from 2-oxoglutarate is described by Kaule and Gunzler (1990; Anal Biochem 184:291-297). Substrate molecules may include HIF α or a fragment thereof, e.g., HIF(556-575). Enzyme may include,

e.g., HIF α prolyl hydroxylase (see, e.g., GenBank Accession No. AAG33965), obtained from any source. Enzyme may also be present in a crude cell lysate or in a partially purified form. Compounds that inhibit hydroxylation of HIF α may be identified by measuring and comparing enzyme activity in the absence and presence of the compound.

5

Additionally and in combination with the above methods, compounds can be identified by any of a variety of screening techniques known in the art. Such screening methods may allow for target polypeptides or the compounds to be free in solution, affixed to a solid support, borne on a cell surface, or located within a cell. For example, microarrays carrying test compounds can be prepared, used, and analyzed using methods available in the art. (See, e.g., Shalon, D. et al., (1995) PCT Application No. WO95/35505; Baldeschweiler et al., (1995) PCT Application No. WO95/251116; Brennan, T.M. et al., (1995) U.S. Patent No. 5,474,796; Heller, M.J. et al., (1997) U.S. Patent No. 5,605,662.)

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These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

15

EXAMPLES

The invention will be further understood by reference to the following examples, which are intended to be purely exemplary of the invention. These examples are provided solely to illustrate the claimed invention. The present invention is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only. Any methods that are functionally equivalent are within the scope of the invention. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

20

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Example 1: HIF α Stabilization in Cells *in vitro*.

Human cells derived from cardiomyocytes, adenovirus-transformed fetal kidney epithelium (293A), cervical epithelial adenocarcinoma (HeLa), hepatocarcinoma (Hep3B), and foreskin fibroblast (HFF) tissues (see, e.g., American Type Culture Collection, Manassas VA; and Qbiogene, Carlsbad CA) were separately seeded into 35 mm culture dishes and grown at 37°C, 20% O₂, 5% CO₂ in media as follows: HeLa cells in Dulbecco's Modification of Eagle's Medium (DMEM), 2% fetal bovine serum (FBS); HFF cells in DMEM, 10%FBS; 293A cells in DMEM, 5%FBS; and Hep3B in Minimal Essential Medium (MEM), Earle's BSS (Mediatech Inc., Herndon VA), 2mM L-glutamine, 0.1mM non-essential amino acids, 1 mM sodium pyruvate, 10% FBS. When cell layers reached confluence, the media was replaced

30

with OPTI-MEM media (Invitrogen Life Technologies, Carlsbad CA) and cell layers were incubated for approximately 24 hours in 20% O₂, 5% CO₂ at 37°C. One of the following was then added to existing medium and incubation was continued overnight: 25 µM 3-carboxy-4-oxo-3,4-dihydro-1,10-phenanthroline (compound A); 0.5, 1, 5, 10 or 25 µM N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B); or 1% DMSO (negative control).

Following incubation, the media was removed, centrifuged, and stored for analysis (see below). The cells were washed two times in cold phosphate buffered saline (PBS) and then lysed in 1 ml of 10 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% IGEPAL (Sigma-Aldrich, St. Louis MO), and a protease inhibitor mix (Roche) for 15 minutes on ice. Cell lysates were centrifuged at 3,000xg for 5 minutes at 4°C, and the cytosolic fraction (supernatant) was collected. The nuclei (pellet) were resuspended and lysed in 100 µl of 20 mM HEPES (pH 7.2), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and a protease mix (Roche), centrifuged at 13,000xg for 5 minutes at 4°C, and the nuclear protein fraction (supernatant) was collected.

Nuclear fractions were normalized based on protein concentration and loaded onto a 4-12% TG gel and fractionated under reducing conditions. Proteins were transferred to a PVDF membrane (Invitrogen Corp., Carlsbad CA) at 500 mA for 1.5 hours. The membrane was blocked in T-TBS, 2% milk for 1 hour at room temperature and incubated overnight with mouse anti-HIF-1α antibody (BD Biosciences, Bedford MA), diluted 1:250 in T-TBS, 2% milk. The blot was developed using SUPERSIGNAL WEST chemiluminescent substrate (Pierce, Rockford IL). As can be seen in Figure 1, the substituted heterocyclic carbonyl glycine N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B) stabilizes HIFα in a normoxic environment in a dose dependent manner, allowing HIFα to accumulate within the cell. As can be seen in Figure 2, cardiomyocytes, transformed kidney epithelial cells (293A), and foreskin fibroblasts show approximately equal levels of HIFα stabilization when treated with 25 µM 3-carboxy-4-oxo-3,4-dihydro-1,10-phenanthroline (compound A) or compound B in a normoxic environment.

Example 2: Expression of HIF-Regulated Genes *in vitro*.

Conditioned medium collected from cell cultures grown as above were analyzed for vascular endothelial growth factor (VEGF) expression using a QUANTIKINE immunoassay (R&D Systems, Inc., Minneapolis MN) according to the manufacturer's instructions. As can be seen in Figure 3, fibroblasts (HFF) and cervical epithelial adenocarcinoma (HeLa) cells treated with compound B show a dose-

dependent increase in VEGF expression. Thus, stabilization of HIF α by compounds of the invention leads to subsequent increase in expression of HIF-regulated genes.

Example 3: Expression of HIF-Regulated Genes *in vivo*.

5 Swiss Webster male mice (30-32 g) were obtained from Charles River Laboratories, Inc (Wilmington MA) and treated by oral gavage two times per day for approximately 50 hours (5 doses) with a 2 ml/kg volume of either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis MO) (control) or 5.0% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC) (compound B). Two and five hours after the final dose, animals were anesthetized with isoflurane and 0.1 ml blood was
10 collected from the orbital sinus into a heparinized tube. Twenty hours after the final dose, animals were subjected to a sub-lethal dose of CO₂ and blood was collected from the abdominal vein into a heparinized tube. All blood samples were stored at -80°C.

Plasma samples were processed and analyzed for vascular endothelial growth factor (VEGF) expression using a QUANTIKINE immunoassay (R&D Systems) according to the manufacturer's instructions. As
15 can be seen in Figure 4, levels of VEGF in the plasma of animals treated with compound B are significantly increased relative to untreated control animals at 2, 5, and 20 hours after the final dose.

Alternatively, tissue-specific HIF-regulated gene expression is measured using a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) procedure as follows. After blood collection (see
20 above), animals from each group are sacrificed and tissues such as heart, brain, lung, kidney, liver, spleen, muscle, etc., are isolated and stored in ice-cold saline. Tissue slices of approximately 150 mg are cut into small pieces, 2 ml of RLT lysis buffer (RNEASY kit; Qiagen Inc., Valencia CA) are added, and the pieces are homogenized using a rotor-stator homogenizer. RNA is isolated from the homogenate using an
25 RNEASY kit (Qiagen) according to the manufacturer's instructions. Genomic DNA is removed from the RNA using the DNA-FREE system (Ambion Inc., Austin TX) according to the manufacturer's instructions. RNA is stored at -80°C. cDNA synthesis is then performed using OMNISCRIPT reverse transcriptase (Qiagen) according to the manufacturer's instructions using random hexamer primers at a concentration of 1 μ M. Resulting cDNA samples are stored at -20°C.

30 Analysis of the relative level of HIF-regulated gene expression is performed by quantitative PCR using FASTSTART DNA MASTER SYBR GREEN I reaction mix (Roche) and gene-specific primers, using a LIGHTCYCLER system (Roche), according to manufacturer's instructions. Each PCR run includes a standard curve and water blank. In addition, a melt curve is run after completion of each PCR run to

assess the specificity of the amplification. Gene specific primers are designed according to the gene of interest; for example, amplification of VEGF may use the primer set and reaction conditions specified in the mouse VEGF RELATIVE RT-PCR kit (Ambion).

5 **Example 4: HIF α Stabilization in Cells *in vivo***

Tissues isolated from animals treated with compounds of the invention as described in Example 3 are analyzed for HIF α protein levels as follows. Tissues are homogenized in 3 ml of 10 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% IGEPAL (Sigma-Aldrich), and a protease inhibitor mix (Roche Molecular Biochemicals, Indianapolis IN) for 15 seconds using a POLYTRON PT-1200 homogenizer
10 (Brinkmann Instruments, Inc., Westbury NY). Cell lysates are centrifuged at 3,000xg for 5 minutes at 4°C, and the cytosolic fraction (supernatant) is collected. The nuclei (pellet) are resuspended and lysed in 100 μ l of 20 mM HEPES (pH 7.2), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and a protease mix (Roche Molecular Biochemicals), centrifuged at 13,000xg for 5 minutes at 4°C, and the nuclear protein fraction (supernatant) is collected.

Nuclear fractions are normalized based on protein concentration and loaded onto a 4-12% TG gel and fractionated under reducing conditions. Proteins are transferred to a PVDF membrane (Invitrogen Corp., Carlsbad CA) at 500 mA for 1.5 hours. The membrane is blocked in T-TBS, 2% milk for 1 hour at room temperature and incubated overnight with anti-HIF-1 α antibody diluted in T-TBS, 2% milk. The blot is developed using SUPERSIGNAL WEST PICO chemiluminescent substrate (Pierce, Rockford IL).
20

Example 5: Effect of HIF α Stabilization on Oxygen Consumption.

Oxygen Sensor cell culture plates (BD Biosciences) contain a ruthenium complex which is more fluorescent in the absence of oxygen. Therefore, the fluorescent read-out is increased by the presence of
25 oxygen-consuming cells in the plate, which change the equilibrium to lower oxygen saturation and higher fluorescence. A compound that stabilizes HIF by inhibiting hydroxylation is expected to decrease oxygen consumption by decreasing oxygen consumed by the hydroxylation event itself and/or by shifting cellular metabolism from aerobic to anaerobic energy production.

30 HeLa and 293A cells were grown to confluence in media (high glucose DMEM (Mediatech), 1% penicillin/streptomycin mixture (Mediatech), 1% FBS). Cells were collected and resuspended in media at a density of 500,000 cells/ml. The cell suspension was distributed at 0.2 ml/well into each well of an Oxygen Biosensor 96-well cell culture plate (BD Biosciences). The following treatments were added in 10 μ l volumes to triplicate sets of wells: 1) 0.5% DMSO (0 μ M compound B); 2) 5, 25, or 50 μ M N-((1-

chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B); 3) 200 μ M SDS; or 4) 5 or 50 μ M desferrioxamine mesylate (DFO). Cultures were incubated at 37°C, 10% CO₂ and at 24, 48, and 72 hours plates were read in an FL600 fluorimeter (Biotek Instruments, Inc., Winooski VT) at an excitation wavelength of 485 nm and emission wavelength of 590 nm. Data was plotted as a function of fluorescence and descriptive statistical analysis was performed using EXCEL software (Microsoft Corp., Bellevue WA). Cell proliferation and viability were assayed using WST-1 reagent (Roche Diagnostics GmbH) according to manufacturer's instructions.

As can be seen in Figure 5A, the control cells (0.5% DMSO; 0 μ M compound B) produced fluorescence levels from 37,000 (HeLa) to 47,000 (293A) units over background levels. However, compound B showed dose-dependent fluorescence quenching in the same dose range that showed HIF stabilization, with maximal effect seen at 50 μ M. As can be seen in Figure 5B, the cells treated with compound B showed little to no loss of viability. Therefore, the decrease in oxygen consumption in the dose range between 5 and 50 μ M was not associated with cytotoxicity.

Example 6: Cardiac Ischemia

Male Wistar rats (100-110 g), aged 4-5 weeks, were kept on a regular diet and a 12 hour day-night cycle. The animals were randomized into treatment regiments as follows: 1) Sham operated animals (n=12), 2) myocardial infarction controls (n=25), and 3) myocardial infarction with N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (compound B) treatment (n=25). Animals were treated for two days prior to surgery and for one week following surgery. Animals were treated by oral gavage two times per day with either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis MO) (control) or 50 mg/kg N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine in 0.5% CMC (compound B). Ligation of the left anterior descending coronary artery was performed in artificially ventilated animals after left thoracotomy. Animals were sacrificed one week after surgery and echocardiography was performed. Fractional shortening, end-diastolic diameters, and end-systolic diameters were determined in a blinded fashion.

As can be seen in Figure 6A, fractional shortening was reduced from 51% in sham-operated animals to 29% in untreated MI controls. Treatment with compound B showed a statistically significant ($p < 0.05$; one-way ANOVA/Turey's test) improvement in fractional shortening, to 41%, relative to the untreated control group. Similarly, Figure 6B shows statistically significant improvement in left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters in treated animals relative to untreated MI controls ($p < 0.005$ and $p < 0.001$, respectively; one-way ANOVA/Turey's test). Animals treated with

compound B showed no increase in left ventricular end-systolic diameter and an 18% increase in end-diastolic diameter over sham operated animals. The untreated controls, however, showed a 15% and 65% increase in LVESD and LVEDD, respectively.

5 **Example 7: Screening Assay**

Compounds that inhibit HIF-specific prolyl hydroxylase activity and thereby stabilize HIF α can be identified and characterized using the following assay. A 50 μ l aliquot of a reaction mix containing 4 mg/ml BSA, 0.1 M Tris HCl (pH 7.2), 2 mM ascorbate, 80 μ M ferrous sulfate, 0.2 mM 2-oxoglutarate, 600 units/ml catalase, with or without 100 μ M HIF α peptide is mixed with 50 μ l HeLa cell extract and
10 incubated 1.5 hours at 37°C. Following incubation, 50 μ l of streptavidin beads are added and the mixture is incubated for 1 hour with agitation at 4°C. The mixture is transferred to tubes and centrifuged at low speed to pellet the beads. The beads are washed three times with 0.5-1 ml 20 mM Tris HCl (pH 7.2). The peptide is then eluted from the beads with 5 μ l 2 mM biotin in 20 mM Tris HCl (pH 7.2) for 1 hour. The tubes are centrifuged to pellet the resin and 40-50 μ l of supernatant is removed and an equal volume of acetonitrile is added. The non-hydroxylated and hydroxylated peptides are then separated by reverse-
15 phase HPLC on a C18 column with UV detection at 214 nm.

Various modifications of the invention, in addition to those shown and described herein, will become
20 apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are hereby incorporated by reference in their entirety.

ABSTRACT

The present invention relates to compounds that stabilize the alpha subunit of hypoxia inducible factor and to the use of the compounds in preventing or treating ischemic conditions including stroke,
5 myocardial infarction, and the like.

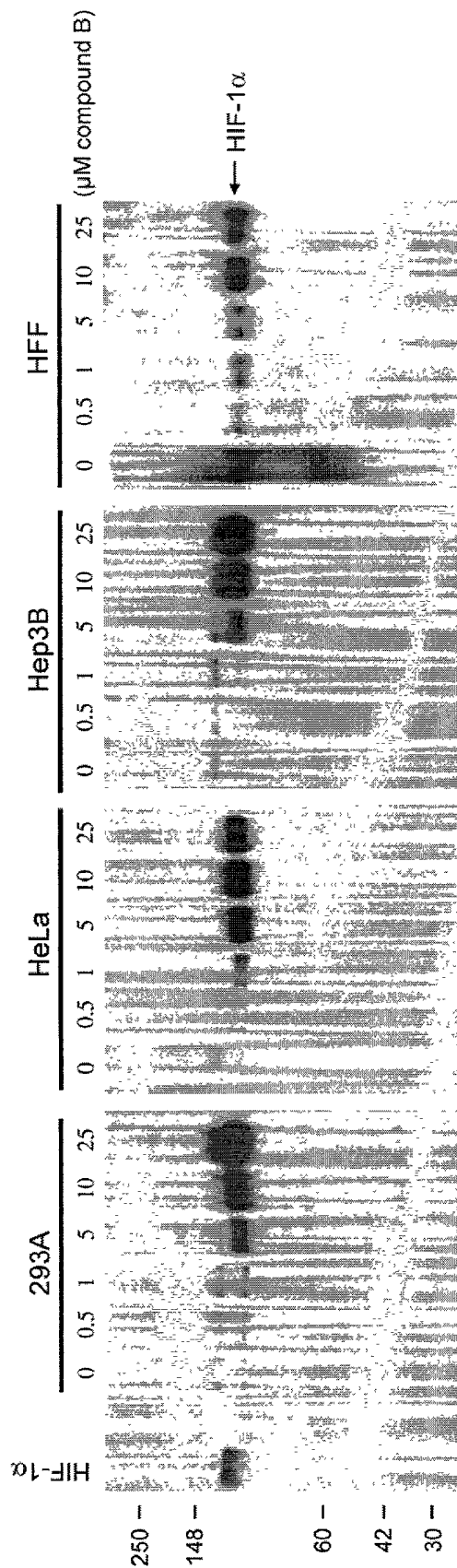


FIGURE 1

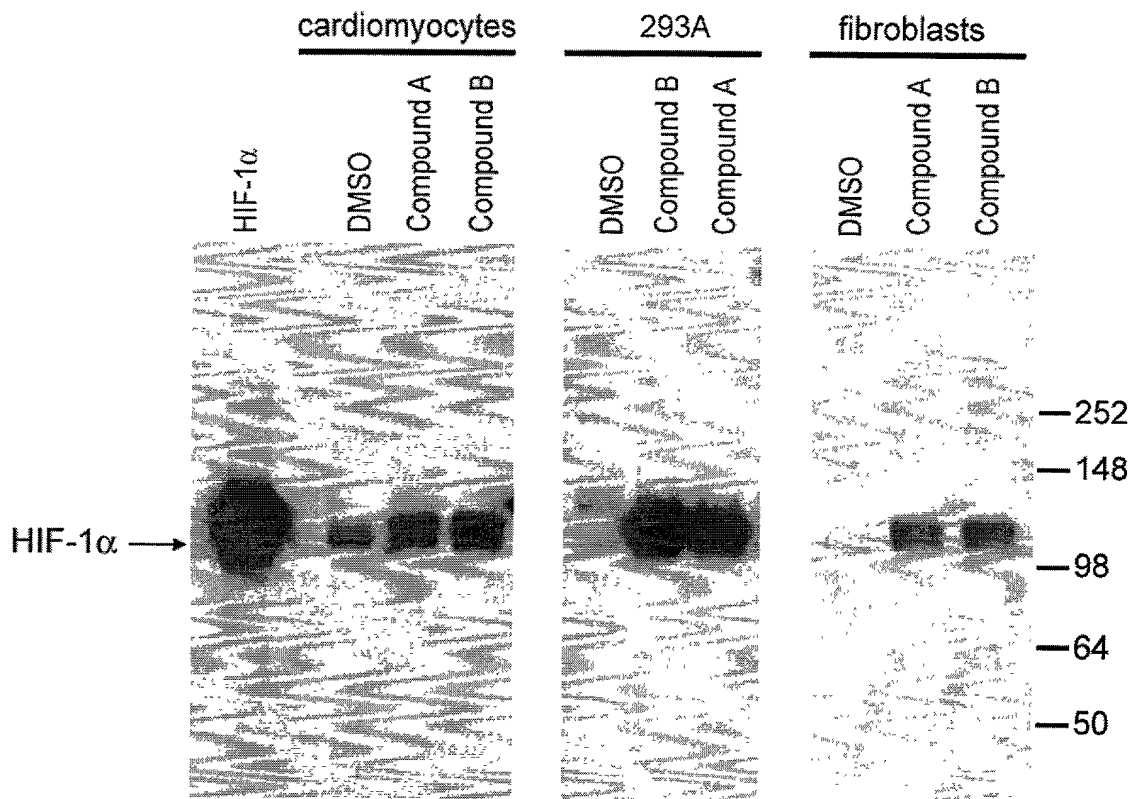


FIGURE 2

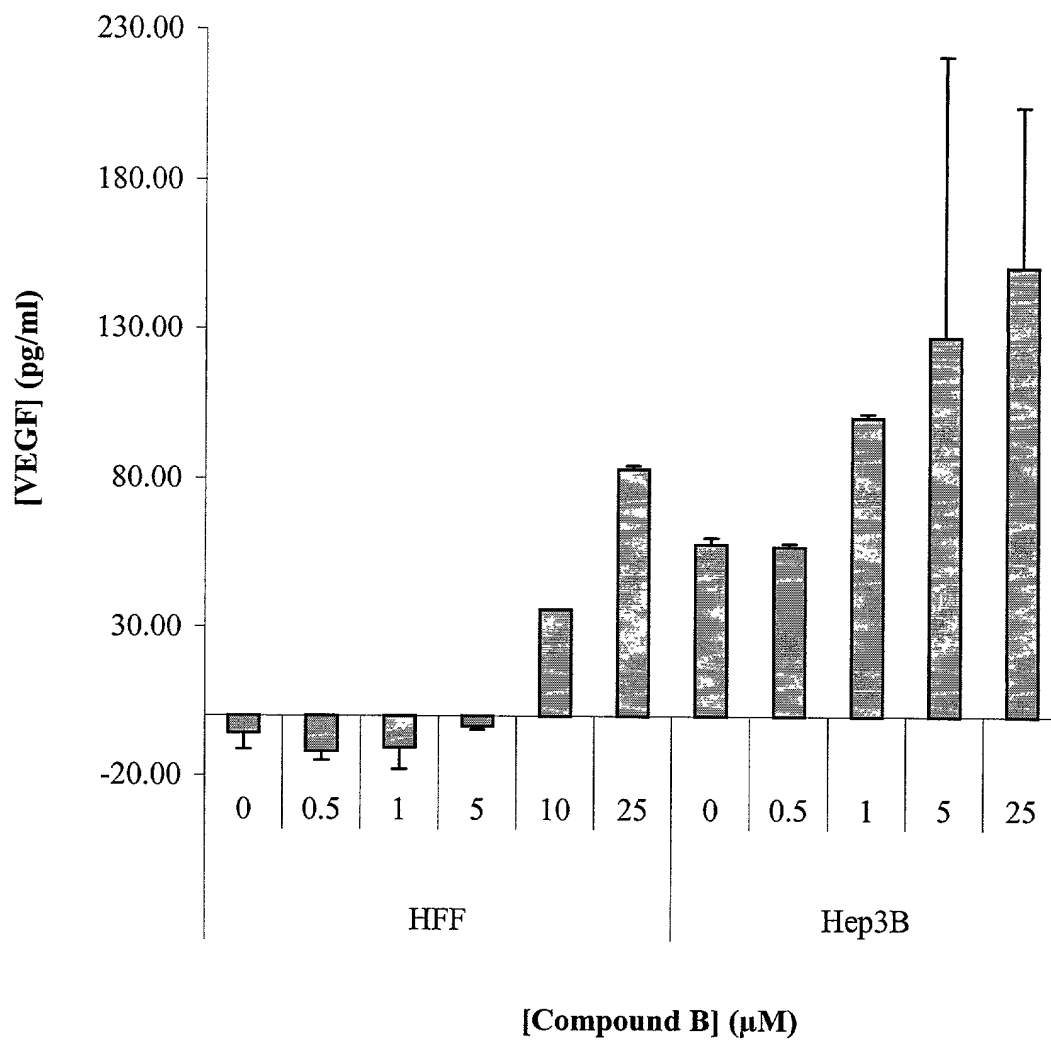


FIGURE 3

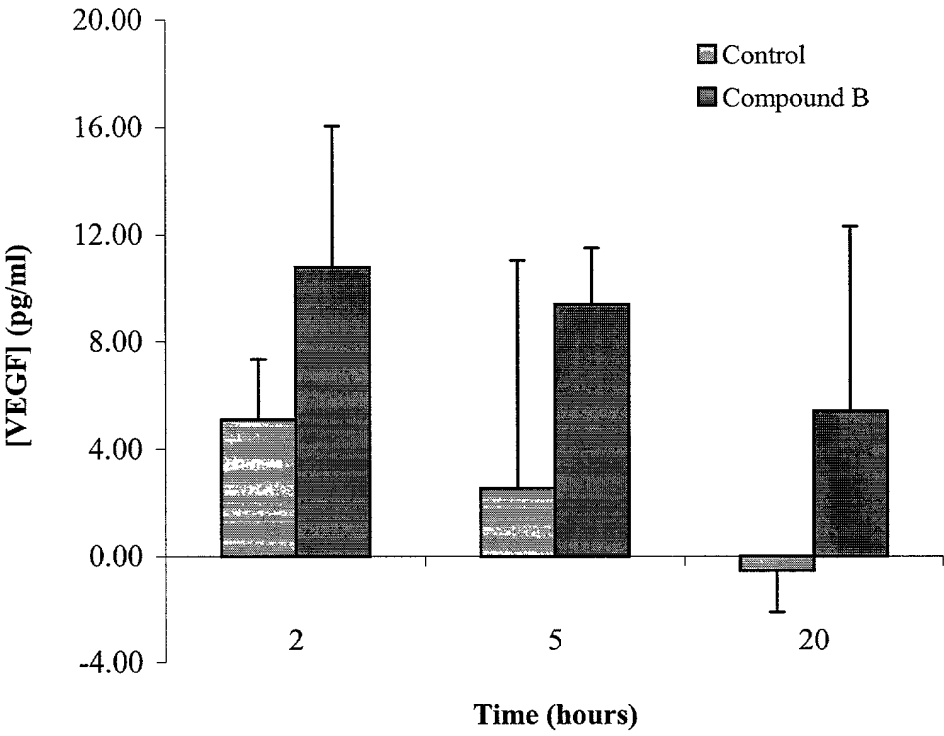
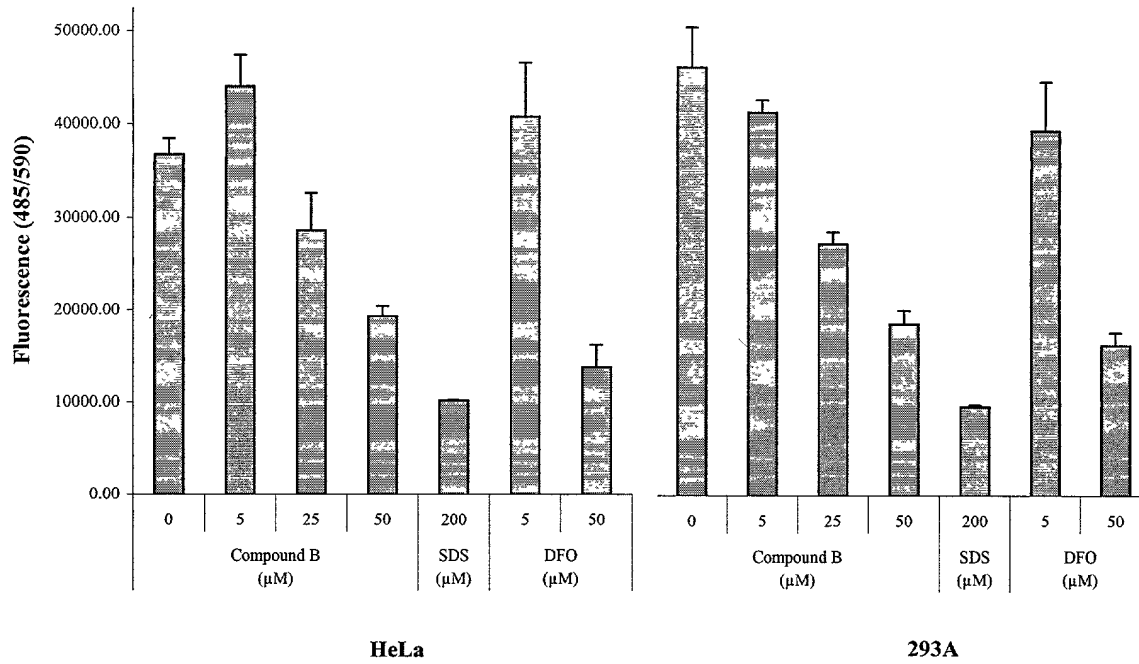


FIGURE 4

A.



B.

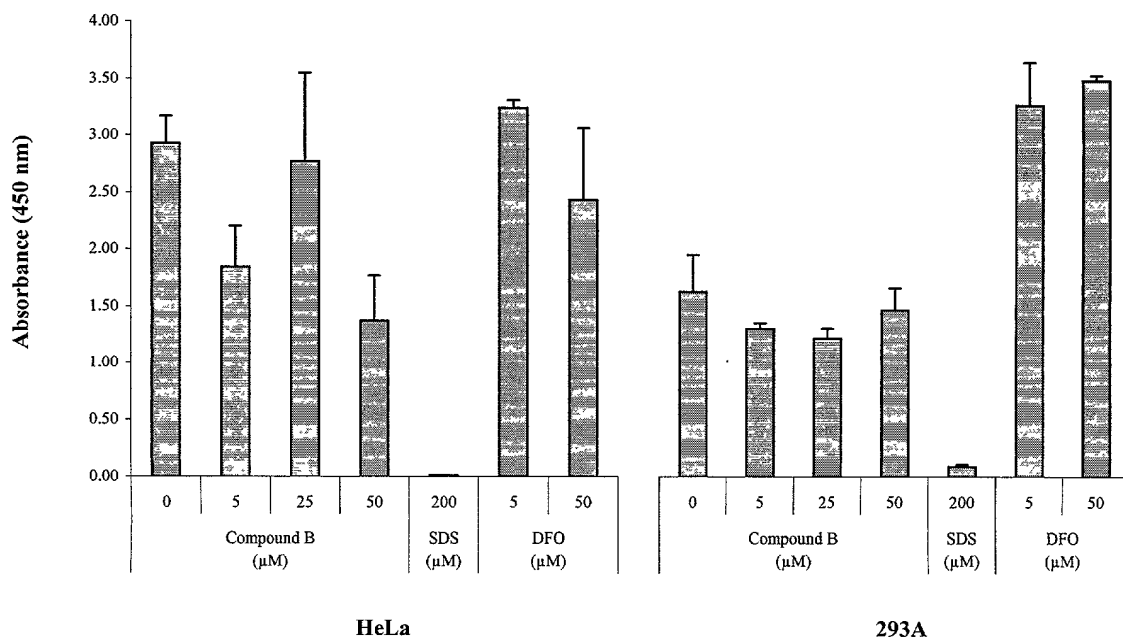
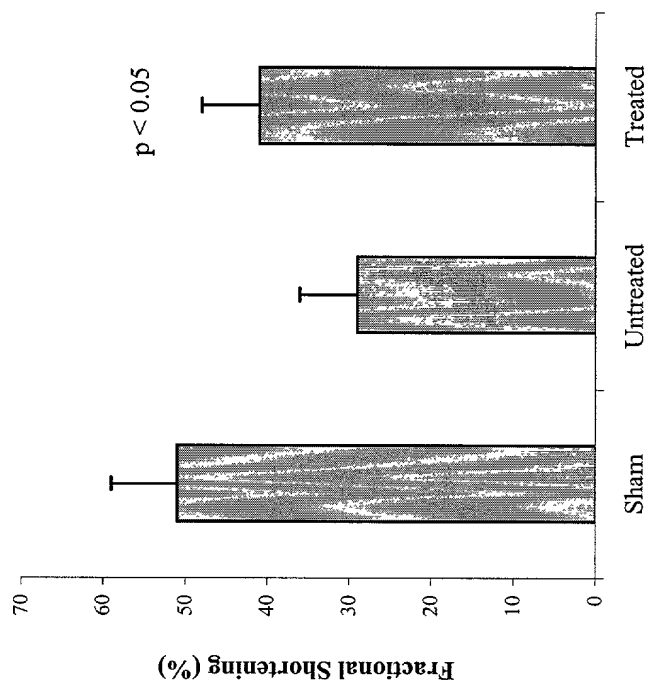


FIGURE 5

A.



B.

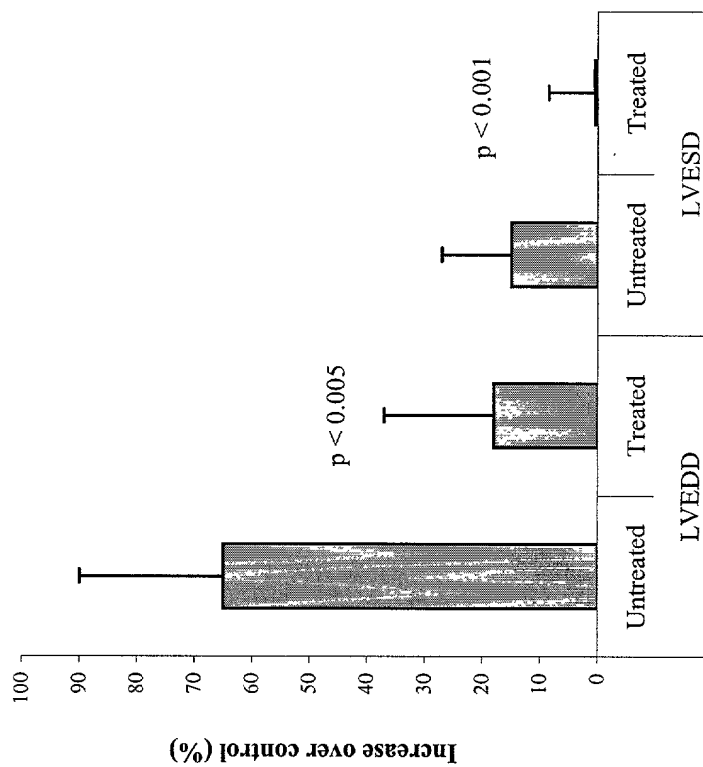


FIGURE 6