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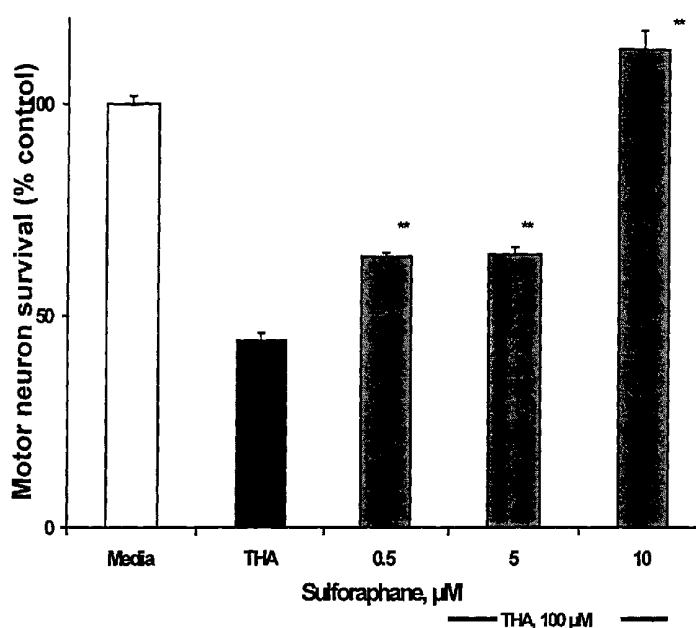
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[Continued on next page]

(54) Title: PREVENTION AND TREATMENT OF DEGENERATIVE DISEASES BY GLUTATHIONE AND PHASE II DETOXIFICATION ENZYMES



(57) Abstract: The present invention generally relates to the field of treating degenerative disease by administering a pharmaceutically effective amount of a compound that elevates glutathione or at least one Phase II detoxification enzyme in diseased tissue. The present invention also relates to a pharmaceutical composition useful for the treatment of degenerative diseases, as well as a method of identifying agents that modulate intracellular levels of glutathione or intracellular levels of at least one Phase II enzyme in neuronal cells.



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1
2 **Prevention and Treatment of Degenerative Diseases By**
3 **Glutathione and Phase II Detoxification Enzymes**

4 **Statement as to the Rights to Inventions Made Under Federally-Sponsored**
5 **Research and Development**

6 Part of the work performed during development of this
7 invention utilized U.S. Government funds. The U.S. Government has
8 certain rights in this invention.

9 **Background of the Invention**

10 Field of the Invention

11 The present invention generally relates to the field of treating
12 degenerative disease by administering a pharmaceutically effective
13 amount of a compound that elevates glutathione or at least one Phase II
14 detoxification enzyme in diseased tissue. The present invention also
15 relates to a pharmaceutical composition useful for the treatment of
16 degenerative diseases, as well as a method of identifying agents that
17 modulate intracellular levels of glutathione or intracellular levels of at least
18 one Phase II enzyme in neuronal cells.

19 Background of the Invention

20 Enzymes of xenobiotic biotransformation belong to two
21 families: Phase I enzymes (cytochromes P-450), which usually oxidize or
22 reduce xenobiotics. Although their primary role is to detoxify xenobiotics,
23 cytochromes P-450 may also generate highly reactive and toxic
24 compounds (Miller, et al., Bioactivation of Foreign Compounds, 3-28

1 (1985)). The second family of xenobiotic transformative enzymes is
2 Phase II enzymes, which usually conjugate an endogenous moiety (e.g.,
3 glutathione, sulfate) to the xenobiotic, and serve primarily a detoxification
4 role (Jakoby, et al., J. Biol. Chem. 265:20715-20718 (1990)). Phase II
5 enzymes are generally induced in a coordinated fashion in response to
6 xenobiotics. Quinone reductase (QR) is also considered a Phase II enzyme
7 because it has protective functions (Prochaska, et al., Oxidative Stress:
8 Oxidants and Antioxidants, 195-211 (1991)), is induced coordinately with
9 other Phase II enzymes, and is regulated by enhancer elements similar to
10 those that control glutathione transferase (Favreau, et al., J. Biol. Chem.
11 266:4556-4561 (1991)).

12 Well-documented evidence established that feeding of certain
13 vegetables (e.g., brussels sprouts and cabbage) modulates both Phase I
14 and Phase II enzymes in animal tissues (Conney, et al., Fed. Proc. Fed.
15 Am. Soc. Exp. Biol. 36:1647-1652 (1977); Sporn, et al., J. Natl.
16 Cancer Inst. 66:769-771 (1981); Sporn, et al., J. Natl. Cancer Inst.
17 68:493-496 (1982); Aspry, et al., Food Chem. Toxicol. 21:133-142
18 (1983); Bradfield, et al., Food Chem. Toxicol. 23:899-904 (1985); Salbe,
19 et al., Food Chem. Toxicol. 24:851-856 (1985); Whitty, et al., Food
20 Chem. Toxicol. 25:581-587 (1987); Ansher, et al., Hepatology 3:932-
21 935 (1983); Ansher, et al., Food Chem. Toxicol. 24:405-415 (1986)) and
22 stimulates the metabolism of drugs in humans (Conney, et al., Fed. Proc.
23 Fed. Am. Soc. Exp. Biol. 36:1647-1652 (1977); Pantuck, et al., Clin.
24 Pharmacol. Ther. 25:88-95 (1979); Pantuck, et al., Clin. Pharmacol. Ther.
25 35:161-169 (1984)). The elevations of enzymes that metabolize
26 xenobiotics and scavenge free-radicals may be highly relevant to the
27 chemoprotective effects of vegetables, since relatively modest dietary
28 changes not only affects the metabolism of drugs (Ansher, et al., Food
29 Chem. Toxicol. 24:405-415 (1986)) but also modifies the ability of
30 carcinogens to cause tumors in rodents (Tannenbaum, et al., Adv. Cancer

1 Res. 1:451-501 (1953); National Research Council, Diet, Nutrition and
2 Cancer (1982); National Research Council, Diet and Health: Implications
3 for Reducing Chronic Disease Risk (1989); Creasey, Diet and Cancer
4 (1985); Knudsen, Genetic Toxicology of the Diet (1986); Longnecker, et
5 al., Cancer 47:1562-1572 (1981); Fullerton, et al., Proc. Am. Assoc.
6 Cancer Res. 29:147 (1988); Li, et al., Cancer Res. 50:3991-3996
7 (1990)).

8 In degenerative diseases, which are generally characterized
9 by progressive morphological changes and progressive loss in normal
10 metabolic activity in the cells of the tissue, it is believed that oxidative
11 stress may play an important role in the etiology of the diseases. Thus
12 anti-oxidants, which function to scavenge free-radicals and thereby
13 prevent oxidative damages to cells, may provide an opportunity to treat or
14 prevent the progression of degenerative diseases. Additionally, if a
15 treatment can act as an anti-oxidant to the cells, as well as induce Phase
16 II enzymes, which also protect the cell from free radicals, this treatment
17 would provide a two-pronged attack on the progression of degenerative
18 diseases. To date, no such compounds have been identified.

19 **Summary Of The Invention**

20 The invention relates to a method of treating a subject in
21 need of treatment of a degenerative disease, which comprises
22 administering to the subject a pharmaceutically effective amount of a
23 compound that elevates glutathione, or at least one Phase II detoxification
24 enzyme, in the diseased tissue of the subject.

25 The invention also relates to a method of inhibiting cell death
26 in neuronal cells, which comprises treating the neuronal cells with an
27 agent that increases levels of glutathione, or intracellular levels of at least
28 one Phase II detoxification enzyme.

1 The invention also relates to a method of identifying agents
2 that modulate intracellular levels of glutathione or intracellular levels of at
3 least one Phase II enzyme in neuronal cells, which comprises treating the
4 neuronal cells with an agent, assaying for intracellular levels of
5 glutathione or intracellular levels of at least one Phase II detoxification
6 enzyme, and comparing these levels to control levels.

7 The invention also relates to a method of identifying agents
8 that modulate intracellular levels of glutathione or intracellular levels of at
9 least one Phase II enzyme in neuronal cells comprising comparing the
10 levels of the intracellular glutathione or intracellular levels of at least one
11 Phase II detoxification enzyme to determine if the agent modulates
12 intracellular levels of glutathione or intracellular levels of at least one
13 Phase II detoxification enzyme.

14 The invention also relates to a method of modulating
15 intracellular levels of at least one Phase II detoxification enzyme in
16 neuronal cells. The method comprises treating the neuronal cells with an
17 agent that binds to glutathione to form a complex. The complex
18 modulates the intracellular levels of at least one Phase II detoxification
19 enzyme.

20 The invention also relates to a composition for use in the
21 treatment of a degenerative disease, which comprises a pharmaceutical
22 excipient and a pharmaceutically effective amount of an agent that
23 increases intracellular levels of glutathione or at least one Phase II
24 detoxification enzyme.

25 **Brief Description Of The Drawings**

26 *Figure 1* depicts sulforaphane protecting motor neurons from chronic
27 glutamate toxicity. **, $p < 0.01$.

28

1 *Figure 2* depicts sulforaphane elevating organotypic spinal cord
2 glutathione level. **, $p < 0.01$.

3
4 *Figure 3* depicts the ratio of specific enzyme activity or GSH levels in
5 culture mediums treated with sulforaphane for 2 weeks.

6
7 *Figure 4* depicts the ratio of specific enzyme activity or GSH levels in
8 culture mediums treated with sulforaphane for 24 hours.

9
10 *Figure 5* depicts that oral administration of sulforaphane in mice potentially
11 elevates brain glutathione level. **, $p < 0.01$.

12

13 **Detailed Description Of The Preferred Embodiments**

14 The present invention relates to a method of treating a
15 subject in need of treatment of a degenerative disease, the method
16 comprising administering to the subject a pharmaceutically effective
17 amount of a compound that elevates levels intracellular glutathione or
18 intracellular levels of at least one Phase II detoxification enzyme in
19 diseased tissue of said subject.

20 As used herein, the term subject can be used to mean an
21 animal, preferably a mammal including a human or non-human. The term
22 patient is used to indicate a subject in need of treatment of a
23 degenerative disease. Furthermore, the terms "disease," "condition" and
24 "disorder" may all be used interchangeably.

25 The current invention can be useful in treating a subject in
26 need of treatment of a degenerative disease where there may be aberrant
27 levels of glutathione or any Phase II enzyme present in the diseased cells
28 or tissue. These abnormal levels may be either causal or symptomatic of
29 the degenerative disease. The phrase "degenerative disease," as used in

1 the current context, should be obvious to one of ordinary skill in the art,
2 but is used to mean any physiological condition that may be characterized
3 by the death of normal cells in the affected tissue. As used herein a
4 degenerative disease is not used to mean a disease where the death of
5 the normal cells is caused by tumor growth or acute toxic insult.
6 Examples of degenerative disorders for which the current invention can be
7 used preferably include, but are not limited to, diabetes, chronic liver
8 failure, chronic kidney failure, Wilson's disease, congestive heart failure
9 and atherosclerosis and any neurodegenerative disease.

10 A neurodegenerative disease, as used in the current context,
11 should be obvious to one skilled in the art, but is meant to include any
12 abnormal physical or mental behavior or experience where the death of
13 neuronal cells is involved in the etiology of the disorder, or is affected by
14 the disorder. As used herein, neurodegenerative diseases encompass
15 disorders affecting the central and peripheral nervous systems, and
16 include such afflictions as memory loss, stroke, dementia, personality
17 disorders, gradual, permanent or episodic loss of muscle control.
18 Examples of neurodegenerative diseases for which the current invention
19 can be used preferably include, but are not limited to, Alzheimer's
20 Disease, Parkinson's Disease, Huntington's Disease, amyotrophic lateral
21 sclerosis, epilepsy, myasthenia gravis, neuropathy, ataxia, dementia,
22 chronic axonal neuropathy and stroke.

23 The treatment envisioned by the current invention can be
24 used for patients with a pre-existing degenerative condition, or for
25 patients pre-disposed to a degenerative disorder. Additionally, the
26 method of the current invention can be used to correct cellular or
27 physiological abnormalities involved with a degenerative disorder in
28 patients, and/or to alleviate symptoms of a degenerative disorder in
29 patients, or as a preventative measure in patients.

1 As used herein, the term agent or compound is intended to
2 mean a protein, nucleic acid, carbohydrate, lipid, polymer or a small
3 molecule, such as a drug. The types of agents or compounds which can
4 be envisioned are limited only by their ability to elevate intracellular levels
5 of glutathione or Phase II enzymes.

6 As used herein, "a pharmaceutically effective amount" is
7 intended used to mean an amount effective to elicit a cellular response
8 that is clinically significant, without excessive levels of side effects.

9 The present invention relates to methods of elevating
10 intracellular glutathione (GSH) as a way to prevent or treat degenerative
11 diseases. GSH is a tripeptide composed of glycine, cysteine and
12 glutamate, with the glutamate being linked to cysteine via the gamma-
13 carboxyl group (as opposed to an alpha-carboxyl linkage that normally
14 occurs in a peptide). GSH is a detoxifying peptide that the body
15 conjugates to xenobiotics (foreign chemical or compound) to render the
16 xenobiotic more hydrophilic, thus promoting their excretion from the
17 body. The synthesis of GSH involves a two-step reaction, with the first
18 being catalyzed by gamma-glutamylcysteine synthetase. Glutathione
19 synthetase catalyzes the second reaction. In turn, the conjugation of
20 GSH to the xenobiotic is catalyzed by glutathione S-transferase (also
21 referred to as GSH transferase), which can be a dimer of identical
22 (homodimer) or different (heterodimer) subunits, although some
23 heterodimers do exist. To date, at least four classes of GSH transferases
24 have been identified, with each class having two or more types of
25 subunits therein. Most GSH transferases are cytosolic (or soluble),
26 meaning they are found in a cell's cytosol, although at least two
27 microsomal GSH transferases exist. Based on such factors as amino acid
28 similarity and biological activity, one of ordinary skill in the art will be able
29 to appreciate and recognize the many types of GSH transferases that
30 exist, as well as any that may not yet be identified. Thus, as used herein,

1 the phrases "increase in intracellular GSH" or "elevation in intracellular
2 GSH" is intended to mean the GSH tripeptide itself, as well as the
3 enzymes responsible for its synthesis and conjugation to xenobiotics.

4 The present invention also relates to a method increasing the
5 intracellular levels of at least one Phase II detoxification enzyme. The
6 phrases "Phase II detoxification enzyme" and "Phase II enzyme" are used
7 interchangeably herein. As used herein, a Phase II enzyme is any enzyme
8 that is involved in any of the Phase II reactions which are responsible for
9 the biotransformation of xenobiotics and/or prevention of oxidative stress.
10 Generally speaking, a Phase II reaction will generally involve the
11 conjugation of a moiety to the xenobiotic that will increase the
12 hydrophilicity of the xenobiotic. This conjugated xenobiotic, which is
13 now more hydrophilic, is more readily excreted from the body, than the
14 unconjugated xenobiotic. There are six types of Phase II conjugation
15 reactions, including glucuronidation, sulfation, methylation, acetylation,
16 amino acid conjugation and glutathione conjugation. The reaction
17 catalyzed by the enzyme rhodanese (the transfer of a sulfur ion to cyanide
18 to form thiocyanate) will also be considered a Phase II reaction herein.

19 Briefly, glucuronidation is a major pathway of xenobiotic
20 transformation and involves the conjugation of glucuronide to the
21 xenobiotic. The reaction is catalyzed by UDP-glucuronosyltransferase
22 (UDPGT), of which multiple forms exist. These multiple forms are
23 encoded by several different genes. One of ordinary skill in the art will be
24 able to recognize and appreciate the different forms of UDPGT, and the
25 reactions they catalyze.

26 Sulfation is a pathway of xenobiotic transformation that
27 involves the conjugation of sulfate to the xenobiotic. The reaction is
28 catalyzed by sulfotransferases, of which more than a dozen forms have
29 been identified. One of ordinary skill in the art will be able to recognize

1 and appreciate the different forms of sulfotransferases, and the reactions
2 they catalyze.

3 Methylation is a pathway of xenobiotic transformation that
4 involves the conjugation of a methyl group to the xenobiotic. There are
5 three different methylation reactions, based on the type of atom on the
6 xenobiotic that is methylated. The three methylation reactions can occur
7 on, sulfur (S), oxygen (O) and nitrogen (N), each of which is catalyzed by
8 a different sets of enzymes. O-methylation is catalyzed by phenol-O-
9 methyltransferase (POMT) or catechol-O-methyltransferase (COMT). It is
10 believed that COMT is encoded by a single gene, with at least two
11 different allelic variants. N-methylation is catalyzed by histamine-N-
12 methyltransferase or nicotinamide-N-methyltransferase. S-methylation is
13 catalyzed by at least two enzymes, including thiopurine methyltransferase
14 (TPMT) and thiol methyltransferase (TMT). One of ordinary skill in the art
15 will be able to recognize and appreciate the different forms of
16 methytransferases, and the reactions they catalyze.

17 Acetylation is a pathway of xenobiotic transformation that
18 involves the conjugation of an acetyl group to the xenobiotic. There are
19 two different acetylation reactions, based on the type of atom (O and N)
20 on the xenobiotic that is acetylated. The two acetylation reactions may
21 or may not be catalyzed by the same set of enzymes. N-acetylation is
22 catalyzed by N-acetyltransferase (NAT), of which two forms exist in
23 humans. These forms are encoded by two different genes. O-acetylation
24 is catalyzed by O-acetyltransferase, but may also be catalyzed by NAT.
25 One of ordinary skill in the art will be able to recognize and appreciate the
26 different forms of acetyltransferases, and the reactions they catalyze.

27 Amino acid conjugation is a pathway of xenobiotic
28 transformation that involves the conjugation of an amino acid to the
29 xenobiotic. There are two principle pathways where amino acids are
30 conjugated to xenobiotics. The first reaction involves xenobiotics

1 containing a carboxylic acid. This reaction takes place by acyl-CoA
2 synthetase catalyzing the conjugation of CoA to the xenobiotic to form a
3 thioester. The thioester is subsequently conjugated to the amino acid via
4 the acyl-CoA:amino acid N-transferase enzyme. The second principle
5 pathway where xenobiotics are conjugated to amino acids involves
6 xenobiotics containing an aromatic hydroxylamine. This reaction involves
7 the activation of an amino acid with the aminoacyl-tRNA-synthetase. The
8 activated amino acid subsequently reacts with the aromatic
9 hydroxylamine on the xenobiotic to form a reactive N-ester. One of
10 ordinary skill in the art will be able to recognize and appreciate the
11 different types of enzymes responsible for the conjugation of amino acids
12 to xenobiotics, and the reactions they catalyze.

13 Quinone reductase (QR) is considered a Phase II enzyme
14 because it has protective functions (Prochaska, et al., *Oxidative Stress:*
15 *Oxidants and Antioxidants*, 195-211 (1991)), is induced coordinately with
16 other phase II enzymes, and is regulated by enhancer elements similar to
17 those that control glutathione transferase (Favreau, et al., *J. Biol. Chem.*
18 *266:4556-4561* (1991)).

19 Heme oxygenase (HO), also considered a Phase II enzyme,
20 catalyzes the conversion of heme to biliverdin, which is subsequently
21 reduced to bilirubin. Thus the HO enzyme is responsible for the
22 production of bilirubin, which itself is a powerful antioxidant.
23 Additionally, the HO enzyme is induced by many of the same compounds
24 that induce other Phase II enzymes.

25 Examples of additional Phase II enzymes include, but are not
26 limited to, such enzymes as glutathione reductase, glutathione
27 peroxidase, catalase and superoxide dismutase.

28 The present invention relates to increasing the levels of at
29 least one Phase II enzyme in diseased tissue. It is possible that the
30 compounds contemplated in this invention may be responsible for the

1 increase levels of only one of the Phase II enzymes, or more than one
2 Phase II enzyme. As used herein, the phrase "increase in levels of Phase
3 II enzymes" is used to mean an increase in the quantity of Phase II
4 enzymes present in the cell, compared to control (non-stimulated) levels.
5 The phrase is also used to mean an increase in the activity or specificity
6 of the enzymes present in the cell.

7 As used herein, the term diseased tissue may be used to
8 mean individual cells, as cultured *in vitro*, or excised tissue in whole or in
9 part. Diseased tissue may also be used to mean tissue in the subject that
10 is undergoing the degenerative process, or tissue within the same organ
11 that may not yet be affected by the degenerative process. The normal
12 tissue may or may not be adjacent to the degenerative tissue.

13 As a preferred embodiment, the compounds used in the
14 methods of the current invention that elevate glutathione or any Phase II
15 enzyme, are selected from the group consisting of a glutathione
16 precursor, an isothiocyanate and a glucosinolate. Glutathione precursors
17 include N-acetylcysteine.

18 Isothiocyanates are compounds containing the thiocyanate
19 (SCN⁻) moiety and are easily identifiable by one of ordinary skill in the art.
20 An example of an isothiocyanate includes, but is not limited to
21 sulforaphane or its analogs. The description and preparation of
22 isothiocyanate analogs is described in United States Reissue Patent
23 36,784, and is hereby incorporated by reference in its entirety. In a
24 preferred embodiment, the sulforaphane analogs used in the present
25 invention include 6-isothiocyanato-2-hexanone, exo-2-acetyl-6-
26 isothiocyanatonorbornane, exo-2-isothiocyanato-6-
27 methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-
28 4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
29 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
30 isothiocyanato-5-methylsulfonylpentane, cis-3-

1 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
2 (methylsulfonyl)cyclohexylmethylisothiocyanate.

3 Glucosinolates, which are well-known in the art, are
4 precursors to isothiocyanates. Glucosinolates are easily recognizable and
5 appreciated by one of ordinary skill in the art and are reviewed in Fahey *et*
6 *al.* Phytochemistry, 56:5-51 (2001), the entire contents of which are
7 hereby incorporated by reference.

8 Other compounds contemplated by the present invention
9 include compounds that are known to induce (increase) levels of Phase II
10 enzymes. Preferably, these compounds include resveratrol, oltipraz,
11 dimethylfumarate, 2(3)-tert-butyl-4-hydroxyanisole, 3,5-di-tert-butyl-4-
12 hydroxytoluene and an analog thereof.

13 The present invention also provides a method of inhibiting
14 cell death in neuronal cells comprising treating said neuronal cells with an
15 agent that increases intracellular levels of glutathione or intracellular levels
16 of at least one Phase II detoxification enzyme. As contemplated by the
17 current invention, "cell death" is used to mean a process or event that
18 causes the cell to cease or diminish normal metabolism *in vivo* or *in vitro*.
19 The various forms and signs of cell death are obvious to those skilled in
20 the art, but examples of cell death include, but are not limited to,
21 programmed cell death (i.e., apoptosis), gradual death of the cells as
22 occurs in diseased states (i.e., necrosis), and more immediate cell death
23 such as acute toxicity. The inhibition of cell death for which the current
24 invention provides can be a complete or partial inhibition of cell death.
25 Likewise, the inhibition of cell death for which the current invention
26 provides can be a complete or partial reversal of the process of cell death.

27 As used herein, neuronal cells include cells of the central and
28 peripheral nervous systems, including the two classes of neurons and glial
29 cells. Within each class of cells, there are unique types of cells that one

1 of ordinary skill in the art would recognize such as oligodendrocytes,
2 astrocytes and Schwann cells belonging to the glial cell class.

3 As a preferred embodiment, the method of inhibiting
4 neuronal cell death of the current invention can be accomplished by
5 treating the cell with an agent that is selected from the group consisting
6 of a glutathione precursor, an isothiocyanate and a glucosinolate. More
7 preferably, the isothiocyanate is selected from the group consisting of
8 sulforaphane and a sulforaphane analog.

9 Preferably, the sulforaphane analogs to be used for
10 preventing neuronal cell death are selected from the group consisting of
11 6-isothiocyanato-2-hexanone, exo-2-acetyl-6-isothiocyanatonorbornane,
12 exo-2-isothiocyanato-6-methylsulfonylnorbornane, 6-isothiocyanato-2-
13 hexanol, 1-isothiocyanato-4-dimethylphosphonylbutane, exo-2-(1'-
14 hydroxyethyl)-5-isothiocyanatonorbornane, exo-2-acetyl-5-
15 isothiocyanatonorbornane, 1-isothiocyanato-5-methylsulfonylpentane, cis-
16 3-(methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
17 (methylsulfonyl)cyclohexylmethylisothiocyanate.

18 Other compounds may be used to prevent neuronal cell
19 death by increasing the levels of glutathione or Phase II enzymes. In
20 another preferred embodiment, those compounds are selected from the
21 group consisting of resveratrol, oltipraz, dimethylfumarate, 2(3)-tert-butyl-
22 4-hydroxyanisole, 3,5-di-tert-butyl-4-hydroxytoluene and an analog
23 thereof.

24 The Phase II enzymes that are to be elevated by the methods
25 of the current invention to prevent neuronal cell death have been
26 previously described herein.

27 The current invention also provides a method of identifying
28 agents that modulate intracellular levels of glutathione or intracellular
29 levels of at least one Phase II enzyme in neuronal cells comprising (a)
30 treating said neuronal cells with an agent, and assaying for intracellular

1 levels of glutathione or intracellular levels of at least one Phase II
2 detoxification enzyme; (b) treating said neuronal cells with a known
3 modulator of intracellular levels of glutathione or intracellular levels of at
4 least one Phase II detoxification enzyme (control substance), and assaying
5 for intracellular levels of glutathione or intracellular levels of at least one
6 Phase II detoxification enzyme and (c) comparing the levels of said
7 intracellular glutathione or intracellular levels of at least one Phase II
8 detoxification enzyme in (a) and (b) to determine if said agent modulates
9 intracellular levels of glutathione or intracellular levels of at least one
10 Phase II detoxification enzyme.

11 In one embodiment of the current invention, the method of
12 identifying an agent that modulates intracellular levels of glutathione or
13 Phase II enzymes in neuronal cells is performed on a single population of
14 cells, and (b) is performed on the identical population after the agent in (a)
15 is removed. In another embodiment of the invention, the method of
16 identifying an agent that modulates intracellular levels of glutathione or
17 Phase II enzymes in cells is performed on two nearly identical populations
18 of cells, under the same culture conditions, where (a) is performed on one
19 population and (b) is performed on another population, and (c) is a
20 comparison of the levels of intracellular levels of glutathione or Phase II
21 enzymes between the two populations of cells.

22 As used herein, assaying for intracellular levels of glutathione
23 or Phase II enzymes can be accomplished by using any means that can
24 detect differences in intracellular or extracellular intracellular levels of
25 glutathione or Phase II enzymes. Such means, which should be obvious
26 to one of ordinary skill on the art.

27 As used herein, modulation of intracellular glutathione or any
28 intracellular Phase II enzyme of the affected cell includes such responses
29 as increasing or decreasing the levels of glutathione or any Phase II
30 enzyme that are normally up-regulated or down-regulated in the cell, in

1 response to another stimulus. Likewise, modulation of intracellular
2 glutathione or any intracellular Phase II enzyme in the affected cell also
3 includes such responses as increasing or decreasing the levels of
4 glutathione or any Phase II enzyme that are normally present in the cell, in
5 the absence of another stimulus. Additionally, modulation of intracellular
6 glutathione or any intracellular Phase II enzyme is meant to encompass
7 increasing or decreasing the quantity of release or uptake of glutathione
8 or any Phase II enzyme from or to intracellular stores, such as
9 mitochondria, in conjunction with another stimulus. Likewise, modulation
10 of intracellular glutathione or any intracellular Phase II enzyme is also
11 meant to encompass increasing or decreasing the quantity of release or
12 uptake of glutathione or any Phase II enzyme from or to intracellular
13 stores, such as mitochondria, in the absence of another stimulus.
14 Furthermore, modulation of glutathione or any Phase II enzyme is meant
15 to include increased or decreased expression of the enzymes responsible
16 for the production of glutathione, including glutathione transferase and
17 glutamylcysteine synthetase, as well as increased or decreased
18 expression of any Phase II enzyme.

19 As used herein, the phrase "increased or decreased
20 expression" is used to mean an increase or decrease in the transcription
21 rates of the genes responsible for coding the enzymes, resulting in an
22 increase or decrease in the levels of mRNA for each gene, respectively.
23 The phrase is also used to mean an increase or decrease in the levels of
24 the protein or enzyme in the cell, independent of transcription rates. For
25 example, an increase in degradation rate of an mRNA encoding the protein
26 in question, without a change in the transcription rate, may result in a
27 decrease in the levels of protein in the cell.

28 In a preferred embodiment, the methods of identifying agents
29 that modulate intracellular levels of glutathione or Phase II enzymes are
30 performed with an agent known to increase the intracellular levels of

1 glutathione or intracellular levels of at least one Phase II detoxification
2 enzyme.

3 In a more preferred embodiment, the methods of identifying
4 the agents that modulate intracellular glutathione or Phase II enzymes are
5 accomplished by using the control substances isothiocyanate, a
6 glutathione precursor and a glucosinolate.

7 In a still more preferred embodiment, the methods of
8 identifying agents that modulate intracellular glutathione or Phase II
9 enzymes are accomplished by using control substances sulforaphane and
10 a sulforaphane analog.

11 In a preferred embodiment, the methods of identifying agents
12 that modulate intracellular levels of glutathione or Phase II enzymes are
13 performed with an agent known to decrease the intracellular levels of
14 glutathione or intracellular levels of at least one Phase II detoxification
15 enzyme.

16 The present invention also provides a method of modulating
17 intracellular levels of at least one Phase II detoxification enzyme in
18 neuronal cells, said method comprising treating said neuronal cells with an
19 agent that binds to glutathione to form a complex, wherein said complex
20 modulates said intracellular levels of at least one Phase II detoxification
21 enzyme.

22 The current invention also provides for a composition for use
23 in the treatment of a degenerative disease, comprising a pharmaceutical
24 excipient and a pharmaceutically effective amount of an agent that
25 increases intracellular levels of glutathione or at least one Phase II
26 detoxification enzyme.

27 In a preferred embodiment, the composition of the current
28 invention comprises an agent selected from the group consisting of a
29 glutathione precursor, an isothiocyanate and a glucosinolate. In a more

1 preferred embodiment, the composition of the current invention comprises
2 sulforaphane or a sulforaphane analog.

3 In another preferred embodiment, the composition of the
4 current invention comprises an agent selected from the group consisting
5 of 6-isothiocyanato-2-hexanone, exo-2-acetyl-6-
6 isothiocyanatonorbornane, exo-2-isothiocyanato-6-
7 methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-
8 4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
9 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
10 isothiocyanato-5-methylsulfonylpentane, cis-3-
11 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
12 (methylsulfonyl)cyclohexylmethylisothiocyanate.

13 In still another preferred embodiment, the composition of the
14 current invention comprises an agent selected from the group consisting
15 of resveratrol, oltipraz, dimethylfumarate, 2(3)-tert-butyl-4-
16 hydroxyanisole, 3,5-di-tert-butyl-4-hydroxytoluene and an analog thereof.

17 In a preferred embodiment, the composition of the current
18 invention that is used to treat a degenerative disease is used to treat
19 diabetes, chronic liver failure, Wilson's disease, congestive heart failure,
20 atherosclerosis, and a neurological disorder.

21 In a more preferred embodiment, the composition of current
22 invention is used to treat Alzheimer's Disease, Parkinson's Disease,
23 Huntington's Disease, amyotrophic lateral sclerosis, epilepsy, myasthenia
24 gravis, neuropathy, ataxia, dementia, chronic axonal neuropathy and
25 stroke.

26 In another preferred embodiment, the composition of the
27 current invention, which is used to treat degenerative diseases by
28 increasing the levels of glutathione or any Phase II enzyme, is used to
29 increase the enzymes selected from the group consisting of UDP-
30 glucuronosyltransferases, sulfotransferases, phenol-O-methyltransferase,

1 catechol-O-methyltransferase, histamine N-methyltransferase,
2 nicotinamide N-methyltransferase, thiopurine methyltransferase, thiol
3 methyltransferase, N-acetyltransferases, acyl-CoA synthetases, acyl-
4 CoA:amino acid N-acyltransferases, aminoacyl-tRNA synthetases,
5 glutathione synthetases, gamma glutamylcysteine synthetases,
6 glutathione S-transferases, quinone reductases, heme oxygenases and
7 rhodanases.

8 Formulations and Methods of Administration

9 A pharmaceutical composition of the invention is provided
10 comprising an agent of the invention useful for treatment of a neurological
11 disorder and a pharmaceutically acceptable carrier or excipient.

12 It will be desirable or necessary to introduce the
13 pharmaceutical compositions directly or indirectly to the brain. Direct
14 techniques usually involve placement of a drug delivery catheter into the
15 host's ventricular system to bypass the blood-brain barrier. Indirect
16 techniques, which are generally preferred, involve formulating the
17 compositions to provide for drug latentiation by the conversion of
18 hydrophilic drugs into lipid-soluble drugs. Latentiation is generally
19 achieved through blocking of the hydroxyl, carboxyl, and primary amine
20 groups present on the drug to render the drug more lipid-soluble and
21 amenable to transportation across the blood-brain barrier. Alternatively,
22 the delivery of hydrophilic drugs can be enhanced by intra-arterial infusion
23 of hypertonic solutions which can transiently open the blood-brain barrier.

24 The blood-brain barrier (BBB) is a single layer of brain
25 capillary endothelial cells that are bound together by tight junctions. The
26 BBB excludes entry of many blood-borne molecules. In the invention, the
27 agent can be modified for improved penetration of the blood-brain barrier
28 using methods known in the art. Alternatively, a compound that
29 increases permeability of the BBB can be administered to the subject.

1 RMP-7, a synthetic peptidergic bradykinin agonist was reported to
2 increase the permeability of the blood-brain barrier by opening the tight
3 junctions between the endothelial cells of brain capillaries (Elliott, P.J. *et*
4 *al.*, *Exptl. Neurol.* 141:214-224 (1996)).

5 The invention further contemplates the use of prodrugs
6 which are converted *in vivo* to the therapeutic compounds of the
7 invention (Silverman, R.B., "The Organic Chemistry of Drug Design and
8 Drug Action," Academic Press, Ch. 8 (1992)). Such prodrugs can be
9 used to alter the biodistribution (e.g., to allow compounds which would
10 not typically cross the blood-brain barrier to cross the blood-brain barrier)
11 or the pharmacokinetics of the therapeutic compound. For example, an
12 anionic group, e.g., a sulfate or sulfonate, can be esterified, e.g., with a
13 methyl group or a phenyl group, to yield a sulfate or sulfonate ester.
14 When the sulfate or sulfonate ester is administered to a subject, the ester
15 is cleaved, enzymatically or non-enzymatically, to reveal the anionic
16 group. Such an ester can be cyclic, e.g., a cyclic sulfate or sultone, or
17 two or more anionic moieties can be esterified through a linking group.
18 An anionic group can be esterified with moieties (e.g., acyloxymethyl
19 esters) which are cleaved to reveal an intermediate compound which
20 subsequently decomposes to yield the active compound. Furthermore, an
21 anionic moiety can be esterified to a group which is actively transported
22 *in vivo*, or which is selectively taken up by target organs. The ester can
23 be selected to allow specific targeting of the therapeutic moieties to
24 particular organs, as described below for carrier moieties.

25 The therapeutic compounds or agents of the invention can
26 be formulated to cross the blood-brain-barrier, for example, in liposomes.
27 For methods of manufacturing liposomes, see, e.g., U.S. Pat. Nos.
28 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise
29 one or more moieties which are selectively transported into specific cells
30 or organs thus providing targeted drug delivery (Ranade, J., *Clin.*

1 *Pharmacol.* 29:685 (1989)). Exemplary targeting moieties include folate
2 or biotin (U.S. Pat. No. 5,416,016), mannosides (Umezawa *et al.*,
3 *Biochem. Biophys. Res. Comm.* 153:1038 (1988)), antibodies (Bloeman
4 *et al.*, *FEBS Lett.* 357:140 (1995); Owais *et al.*, *Antimicrob. Agents*
5 *Chemother.* 39:180 (1995)), surfactant protein A receptor (Briscoe *et al.*,
6 *Am. J. Physiol.* 1233:134 (1995)), gp 120 (Schreier *et al.*, *J. Biol. Chem.*
7 269:9090 (1994); Killion and Fidler, *Immunomethods* 4:273 (1994)).

8 The pharmaceutical composition can be administered orally,
9 nasally, parenterally, intrasystemically, intraperitoneally, topically (as by
10 drops or transdermal patch), buccally, or as an oral or nasal spray. By
11 "pharmaceutically acceptable carrier" is intended, but not limited to, a
12 non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or
13 formulation auxiliary of any type. The term "parenteral" as used herein
14 refers to modes of administration which include intravenous,
15 intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular
16 injection and infusion.

17 A pharmaceutical composition of the present invention for
18 parenteral injection can comprise pharmaceutically acceptable sterile
19 aqueous or nonaqueous solutions, dispersions, suspensions or emulsions
20 as well as sterile powders for reconstitution into sterile injectable
21 solutions or dispersions just prior to use. Examples of suitable aqueous
22 and nonaqueous carriers, diluents, solvents or vehicles include water,
23 ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol,
24 and the like), carboxymethylcellulose and suitable mixtures thereof,
25 vegetable oils (such as olive oil), and injectable organic esters such as
26 ethyl oleate. Proper fluidity can be maintained, for example, by the use of
27 coating materials such as lecithin, by the maintenance of the required
28 particle size in the case of dispersions, and by the use of surfactants.

29 The compositions of the present invention can also contain
30 adjuvants such as, but not limited to, preservatives, wetting agents,

1 emulsifying agents, and dispersing agents. Prevention of the action of
2 microorganisms can be ensured by the inclusion of various antibacterial
3 and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic
4 acid, and the like. It can also be desirable to include isotonic agents such
5 as sugars, sodium chloride, and the like. Prolonged absorption of the
6 injectable pharmaceutical form can be brought about by the inclusion of
7 agents which delay absorption such as aluminum monostearate and
8 gelatin.

9 In some cases, in order to prolong the effect of the drugs, it
10 is desirable to slow the absorption from subcutaneous or intramuscular
11 injection. This can be accomplished by the use of a liquid suspension of
12 crystalline or amorphous material with poor water solubility. The rate of
13 absorption of the drug then depends upon its rate of dissolution which, in
14 turn, can depend upon crystal size and crystalline form. Alternatively,
15 delayed absorption of a parenterally administered drug form is
16 accomplished by dissolving or suspending the drug in an oil vehicle.

17 Injectable depot forms are made by forming microencapsule
18 matrices of the drug in biodegradable polymers such as polylactide-
19 polyglycolide. Depending upon the ratio of drug to polymer and the
20 nature of the particular polymer employed, the rate of drug release can be
21 controlled. Examples of other biodegradable polymers include
22 poly(orthoesters) and poly(anhydrides). Depot injectable formulations are
23 also prepared by entrapping the drug in liposomes or microemulsions
24 which are compatible with body tissues.

25 The injectable formulations can be sterilized, for example, by
26 filtration through a bacterial-retaining filter, or by incorporating sterilizing
27 agents in the form of sterile solid compositions which can be dissolved or
28 dispersed in sterile water or other sterile injectable medium just prior to
29 use.

1 Solid dosage forms for oral administration include, but are
2 not limited to, capsules, tablets, pills, powders, and granules. In such
3 solid dosage forms, the active compounds are mixed with at least one
4 item pharmaceutically acceptable excipient or carrier such as sodium
5 citrate or dicalcium phosphate and/or a) fillers or extenders such as
6 starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders
7 such as, for example, carboxymethylcellulose, alginates, gelatin,
8 polyvinylpyrrolidone, sucrose, and acacia, c) humectants such as glycerol,
9 d) disintegrating agents such as agar-agar, calcium carbonate, potato or
10 tapioca starch, alginic acid, certain silicates, and sodium carbonate, e)
11 solution retarding agents such as paraffin, f) absorption accelerators such
12 as quaternary ammonium compounds, g) wetting agents such as, for
13 example, acetyl alcohol and glycerol monostearate, h) absorbents such as
14 kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate,
15 magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and
16 mixtures thereof. In the case of capsules, tablets and pills, the dosage
17 form can also comprise buffering agents.

18 Solid compositions of a similar type can also be employed as
19 fillers in soft and hardfilled gelatin capsules using such excipients as
20 lactose or milk sugar as well as high molecular weight polyethylene
21 glycols and the like.

22 The solid dosage forms of tablets, dragees, capsules, pills,
23 and granules can be prepared with coatings and shells such as enteric
24 coatings and other coatings well known in the pharmaceutical formulating
25 art. They can optionally contain opacifying agents and can also be of a
26 composition that they release the active ingredient(s) only, or
27 preferentially, in a certain part of the intestinal tract, optionally, in a
28 delayed manner. Examples of embedding compositions which can be
29 used include polymeric substances and waxes.

1 The active compounds can also be in micro-encapsulated
2 form, if appropriate, with one or more of the above-mentioned excipients.

3 Liquid dosage forms for oral administration include, but are
4 not limited to, pharmaceutically acceptable emulsions, solutions,
5 suspensions, syrups and elixirs. In addition to the active compounds, the
6 liquid dosage forms can contain inert diluents commonly used in the art
7 such as, for example, water or other solvents, solubilizing agents and
8 emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl
9 acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene
10 glycol, dimethyl formamide, oils (in particular, cottonseed, groundnut,
11 corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl
12 alcohol, polyethylene glycols and fatty acid esters of sorbitan, and
13 mixtures thereof.

14 Besides inert diluents, the oral compositions can also include
15 adjuvants such as wetting agents, emulsifying and suspending agents,
16 sweetening, flavoring, and perfuming agents.

17 Suspensions, in addition to the active compounds, can
18 contain suspending agents as, for example, ethoxylated isostearyl
19 alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline
20 cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth,
21 and mixtures thereof.

22 Topical administration includes administration to the skin or
23 mucosa, including surfaces of the lung and eye. Compositions for topical
24 administration, including those for inhalation, can be prepared as a dry
25 powder which can be pressurized or non-pressurized. In nonpressurized
26 powder compositions, the active ingredients in finely divided form can be
27 used in admixture with a larger-sized pharmaceutically acceptable inert
28 carrier comprising particles having a size, for example, of up to 100 μm in
29 diameter. Suitable inert carriers include sugars such as lactose.

1 Desirably, at least 95% by weight of the particles of the active ingredient
2 have an effective particle size in the range of 0.01 to 10 μm .

3 Alternatively, the composition can be pressurized and contain
4 a compressed gas, such as nitrogen or a liquefied gas propellant. The
5 liquefied propellant medium and indeed the total composition is preferably
6 such that the active ingredients do not dissolve therein to any substantial
7 extent. The pressurized composition can also contain a surface active
8 agent. The surface active agent can be a liquid or solid non-ionic surface
9 active agent or can be a solid anionic surface active agent. It is preferred
10 to use the solid anionic surface active agent in the form of a sodium salt.

11 The compositions of the present invention can also be
12 administered in the form of liposomes. As is known in the art, liposomes
13 are generally derived from phospholipids or other lipid substances.
14 Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals
15 that are dispersed in an aqueous medium. Any non-toxic, physiologically
16 acceptable and metabolizable lipid capable of forming liposomes can be
17 used. The present compositions in liposome form can contain, in addition
18 to the compounds of the invention, stabilizers, preservatives, excipients,
19 and the like. The preferred lipids are the phospholipids and the
20 phosphatidyl cholines (lecithins), both natural and synthetic. Methods to
21 form liposomes are known in the art (*see, for example, Prescott, Ed.,*
22 *Meth. Cell Biol. 14:33 et seq (1976)*).

23 Dosaging

24 One of ordinary skill will appreciate that effective amounts of
25 the agents of the invention can be determined empirically and can be
26 employed in pure form or, where such forms exist, in pharmaceutically
27 acceptable salt, ester or prodrug form. The agents can be administered to
28 a subject, in need of treatment of a neurological disorder, as
29 pharmaceutical compositions in combination with one or more

1 pharmaceutically acceptable excipients. It will be understood that, when
2 administered to a human patient, the total daily usage of the agents or
3 composition of the present invention will be decided by the attending
4 physician within the scope of sound medical judgement. The specific
5 therapeutically effective dose level for any particular patient will depend
6 upon a variety of factors: the type and degree of the cellular response to
7 be achieved; activity of the specific agent or composition employed; the
8 specific agents or composition employed; the age, body weight, general
9 health, sex and diet of the patient; the time of administration, route of
10 administration, and rate of excretion of the agent; the duration of the
11 treatment; drugs used in combination or coincidental with the specific
12 agent; and like factors well known in the medical arts. For example, it is
13 well within the skill of the art to start doses of the agents at levels lower
14 than those required to achieve the desired therapeutic effect and to
15 gradually increase the dosages until the desired effect is achieved.

16 For example, satisfactory results are obtained by oral
17 administration of the compounds at dosages on the order of from 0.05 to
18 10 mg/kg/day, preferably 0.1 to 7.5 mg/kg/day, more preferably 0.1 to 2
19 mg/kg/day, administered once or, in divided doses, 2 to 4 times per day.
20 On administration parenterally, for example by i.v. drip or infusion,
21 dosages on the order of from 0.01 to 5 mg/kg/day, preferably 0.05 to 1.0
22 mg/kg/day and more preferably 0.1 to 1.0 mg/kg/day can be used.
23 Suitable daily dosages for patients are thus on the order of from 2.5 to
24 500 mg p.o., preferably 5 to 250 mg p.o., more preferably 5 to 100 mg
25 p.o., or on the order of from 0.5 to 250 mg i.v., preferably 2.5 to 125 mg
26 i.v. and more preferably 2.5 to 50 mg i.v.

27 Dosaging can also be arranged in a patient specific manner
28 to provide a predetermined concentration of the agents in the blood, as
29 determined by techniques accepted and routine in the art (HPLC is
30 preferred). Thus patient dosaging can be adjusted to achieve regular on-

1 going blood levels, as measured by HPLC, on the order of from 50 to
2 1000 ng/ml, preferably 150 to 500 ng/ml.

3 It will be readily apparent to one of ordinary skill in the
4 relevant arts that other suitable modifications and adaptations to the
5 methods and applications described herein can be made without departing
6 from the scope of the invention or any embodiment thereof.

7 The following Examples serves only to illustrate the
8 invention, and are not to be construed as in any way to limit the
9 invention.

10 Examples

11 Chronic excitotoxicity is thought to contribute to the
12 characteristic motor neuron degeneration observed in amyotrophic lateral
13 sclerosis (ALS). Organotypic spinal cord cultures defective in glutamate
14 transport have been previously used to model chronic excitotoxicity in
15 vitro to produce slow motor neuron degeneration. This system has proven
16 to be effective as a preclinical screen for evaluating potential
17 neuroprotective agents such as riluzole, insulin growth factor (IGF-1) and
18 gabapentin, which were all shown to be protective, whereas ciliary
19 neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF)
20 both failed to protect against chronic motor neuron degeneration.

21

22 **Example 1: Sulforaphane Protects Motor Neurons from Chronic Glutamate** 23 **Excitotoxicity**

24 The general experimental procedure was previously described
25 (Ho T et al., Experimental Neurology, 161, 664-675, 2000), the contents
26 of which are incorporated herein by reference. Rodent spinal cord tissue
27 slices were treated with sulforaphane at 0, 0.5 μ M, 5 μ M and 10 μ M (for
28 3 weeks, changing medium every three days). At the end of the

1 experiment, each tissue slice was fixed and stained, and remaining
2 neurons were counted.

3 Sulforaphane significantly prevented THA-induced motor
4 neuron death in the spinal cord explant (Figure 1).

5

6 **Example 2: Sulforaphane Elevates Spinal Cord Glutathione Levels**

7 Based on the above results, the level of GSH in spinal cord
8 tissue treated with sulforaphane was investigated. Rat spinal cord slices
9 were similarly prepared and cultured as in the above example but without
10 THA treatment. The tissues were cultured for one or two weeks and then
11 incubated with 1 μ M, 5 μ M, or 10 μ M, of sulphoraphane for 12 h, 24 h or
12 48 h. The tissue slices were then harvested and sonicated and assayed
13 for total GSH content (Richie JP et al., Clinical Chemistry, 42, 64-70,
14 1996, the contents of which are incorporated herein by reference). GSH
15 level in the sulforaphane-treated tissues was raised as much as 160%
16 (Figure2).

17 These experiments indicate that sulforaphane is protective of
18 neuronal cells and will be effective in ameliorating various neurological
19 disorders.

20

21

22 **Example 3: The effects of sulforaphane on Phase II enzyme** 23 **activity in spinal cord cultures**

24 Mouse spinal cord cultures were prepared and cultured as
25 above. Cultures were treated with sulforaphane for 24 hours at
26 concentrations of 0,1 μ m, 5 μ m, 10 μ m and 20 μ m. After 24 hours the
27 tissues were harvested and homogenized, and glutathione transferase
28 activity was measured by the protocol as set forth in Richie, J.P. et al.,
29 Clinical Chemistry, (1996) 42:64-70 and quinone reductase activity was

1 measured by the protocol as set forth in Zhang, et al., Proc. Natl. Acad.
2 Sci. USA 89:2399-2403 (1992).

3

4

5 **Example 4: The effects of sulforaphane on Phase II enzyme**
6 **activity in spinal cord cultures**

7 Mouse spinal cord cultures were prepared and cultured as
8 above. Cultures were treated with sulforaphane for 2 weeks, twice a
9 week, at concentrations of 1 μ m, 5 μ m and 10 μ m. After 2 weeks the
10 tissues were harvested and homogenized, and glutathione transferase
11 activity was measured by the protocol as set forth in Richie, J.P. et al.,
12 Clinical Chemistry, (1996) 42:64-70 and quinone reductase activity was
13 measured by the protocol as set forth in Zhang, et al., Proc. Natl. Acad.
14 Sci. USA 89:2399-2403 (1992).

15

16

17 **Example 5: The effect of sulforaphane in transgenic models**
18 **of Alzheimer's Disease.**

19 **Method:** Transgenic animals will be divided into four groups
20 of 10 animals per group. The first group will receive 15 μ m of
21 sulforaphane orally every day. The second group will receive 15 μ m of
22 sulforaphane and N-acetylcysteine orally every day. The third group will
23 receive N-acetylcysteine alone and a fourth group will receive vehicle
24 control (CMC). Groups receiving N-acetylcysteine are required in this
25 study because sulforaphane may transiently deplete intracellular
26 glutathione levels before the induction of Phase II enzymes. Addition of N-
27 acetylcysteine will boost the precursor for glutathione during this
28 transitional induction period. Animals will begin treatment at weaning, and
29 continue oral daily gavage of drug for a period of 8 to 10 months.
30 Animals will be monitored with weekly weights. If selected transgenic

1 animals have reliable behavior changes, complete behavioral testing will
2 follow completion of the protocol. Animals will then be sacrificed and
3 perfused. The brains will be harvested and amyloid deposits detected
4 using immunohistochemistry and/or silver staining. Plaque burden in brain
5 will be quantified using non-biased stereological methods. Brain GSH
6 levels will also be determined.

7 **Histopathology:** One hemisphere of brains of a subset of
8 mice ($n = 27$; young, $n = 6$; middle-aged, $n = 14$; old, $n = 7$) will be
9 drop-fixed in formalin. Tissue will be mounted coronally and 30- μ m
10 sections collected using a cryostat. Every eighth section will be stored
11 throughout the extent of the brain in antifreeze solution (30%
12 glycerol/30% ethylene glycol in 40 mM NaPO₄) at -20 °C before
13 immunostaining. Sections will be incubated with 3D6-biotinylated
14 antibody overnight at 4°C and then reacted with the horseradish
15 peroxidase–avidin–biotin complex (Vector Laboratories) before
16 development using 3,3'-diaminobenzidine as the chromagen. Amyloid
17 deposition will be quantified automatically using an Image-Pro image
18 analysis system. Video images of the brain will be captured, and outlines
19 around the area of the hippocampus or cortex will be made to obtain a
20 threshold optical density to discriminate staining from background. The
21 amyloid 'plaque burden' is defined as the average percentage of
22 hippocampal or cortical area covered by amyloid depositions over six
23 sections.

24 **ELISA:** Cerebral A β levels will be assayed from formic-acid-
25 extracted hemi-brain sucrose homogenates using an ELISA method in
26 which A β is trapped with either monoclonal antibody to A β ₄₀
27 (JRF/cAb40/10) or A β ₄₂ (JRF/cAb42/26) and then detected with
28 horseradish peroxidase (HRP)-conjugated JRF/Abtot/17. The dilution of
29 JRF/Abtot/17 and samples will be optimized to detect A β in the range of
30 50 to 800 fmol ml⁻¹. ELISA signals will be reported as the mean s.e.m. of

1 four replica wells in fmol A β per mg total protein (determined with the
2 BioRad DC protein assay), based on standard curves using synthetic A β ₁₋₄₀
3 and A β ₁₋₄₂ peptide standards (American Peptide Co. Sunnyvale, CA).
4 Cerebral β APP levels will be analyzed in supernatant of brain as described.

5 **Behavioral tests and data analysis:** The cognitive status of
6 the animals will be evaluated via a battery of tests including the modified
7 Morris water maze or radial-arm water maze test, object recognition and
8 Y-maze performance. The details of the behavioral tests will roughly
9 follow those referenced. Animals will be tested prior to treatment as
10 defined by each protocol, during treatment and just prior to termination of
11 the mice.

12
13 **Example 6: BBB permeability and bioavailability of Sulforaphane**
14 **compounds.**

15 Blood-brain barrier permeability of sulforaphane has been
16 determined. Briefly, animals were dosed via daily oral gavage of the test
17 or control compounds. After one week, the animals' whole brain were
18 snap frozen and homogenized, and GSH levels were subsequently
19 measured according to the protocol of Zhang, et al., Proc. Natl. Acad.
20 Sci. USA 89:2399-2403 (1992). Figure 5 shows the results of these
21 experiments, demonstrating that sulforaphane was able to cross the BBB
22 and elevate GSH levels in the brain.

23 To test for oral bio-availability, animals will receive a daily
24 oral gavage of sulforaphane or control compounds. Blood will be collected
25 and then mice will be euthanized at set intervals (1 weeks, 2 weeks, 1
26 month, 2 months, 6 months) after treatment begins. Brain, liver, heart
27 tissues will also be harvested and analyzed for elevation of Phase II
28 enzymes and GSH levels.

29

30

Example 7: The Effect of sulforaphane in transgenic models of amyotrophic lateral sclerosis (ALS):

SOD-G93 mice are transgenic mice that carry the same genetic mutation as the familial ALS patients. These mice are normal at birth but slowly develop motor neuron degeneration over time. By approximately 160 days, they will have developed severe paralysis because of the progressive loss of the motor neurons.

Method: Transgenic animals will be divided into four groups of 10 animals per group. The first group will receive 15 μ m of sulforaphane orally every day. The second group will receive 15 μ m of sulforaphane and N-acetylcysteine orally every day. The third group will receive N-acetylcysteine alone, and a fourth group will receive vehicle control (CMC). Groups receiving N-acetylcysteine are required in this study because sulforaphane may transiently deplete intracellular glutathione levels before the induction of Phase II enzymes. Addition of N-acetylcysteine will boost the precursor for glutathione during this transitional induction period. Animals will begin treatment at weaning, and continue oral daily gavage of the drugs. Onset of disease will be monitored by alteration on rotorod performance. Animals will be studied twice weekly starting at 1 month of age. A surrogate survival measure will be determined by the ability of the animals to right themselves after being placed on the cage on their backs. If they are unable to right themselves after 30 seconds, they will be considered dead. The survival and rotorod performance of each group of the animal will be compared. It is expected that sulforaphane or sulforaphane and N-acetylcysteine will improve the survival of the animals compared to control. The brains will be harvested and brain GSH levels will also be determined.

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WHAT IS CLAIMED IS:

- 1 1. A method of treating a subject in need of treatment of a
2 neurodegenerative disease, said method comprising administering to said
3 subject a pharmaceutically effective amount of a compound that elevates
4 intracellular levels of glutathione or intracellular levels of at least one
5 Phase II detoxification enzyme in diseased tissue of said subject.
- 1 2. The method of claim 1, wherein said compound is selected
2 from the group consisting of a glutathione precursor, an isothiocyanate
3 and a glucosinolate.
- 1 3. The method of claim 2, wherein said isothiocyanate is
2 selected from the group consisting of sulforaphane and a sulforaphane
3 analog.
- 1 4. The method of claim 3, wherein said sulforaphane analog is
2 selected from the group consisting of 6-isothiocyanato-2-hexanone, exo-
3 2-acetyl-6-isothiocyanatonorbornane, exo-2-isothiocyanato-6-
4 methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-
5 4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
6 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
7 isothiocyanato-5-methylsulfonylpentane, cis-3-
8 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
9 (methylsulfonyl)cyclohexylmethylisothiocyanate.
- 1 5. The method of claim 1, wherein said compound is selected
2 from the group consisting of resveratrol, oltipraz, dimethylfumarate, 2(3)-
3 tert-butyl-4-hydroxyanisole, 3,5-di-tert-butyl-4-hydroxytoluene and an
4 analog thereof.

1 6. The method of claim 1, wherein said neurological disorder is
2 selected from the group consisting Alzheimer's Disease, Parkinson's
3 Disease, Huntington's Disease, amyotrophic lateral sclerosis, epilepsy,
4 myasthenia gravis, neuropathy, ataxia, dementia, chronic axonal
5 neuropathy and stroke.

1 7. The method of claim 1, wherein said Phase II detoxification
2 enzyme is selected from the group consisting of UDP-
3 glucuronosyltransferases, sulfotransferases, phenol-O-methyltransferase,
4 catechol-O-methyltransferase, histamine N-methyltransferase,
5 nicotinamide N-methyltransferase, thiopurine methyltransferase, thiol
6 methyltransferase, N-acetyltransferases, O-acetyltransferases, acyl-CoA
7 synthetases, acyl-CoA:amino acid N-acyltransferases, aminoacyl-tRNA
8 synthetases, glutathione synthetases, gamma glutamylcysteine
9 synthetases, glutathione S-transferases, quinone reductases, heme
10 oxygenases, rhodanases, glutathione reductase, glutathione peroxidase,
11 catalase and superoxide dismutase.

1 8. A method of inhibiting cell death in neuronal cells comprising
2 treating said neuronal cells with an agent that increases intracellular levels
3 of glutathione or intracellular levels of at least one Phase II detoxification
4 enzyme.

1 9. The method of claim 8, wherein said agent is selected from
2 the group consisting of a glutathione precursor, an isothiocyanate and a
3 glucosinolate.

1 10. The method of claim 9, wherein said isothiocyanate is
2 selected from the group consisting of sulforaphane and a sulforaphane
3 analog.

1 11. The method of claim 10, wherein said sulforaphane analog is
2 selected from the group consisting of 6-isothiocyanato-2-hexanone, exo-
3 2-acetyl-6-isothiocyanatonorbornane, exo-2-isothiocyanato-6-
4 methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-
5 4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
6 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
7 isothiocyanato-5-methylsulfonylpentane, cis-3-
8 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
9 (methylsulfonyl)cyclohexylmethylisothiocyanate.

1 12. The method of claim 8, wherein said compound is selected
2 from the group consisting of resveratrol, oltipraz, dimethylfumarate, 2(3)-
3 tert-butyl-4-hydroxyanisole, 3,5-di-tert-butyl-4-hydroxytoluene and an
4 analog thereof.

1 13. The method of claim 8, wherein said Phase II detoxification
2 enzyme is selected from the group consisting of UDP-
3 glucuronosyltransferases, sulfotransferases, phenol-O-methyltransferase,
4 catechol-O-methyltransferase, histamine N-methyltransferase,
5 nicotinamide N-methyltransferase, thiopurine methyltransferase, thiol
6 methyltransferase, N-acetyltransferases, O-acetyltransferases, acyl-CoA
7 synthetases, acyl-CoA:amino acid N-acyltransferases, aminoacyl-tRNA
8 synthetases, glutathione synthetases, gamma glutamylcysteine
9 synthetases, glutathione S-transferases, quinone reductases, heme
10 oxygenases rhodanases, glutathione reductase, glutathione peroxidase,
11 catalase and superoxide dismutase.

1 14. A method of identifying agents that modulate intracellular
2 levels of glutathione or intracellular levels of at least one Phase II enzyme
3 in neuronal cells comprising

- 4 a. treating said neuronal cells with an agent, and
5 assaying for intracellular levels of glutathione or intracellular levels
6 of at least one Phase II detoxification enzyme;
- 7 b. treating said neuronal cells with a known modulator of
8 intracellular levels of glutathione or intracellular levels of at least
9 one Phase II detoxification enzyme, and assaying for intracellular
10 levels of glutathione or intracellular levels of at least one Phase II
11 detoxification enzyme;
- 12 c. comparing the levels of said intracellular glutathione or
13 intracellular levels of at least one Phase II detoxification enzyme in
14 (a) and (b) to determine if said agent modulates intracellular levels
15 of glutathione or intracellular levels of at least one Phase II
16 detoxification enzyme.

1 15. The method of claim 14, wherein (b) is performed on said
2 neuronal cells of (a) after said agent in (a) is removed.

1 16. The method of claim 14, wherein said known modulator of
2 intracellular levels of glutathione or intracellular levels of at least one
3 Phase II detoxification enzyme increases intracellular levels of glutathione
4 or intracellular levels of at least one Phase II detoxification enzyme.

1 17. The method of claim 14 wherein said known modulator of
2 intracellular levels of glutathione or intracellular levels of at least one
3 Phase II detoxification enzyme decreases intracellular levels of glutathione
4 or intracellular levels of at least one Phase II detoxification enzyme.

1 18. The method of claim 14, wherein said known modulator of
2 intracellular levels of glutathione or intracellular levels of at least one
3 Phase II detoxification enzyme is selected from the group consisting of an
4 isothiocyanate, a glutathione precursor and a glucosinolate.

1 19. The method of claim 18, wherein said isothiocyanate is
2 selected from the group consisting of sulforaphane and a sulforaphane
3 analog.

1 20. The method of claim 19, wherein said sulforaphane analog is
2 selected from the group consisting of 6-isothiocyanato-2-hexanone, exo-
3 2-acetyl-6-isothiocyanatonorbornane, exo-2-isothiocyanato-6-
4 methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-
5 4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
6 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
7 isothiocyanato-5-methylsulfonylpentane, cis-3-
8 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
9 (methylsulfonyl)cyclohexylmethylisothiocyanate.

1 21. A method of modulating intracellular levels of at least one
2 Phase II detoxification enzyme in neuronal cells, said method comprising
3 treating said neuronal cells with an agent that binds to glutathione to form
4 a complex, wherein said complex modulates said intracellular levels of at
5 least one Phase II detoxification enzyme.

1 22. The method of claim 21, wherein said agent is selected from
2 the group consisting of a glutathione precursor, an isothiocyanate and a
3 glucosinolate.

1 23. The method of claim 22, wherein said isothiocyanate is
2 selected from the group consisting of sulforaphane and a sulforaphane
3 analog.

1 24. The method of claim 23, wherein said sulforaphane analog is
2 selected from the group consisting of 6-isothiocyanato-2-hexanone, exo-
3 2-acetyl-6-isothiocyanatonorbornane, exo-2-isothiocyanato-6-

4 methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol, 1-isothiocyanato-
5 4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
6 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
7 isothiocyanato-5-methylsulfonylpentane, cis-3-
8 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
9 (methylsulfonyl)cyclohexylmethylisothiocyanate.

1 25. The method of claim 21, wherein said compound is selected
2 from the group consisting of resveratrol, oltipraz, dimethylfumarate, 2(3)-
3 tert-butyl-4-hydroxyanisole, 3,5-di-tert-butyl-4-hydroxytoluene and an
4 analog thereof.

1 26. The method of claim 21, wherein said Phase II detoxification
2 enzyme is selected from the group consisting of UDP-
3 glucuronosyltransferases, sulfotransferases, phenol-O-methyltransferase,
4 catechol-O-methyltransferase, histamine N-methyltransferase,
5 nicotinamide N-methyltransferase, thiopurine methyltransferase, thiol
6 methyltransferase, N-acetyltransferases, O-acetyltransferases, acyl-CoA
7 synthetases, acyl-CoA:amino acid N-acyltransferases, aminoacyl-tRNA
8 synthetases, glutathione synthetases, gamma glutamylcysteine
9 synthetases, glutathione S-transferases, quinone reductases, and heme
10 oxygenases rhodanases, glutathione reductase, glutathione peroxidase,
11 catalase and superoxide dismutase.

1 27. A composition for use in the treatment of a degenerative
2 disease, comprising a pharmaceutical excipient and a pharmaceutically
3 effective amount of an agent that increases intracellular levels of
4 glutathione or at least one Phase II detoxification enzyme.

1 28. The composition of claim 27, wherein said agent is selected
2 from the group consisting of a glutathione precursor, an isothiocyanate
3 and a glucosinolate.

1 29. The composition of claim 28, wherein said isothiocyanate is
2 selected from the group consisting of sulforaphane and a sulforaphane
3 analog.

1 30. The composition of claim 29, wherein said sulforaphane
2 analog is selected from the group consisting of 6-isothiocyanato-2-
3 hexanone, exo-2-acetyl-6-isothiocyanatonorbornane, exo-2-
4 isothiocyanato-6-methylsulfonylnorbornane, 6-isothiocyanato-2-hexanol,
5 1-isothiocyanato-4-dimethylphosphonylbutane, exo-2-(1'-hydroxyethyl)-5-
6 isothiocyanatonorbornane, exo-2-acetyl-5-isothiocyanatonorbornane, 1-
7 isothiocyanato-5-methylsulfonylpentane, cis-3-
8 (methylsulfonyl)cyclohexylmethylisothiocyanate and trans-3-
9 (methylsulfonyl)cyclohexylmethylisothiocyanate.

1 31. The composition of claim 27, wherein said agent is selected
2 from the group consisting of resveratrol, oltipraz, dimethylfumarate, 2(3)-
3 tert-butyl-4-hydroxyanisole, 3,5-di-tert-butyl-4-hydroxytoluene and an
4 analog thereof.

1 32. The composition of claim 27, wherein said neurological
2 disorder is selected from the group consisting of Alzheimer's Disease,
3 Parkinson's Disease, Huntington's Disease, amyotrophic lateral sclerosis,
4 epilepsy, myasthenia gravis, neuropathy, ataxia, dementia, chronic axonal
5 neuropathy and stroke.

1 33. The composition of claim 27 , wherein said Phase II
2 detoxification enzyme is selected from the group consisting of UDP-
3 glucuronosyltransferases, sulfotransferases, phenol-O-methyltransferase,
4 catechol-O-methyltransferase, histamine N-methyltransferase,
5 nicotinamide N-methyltransferase, thiopurine methyltransferase, thiol
6 methyltransferase, N-acetyltransferases, O-acetyltransferases, acyl-CoA

- 7 synthetases, acyl-CoA:amino acid N-acyltransferases, aminoacyl-tRNA
- 8 synthetases, glutathione synthetases, gamma glutamylcysteine
- 9 synthetases, glutathione S-transferases, quinone reductases, heme
- 10 oxygenases, rhodanases, glutathione reductase, glutathione peroxidase,
- 11 catalase and superoxide dismutase.

Figure 1

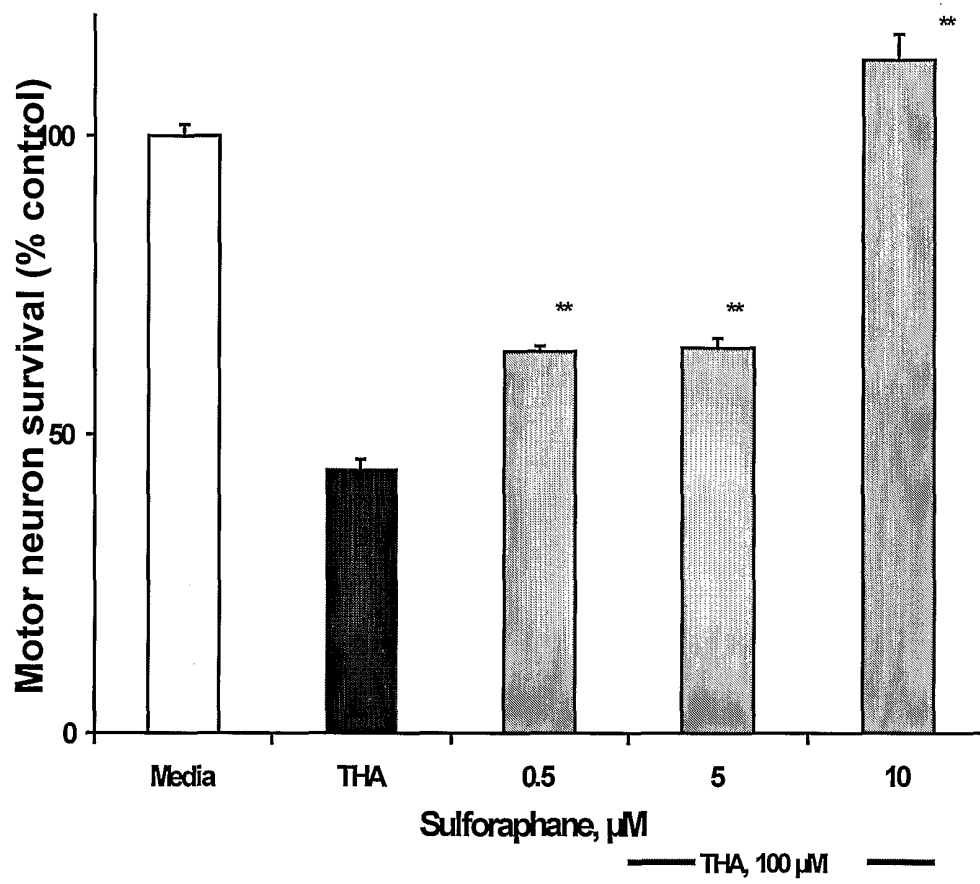
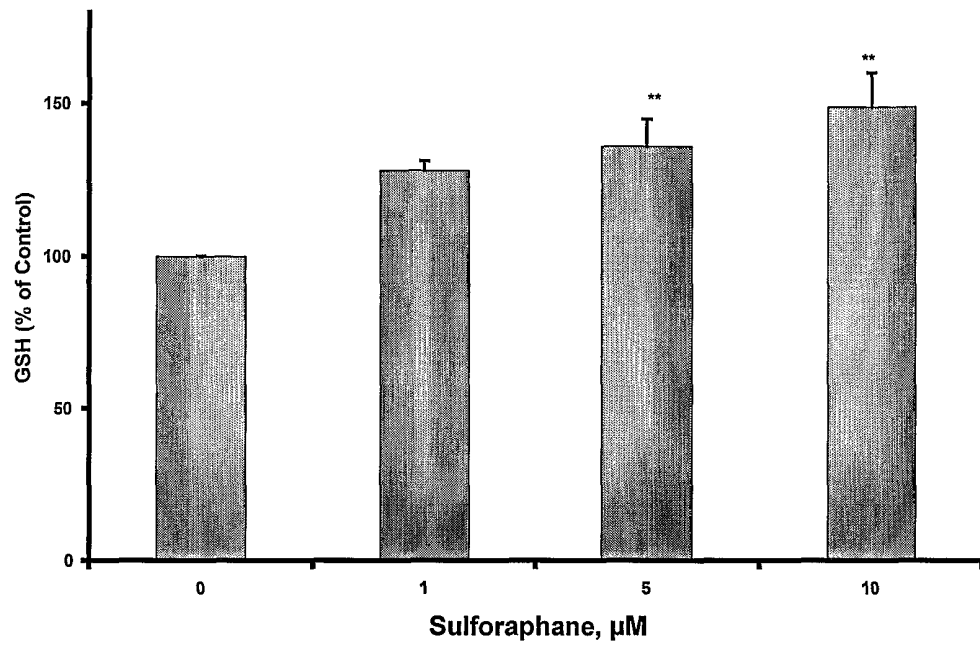


Figure 2



Mouse Spinal Cord Culture without THA

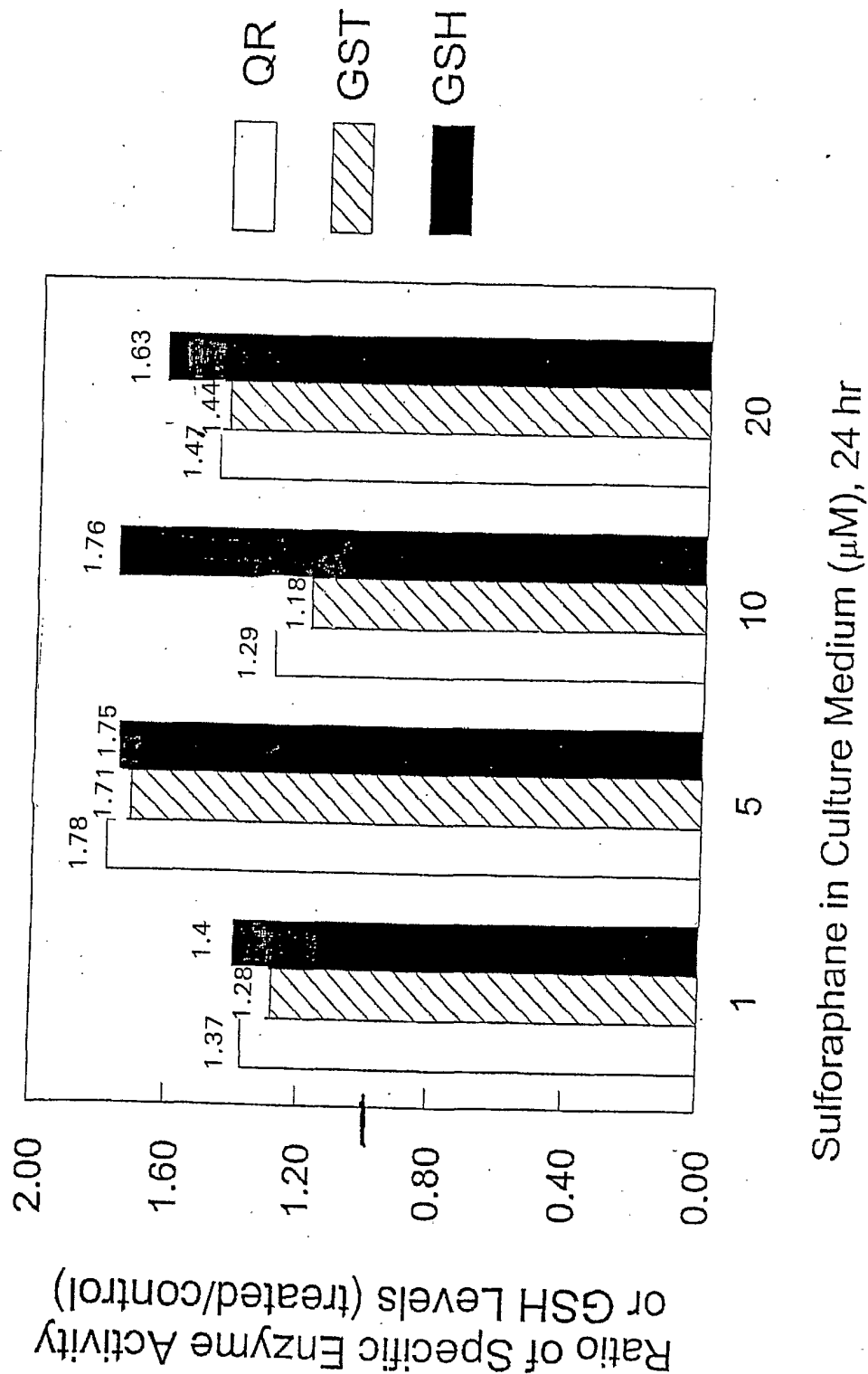


Figure 3

Mouse Spinal Cord Culture without THA

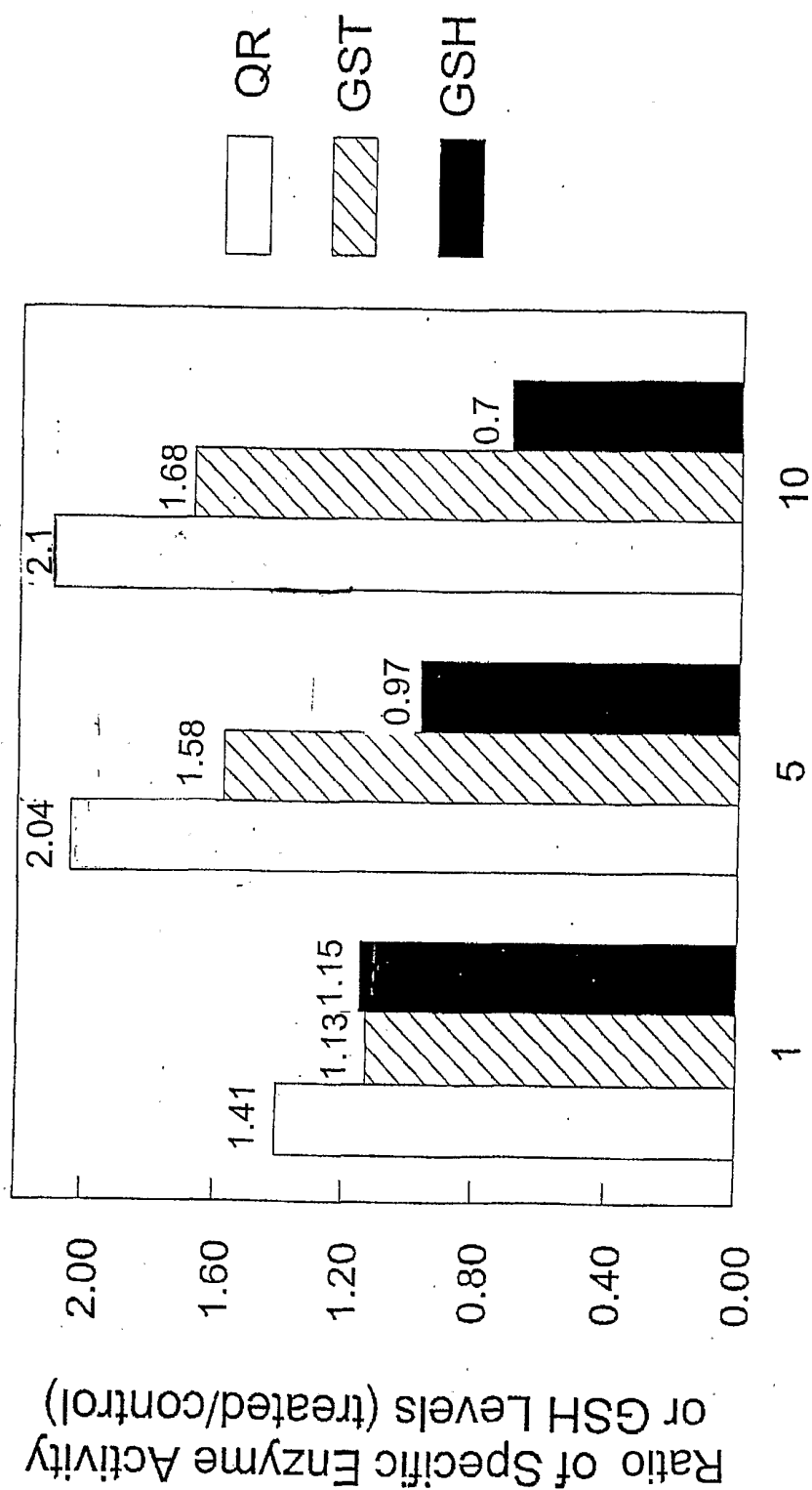


Figure 4

Sulforaphane in Culture Medium (μ M)
Twice a week (Tue. and Fri.) for two weeks

Figure 5

