Title: N-ACETYLCYSTEINE AMIDE (NAC AMIDE) FOR THE TREATMENT OF DISEASES AND CONDITIONS

Abstract: Methods and compositions comprising N-acetylcysteine amide (NAC amide) and derivatives thereof are used in treatments and prophylactic therapies for human and non-human mammalian diseases, disorders, conditions and pathologies associated with bomb blast or other high energy noise or impulse blasts. Pharmacologically or physiologically acceptable compositions of NAC amide or derivatives thereof are also provided, which may be administered alone, or in combination with other suitable agents.

N-Acetylcysteine amide (AD4)

FIG. IB
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N-ACETYLCYSTEINE AMIDE (NAC AMIDE) FOR THE TREATMENT OF DISEASES AND CONDITIONS

FIELD OF THE INVENTION

The present invention generally relates to the treatment of mammalian, including human, diseases with antioxidants. More particularly, the invention relates to treatments and therapies of a variety of diseases and conditions involving the administration of N-acetylcysteine amide (NAC amide) or a derivative thereof, alone or in combination with another agent, to a mammal in need thereof.

BACKGROUND OF THE INVENTION

Oxidative stress plays an important role in the progression of neurodegenerative and age-related diseases, causing damage to proteins, DNA, and lipids. Low molecular weight, hydrophobic antioxidant compounds are useful in preventing conditions of peripheral tissues, such as acute respiratory distress syndrome, amyotrophic lateral sclerosis, atherosclerotic cardiovascular disease, multiple organ dysfunctions and central nervous system neurodegenerative disorders, e.g., Parkinson's disease, Alzheimer's disease and Creutzfeldt-Jakob's disease. Oxidative stress has been causally linked to the pathogenesis of Parkinson's disease, Alzheimer's disease and Creutzfeldt-Jakob's disease, as well as other types of disorders. (U.S. Patent No. 6,420,429 to D. Atlas et al).

A deficiency of cellular antioxidants may lead to excess free radicals, which cause macromolecular breakdown, lipid peroxidation, buildup of toxins and ultimately cell death. Because of the importance of antioxidant compounds in preventing this cellular oxidation, natural antioxidants, such as glutathione (GSH) (γ-glutamyl cysteinyl glycine) are continuously supplied to the tissues. GSH is synthesized by most cells and is one of the primary cellular antioxidants responsible for maintaining the proper oxidation state within the body. When oxidized, GSH forms a dimer, GSSG, which may be recycled in organs producing glutathione reductase. In human adults, reduced GSH is produced from GSSG, primarily in the liver, and to a smaller extent, by skeletal muscle and red and white blood cells, and is distributed through the blood stream to other tissues in the body.

However, under certain conditions, the normal, physiologic supplies of GSH are insufficient, its distribution is inadequate or local oxidative demands are too high to prevent
cellular oxidation. Under other conditions, the production of and demand for cell antioxidants, such as GSH, are mismatched, thus leading to insufficient levels of these molecules in the body. In other cases, certain tissues or biological processes consume the antioxidants so that their intracellular levels are suppressed. In either case, increased serum levels of antioxidant, e.g., glutathione, leads to increased amounts of the antioxidant that can be directed into cells. In facilitated transport systems for cellular uptake, the concentration gradient that drives uptake is increased.

Glutathione N-acetylcysteine amide (NAC amide), the amide form of N-acetylcysteine (NAC), is a low molecular weight thiol antioxidant and a Cu²⁺ chelator. NAC amide provides protective effects against cell damage. NAC amide was shown to inhibit tert.-butylhydroperoxide (BuOOH)-induced intracellular oxidation in red blood cells (RBCs) and to retard BuOOH-induced thiol depletion and hemoglobin oxidation in the RBCs. This restoration of thiol-depleted RBCs by externally applied NAC amide was significantly greater than that found using NAC. Unlike NAC, NAC amide protected hemoglobin from oxidation. (L. Grinberg et al, Free Radic Biol Med., 2005 Jan 1, 38(1): 136-45). In a cell-free system, NAC amide was shown to react with oxidized glutathione (GSSG) to generate reduced glutathione (GSH). NAC amide readily permeates cell membranes, replenishes intracellular GSH, and, by incorporating into the cell's redox machinery, protects the cell from oxidation. Because of its neutral carboxyl group, NAC amide possesses enhanced properties of lipophilicity and cell permeability. (See, e.g., U.S. Patent No. 5,874,468 to D. Atlas et al). NAC amide is also superior to NAC and GSH in crossing the cell membrane, as well as the blood-brain barrier.

NAC amide may function directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells from free-radical mediated damage. NAC amide is a potent cellular antioxidant responsible for maintaining the proper oxidation state within the body. NAC amide can recycle oxidized biomolecules back to their active reduced forms and may be as effective, if not more effective, than GSH as an antioxidant.

Glutamate, an excitatory amino acid, is one of the major neurotransmitters in the central nervous system (CNS). Elevated levels of extracellular glutamate have been shown to be responsible for acute neuronal damage as well as many CNS disorders, including hyperglycemia, ischemia, hypoxia (Choi, D.W., Neuron, 1(8):623-34, 1988), and chronic disorders such as Huntington's, Alzheimer's, and Parkinson's diseases (Meldrum B. and
Garthwaite J., Trends Pharmacol Sci., 11(9):379-87, 1990; and Coyle J.T. and Puttfarcken P., Science, 262(5134):689-95, 1993. Two mechanisms have been proposed for glutamate toxicity. The first mechanism explains the excitotoxicity of glutamate as being mediated through three types of excitatory amino acid receptors (Monaghan D.T. et al., Annu Rev Pharmacol Toxicol, 29:365-402, 1989). In addition to receptor-mediated glutamate excitotoxicity, it has also been proposed that elevated levels of extracellular glutamate inhibits cystine uptake, which leads to a marked decrease in cellular GSH levels, resulting in the induction of oxidative stress (Murphy T.H. et al., Neuron, 2(6):1547-58, 1989).

Cysteine is a critical component for intracellular GSH synthesis. Because of redox instability, almost all of the extracellular cysteine is present primarily in its oxidized state, cystine, which is taken up by cells via a cystine/glutamate transporter, the Xc — system. Studies indicate that glutamate and cystine share the same transporter; therefore, elevated levels of extracellular glutamate competitively inhibit cystine transport, which leads to depletion of intracellular GSH. (Bannai S. and Kitamura E., J Biol Chem. 255(6):2372-6, 1980; and Bannai S., Biochem Biophys Acta., 779(3):289-306, 1984). Depletion of reduced glutathione results in decreased antioxidant capacity of the cell, accumulation of ROS (reactive oxygen species), and ultimately apoptotic cell death. Several studies have demonstrated the induction of oxidative stress by glutamate in various cell lines including immature cortical neurons (Murphy T.H. et al., FASEB J., 4(6): 1624-33, 1990; and Sagara J. et al., J Neurochem., 61(5): 1667-71, 1993), oligodendroglia (Oka A. et al., J Neurosci., 13(4): 1441-53, 1993), cultured rat astrocytes (Cho Y. and Bannai S., J Neurochem., 55(6): 2091-7, 1990), neuroblastoma cells (Murphy T.H. et al, Neuron., 2(6):1547-58, 1989), and PC12 cells (Froissard P. and Duval D., Neurochem Int., 24(5): 485-93, 1994).

Certain antioxidants such as NAC, lipoic acid (LA), (Han D. et al., Am J Physiol, 273:1771-8, 1997), tocopherol (Pereira CM. and Oliveira C.R., Free Radic Biol Med., 23(4):637-47, 1997), and probucol (Naito M. et al, Neurosci Lett., 186(2-3):211-3, 1995) can protect against glutamate cytotoxicity, mostly by replenishing GSH. However, in certain neurological diseases, such as cerebral ischemia and Parkinson's disease, enhancement of tissue GSH in brain regions cannot be attained, because these antioxidant agents have been obstructed by the blood-brain barrier (Panigrahi M. et al, Brain Res., 717(1-2): 184-8, 1996; and Gotz M.E. et al, J Neural Transm Suppl., 29:241-9, 1990).

In addition to neurodegenerative diseases, such as those which affect the brain and/or peripheral nervous tissues, other diseases, such as asthma, respiratory-related diseases and
conditions, e.g., acute respiratory distress syndrome (ARDS), amyotrophic lateral sclerosis (ALS or Lou Gerhig's disease), atherosclerotic cardiovascular disease and multiple organ dysfunction, are related to the overproduction of oxidants or reactive oxygen species by cells of the immune system. A number of other disease states have been specifically associated with reductions in the levels of antioxidants such as GSH. Depressed antioxidant levels, either locally in particular organs or systemically, have been associated with a number of clinically defined diseases and disease states, including HIV/AIDS, diabetes and macular degeneration, all of which progress because of excessive free radical reactions and insufficient antioxidants.

Other chronic conditions may also be associated with antioxidant deficiency, oxidative stress, and free radical formation, including heart failure and associated conditions and pathologies, coronary arterial restenosis following angioplasty, diabetes mellitus and macular degeneration.

Clinical and pre-clinical studies have demonstrated the linkage between a range of free radical disorders and insufficient antioxidant levels. It has been reported that diabetic complications are the result of hyperglycemic episodes that promote glycation of cellular enzymes and thereby inactivate the synthetic pathways of antioxidant compounds. The result is antioxidant deficiency in diabetics, which may be associated with the prevalence of cataracts, hypertension, occlusive atherosclerosis, and susceptibility to infections in these patients.

High levels of antioxidants, such as GSH, have been demonstrated to be necessary for proper functioning of platelets, vascular endothelial cells, macrophages, cytotoxic T-lymphocytes, and other immune system components. Recently it has been discovered that patients infected with the human immunodeficiency virus, HIV, exhibit low GSH levels in plasma, other body fluids, and in certain cell types, such as macrophages. These low GSH levels do not appear to be due to defects in GSH synthesis. Antioxidant deficiency has been implicated in the impaired survival of patients with HIV. (1997, PNAS USA, Vol. 94, pp. 1967-1972). Raising antioxidant levels in cells is widely recognized as being important in HIV/AIDS and other disorders, because the low cellular antioxidant levels in these disease types permit more and more free radical reactions to fuel and exacerbate the disorders.

HIV is known to start pathologic free radical reactions, which lead to the destruction of antioxidant molecules, as well as their exhaustion and the destruction of cellular organelles and macromolecules. In mammalian cells, oxidative stresses, e.g. low intracellular levels of
reduced antioxidants and relatively high levels of free radicals, activate certain cytokines, including NF-κB and TNF-α, which, in turn, activate cellular transcription of the DNA to mRNA, resulting in translation of the mRNA to a polypeptide sequence. In a virus-infected cell, the viral genome is transcribed, resulting in viral RNA production, generally necessary for viral replication of RNA viruses and retroviruses. These processes require a relatively oxidized state of the cell, a condition which results from stress, low antioxidant levels, or the production of reduced cellular products. The mechanism which activates cellular transcription is evolutionarily highly conserved, and therefore it is unlikely that a set of mutations would escape this process, or that an organism in which mutated enzyme and receptor gene products in this pathway would be well adapted for survival. Thus, by maintaining a relatively reduced state of the cell (redox potential), viral transcription, a necessary step in late stage viral replication, is impeded.

The amplification effect of oxidative intracellular conditions on viral replication is compounded by the actions of various viruses and viral products, which degrade antioxidants, such as GSH. For example, gp120, an HIV surface glycoprotein having a large number of disulfide bonds, is normally present on the surface of infected cells. gp120 oxidizes GSH, resulting in reduced intracellular GSH levels. On the other hand, GSH reduces the disulfide bonds of gp120, thus reducing or eliminating its biological activity that is necessary for viral infectivity. Antioxidants such as GSH therefore interfere with the production of such oxidized proteins and degrade them once formed. In a cell that is actively replicating viral gene products, a cascade of events may occur which can allow the cell to pass from a relatively quiescent stage with low viral activity to an active stage with massive viral replication and cell death. This is accompanied by a change in redox potential. By maintaining adequate levels of antioxidant, this cascade may be impeded.

HIV is transmitted through two predominant routes, namely, contaminated blood and/or sexual intercourse. In pediatric cases, approximately one half of the newborn individuals are infected in utero and one half are infected at delivery. This circumstance permits a study of prevention of transmission since the time of spread is known. Initially, there is an intense viral infection simulating a severe case of the flu, with massive replication of the virus. Within weeks, this acute phase passes spontaneously as the body mounts a largely successful immune defense. Thereafter, the individual has no outward manifestations of the infection. However, the virus continues to replicate within immune system cells and
tissues, e.g., lymph nodes, lymphoid nodules, macrophages and certain multidendritic cells that are found in various body cavities.

Such stealthy and widespread infection is not just a viral problem. The virus, in addition to replicating, causes excessive production of various free radicals and various cytokines in toxic or elevated levels. The cytokines are normally occurring biochemical substances that signal numerous reactions and that typically exist in minuscule concentrations. Eventually, after an average of 7-10 years of seemingly quiescent HIV infection, the corrosive free radicals and the toxic levels of cytokines begin to cause outward symptoms in infected individuals and failures in the immune system begin. Substances like 15-HPETE are immunosuppressive and TNF-a causes muscle wasting, among other toxic factors. The numbers of viral particles increase and the patient develops the Acquired Immune Deficiency Syndrome, AIDS, which may last 2 to 4 years before the individual's demise. AIDS, therefore, is not merely a virus infection, although the viral infection is believed to be an integral part of the etiology of the disease.

Further, HIV has a powerful ability to mutate. It is this capability that makes it difficult to create a vaccine or to develop long-term, antiviral pharmaceutical treatments. As more people fail to be successfully treated by the present complex regimens, the number of resistant viral strains is increasing. Resistant strains of HIV are a particularly dangerous population of the virus and pose a considerable health threat. These resistant HIV mutants also add to the difficulties in developing vaccines that will be able to inhibit the activity of highly virulent viral types. Further, the continuing production of free radicals and cytokines that may become largely independent of the virus perpetuate the dysfunctions of the immune system, the gastrointestinal tract, the nervous system, and many other organs in patients with AIDS. The published scientific literature indicates that many of these diverse organ system dysfunctions are due to systemic deficiencies of antioxidant compounds that are engendered by the virus and its free radicals. For example, GSH is consumed in HIV infections because it is the principal, bulwark antioxidant versus free radicals. An additional cause of erosion of GSH levels is the presence of numerous disulfide bonds in HIV proteins, such as the gp120 cell surface protein. Disulfide bonds react with GSH and oxidize it. Thus, there is a need for other antioxidants to be used to replace antioxidants such as GSH whose normal function is adversely affected by HIV infection.

The current HIV/AIDS pharmaceuticals take good advantage of the concept of pharmaceutical synergism, wherein two different targets in one process are affected
simultaneously. The effect is more than additive. The drugs now in use were selected to inhibit two very different points in the long path of viral replication. The pathway of viral replication as understood by skilled practitioners in the art is described in U.S. Patent No. 6,420,429. New anti-HIV/AIDS therapies include additional drugs in the classes of Reverse Transcriptase inhibitors and protease inhibitors. Also, drugs are in development to block the integrase enzyme of the virus, which integrates the HIV DNA into the infected cell's DNA, analogous to splicing a small length of wire into a longer wire. Vaccine development also continues, although prospects seem poor because HIV appears to be a moving target and seems to change rapidly. Vaccine development is also impaired by the immune cell affinity of the virus.

Individuals infected with HIV have lowered levels of serum acid-soluble thiols and antioxidants such as GSH in plasma, peripheral blood monocytes and lung epithelial lining fluid. In addition, it has been shown that CD4+ and CD8+ T cells with high intracellular GSH levels are selectively lost as HIV infection progresses. This deficiency may potentiate HIV replication and accelerate disease progression, especially in individuals with increased concentrations of inflammatory cytokines, because such cytokines stimulate HIV replication more efficiently in cells in which antioxidant compounds are depleted. In addition, the depletion of antioxidants, such as GSH, is also associated with a process known as apoptosis, or programmed cell death. Thus, intercellular processes which artificially deplete GSH may lead to cell death, even if the process itself is not lethal.

Diabetes mellitus ("diabetes") is found in two forms: childhood or autoimmune (Type I, IDDM) and late-onset or non-insulin dependent (Type II, NIDDM). Type I constitutes about 30% of the cases of diabetes. The rest of the cases are represented by Type II. In general, the onset of diabetes is sudden for Type I and insidious or chronic for Type II. Symptoms include excessive urination, hunger and thirst, with a slow and steady loss of weight associated with Type I. Obesity is often associated with Type II and has been thought to be a causal factor in susceptible individuals. Blood sugar is often high and there is frequent spilling of sugar in the urine. If the condition goes untreated, the victim may develop ketoacidosis with a foul-smelling breath similar to some who has been drinking alcohol. The immediate medical complications of untreated diabetes can include nervous system symptoms, and even diabetic coma.

Because of the continuous and pernicious occurrence of hyperglycemia (very high blood sugar levels), a non-enzymatic chemical reaction, called glycation, frequently occurs
inside cells and causes a chronic inactivation of essential enzymes. One of the most critical enzymes, γ-glutamyl-cysteine synthetase, is glycated and readily inactivated. This enzyme is involved in a critical step in the biosynthesis of glutathione in the liver. The net result of this particular glycation is a deficiency in the production of GSH in diabetics.

GSH is in high demand throughout the body for multiple, essential functions, for example, within all mitochondria, to produce chemical energy called ATP. With a deficiency or absence of GSH, brain cells, heart cells, nerve cells, blood cells and many other cell types are not able to function properly and can be destroyed through apoptosis associated with oxidative stress and free radical formation. GSH is the major antioxidant in the human body and the only one that can be synthesized de novo. It is also the most common small molecular weight thiol in both plants and animals. Without GSH the immune system cannot function, and the central and peripheral nervous systems become aberrant and then cease to function. Because of the dependence on GSH as the carrier of nitric oxide, a vasodilator responsible for control of vascular tone, the cardiovascular system does not function well and eventually fails. Since all epithelial cells seem to require GSH, without GSH, intestinal lining cells also do not function properly and valuable micronutrients are lost, nutrition is compromised, and microbes are given portals of entry to cause infections.

In diabetes, the use of GSH precursors cannot help to control GSH deficiency due to the destruction of the rate-limiting enzyme by glycation. As GSH deficiency becomes more profound, the well-known sequelae of diabetes progress in severity. The complications that develop in diabetics are essentially due to runaway free radical damage since the available GSH supplies in diabetics are insufficient. For example, a diabetic individual becomes more susceptible to infections because the immune system approaches collapse when GSH levels fall, analogous to the situation in HIV/AIDS. In addition, peripheral vasculature becomes comprised and blood supply to the extremities is severely diminished because GSH is not available in sufficient amounts to stabilize nitric oxide to effectively exert its vascular dilation (relaxation) property. Gangrene is a common sequel and successive amputations often result in later years. Peripheral neuropathies, the loss of sensation commonly of the feet and lower extremities develop and are often followed by aberrant sensations like uncontrollable burning or itching. Retinopathy and nephropathy are later events that are actually due to microangiopathy, i.e., excessive budding and growth of new blood vessels and capillaries, which often will bleed due to weakness of the new vessel walls. This bleeding causes damage to the retina and kidneys with resulting blindness and renal shutdown, which
requires dialysis treatment. Further, cataracts occur with increasing frequency as the GSH deficiency deepens. Large and medium sized arteries become sites of accelerated severe atherosclerosis, with myocardial infarcts at early ages, and of a more severe degree. If coronary angioplasty is used to treat the severe atherosclerosis, diabetics are much more likely to have re-narrowing of cardiac vessels, termed restenosis.

Macular degeneration as a cause of blindness is a looming problem as the population ages. Age-related macular degeneration (ARMD) is characterized by either a slow (dry form) or rapid (wet form) onset of destruction and irrevocable loss of rods and cones in the macula of the eye. The macula is the approximate center of the retina wherein the lens of the eye focuses its most intense light. The visual cells, known as the rods and cones, are an outgrowth and active part of the central nervous system. They are responsible and essential for the fine visual discrimination required to see clear details such as faces and facial expression, reading, driving, operation of machinery and electrical equipment and general recognition of surroundings. Ultimately, the destruction of the rods and cones leads to functional, legal blindness. Since there is no overt pain associated with the condition, the first warnings of onset are usually noticeable loss of visual acuity. This may already signal late stage events. It is now thought that one of the very first events in this pathologic process is the formation of a material called "drusen", which first appears as either patches or diffuse drops of yellow material deposited upon the surface of the retina in the macula lutea or yellow spot. This is the area of the retina where sunlight is focused by the lens and which contains the highest density of rods for acuity. Although cones, which detect color, are lost as well in this disease, it is believed to be loss of rods, which causes the blindness. Drusen has been chemically analyzed and found to be composed of a mixture of lipids that are peroxidized by free radical reactions.

It is believed that the loss of retinal pigmented epithelial (RPE) cells occurs first in ARMD. Once an area of the retinal macula is devoid of RPE cells, loss of rods, and eventually some cones, occurs. Finally, budding of capillaries begins and typical microangiopathy associated with late stage ARMD occurs. It is also known that RPE cells require large quantities of GSH for their proper functioning. When GSH levels drop severely in cultures of RPE cells, the RPE cells begin to die. When cultures of these cells are supplemented with GSH in the medium, they thrive. There is increasing evidence that progression of the disease is paced by a more profound deficiency in GSH within the retina and probably within these cells, as indicated by cell culture studies.
It is generally believed that "near" ultraviolet (UVB) and visual light of high intensity primarily from sunlight is a strong contributing factor of ARMD. People with light-colored irises constitute a high risk population for macular degeneration, as do those with jobs that keep them outdoors and those in equatorial areas where sunlight is most intense. Additional free radical insults, e.g., smoking, adds to the risk of developing ARMD. Several approaches have been unsuccessfully tested to combat ARMD, including chemotherapy. Currently, there is no effective therapy to treat ARMD. Laser therapy has been developed which has been used widely to slow the damage produced in the slow onset form of the disease by cauterizing neovascular growth. However the eventual outcome of the disease, once it has started to progress, is certain.

The importance of thiols and especially of GSH to lymphocyte function has been known for many years. Adequate concentrations of GSH are required for mixed lymphocyte reactions, T-cell proliferation, T- and B-cell differentiation, cytotoxic T-cell activity, and natural killer cell activity. Adequate GSH levels have been shown to be necessary for microtubule polymerization in neutrophils. Intraperitoneally administered GSH augments the activation of cytotoxic T-lymphocytes in mice, and dietary GSH was found to improve the splenic status of GSH in aging mice, and to enhance T-cell mediated immune responses. The presence of macrophages can cause a substantial increase of the intracellular GSH levels of activated lymphocytes in their vicinity. Macrophages consume cystine via a strong membrane transport system, and generate large amounts of cysteine, which they release into the extracellular space. It has been demonstrated that macrophage GSH levels (and therefore cysteine equivalents) can be augmented by exogenous GSH. T-cells cannot produce their own cysteine, and it is required by T-cells as the rate-limiting precursor of GSH synthesis. The intracellular GSH level and the DNA synthesis activity in mitogenically-stimulated lymphocytes are strongly increased by exogenous cysteine, but not cystine. In T-cells, the membrane transport activity for cystine is ten-fold lower than that for cysteine. As a consequence, T-cells have a low baseline supply of cysteine, even under healthy physiological conditions. The cysteine supply function of the macrophages is an important part of the mechanism which enables T-cells to shift from a GSH-poor to a GSH-rich state.

The importance of the intracellular GSH concentration for the activation of T-cells is well established. It has been reported that GSH levels in T-cells rise after treatment with GSH; it is unclear whether this increase is due to uptake of the intact GSH or via extracellular breakdown, transport of breakdown products, and subsequent intracellular GSH synthesis.
Decreasing GSH by 10-40% can completely inhibit T-cell activation in vitro. Depletion of intracellular GSH has been shown to inhibit the mitogenically-induced nuclear size transformation in the early phase of the response. Cysteine and GSH depletion also affects the function of activated T-cells, such as cycling T-cell clones and activated cytotoxic T-lymphocyte precursor cells in the late phase of the allogeneic mixed lymphocyte culture. DNA synthesis and protein synthesis in IL-2 dependent T-cell clones, as well as the continued growth of preactivated CTL precursor cells and/or their functional differentiation into cytotoxic effector cells are strongly sensitive to GSH depletion.

Glutathione status is a major determinant of protection against oxidative injury. GSH acts on the one hand by reducing hydrogen peroxide and organic hydroperoxides in reactions catalyzed by glutathione peroxidases, and on the other hand by conjugating with electrophilic xenobiotic intermediates capable of inducing oxidant stress. The epithelial cells of the renal tubule have a high concentration of GSH, no doubt because the kidneys function in toxin and waste elimination, and the epithelium of the renal tubule is exposed to a variety of toxic compounds. GSH, transported into cells from the extracellular medium, substantially protects isolated cells from intestine and lung against t-butylhydroperoxide, menadione or paraquat-induced toxicity. Isolated kidney cells also transport GSH, which can supplement endogenous synthesis of GSH to protect against oxidant injury. Hepatic GSH content has also been reported to increase (i.e. to double) in the presence of exogenous GSH. This may be due either to direct transport, as has been reported for intestinal and alveolar cells, or via extracellular degradation, transport, and intracellular resynthesis.

The nucleophilic sulfur atom of the cysteine moiety of GSH serves as a mechanism to protect cells from harmful effects induced by toxic electrophiles. It is well established that glutathione S-conjugate biosynthesis is an important mechanism of drug and chemical detoxification. GSH conjugation of a substrate generally requires both GSH and glutathione-s-transferase activity. The existence of multiple glutathione-S-transferases with specific, but also overlapping, substrate specificities enables the enzyme system to handle a wide range of compounds. Several classes of compounds are believed to be converted by glutathione conjugate formation to toxic metabolites. For example, halogenated alkenes, hydroquinones, and quinones have been shown to form toxic metabolites via the formation of S-conjugates with GSH. The kidney is the main target organ for compounds metabolized by this pathway. Selective toxicity to the kidney is the result of the kidney’s ability to accumulate...
intermediates formed by the processing of S-conjugates in the proximal tubular cells, and to bioactivate these intermediates to toxic metabolites.

The administration of morphine and related compounds to rats and mice results in a loss of up to approximately 50% of hepatic GSH. Morphine is known to be biotransformed into morphinone, a highly hepatotoxic compound, which is 9 times more toxic than morphine in mouse by subcutaneous injection, by morphine 6-dehydrogenase activity. Morphinone possesses an α,β-unsaturated ketone, which allows it to form a glutathione S-conjugate. The formation of this conjugate correlates with loss of cellular GSH. This pathway represents the main detoxification process for morphine. Pretreatment with GSH protects against morphine-induced lethality in the mouse.

The deleterious effects of methylmercury on mouse neuroblastoma cells are largely prevented by co-administration of GSH. GSH may complex with methylmercury, prevent its transport into the cell, and increase cellular antioxidant capabilities to prevent cell damage. Methylmercury is believed to exert its deleterious effects on cellular microtubules via oxidation of tubulin sulphydryls, and by alterations due to peroxidative injury. GSH also protects against poisoning by other heavy metals such as nickel and cadmium.

Because of its known role in renal detoxification and its low toxicity, GSH has been explored as an adjunct therapy for patients undergoing cancer chemotherapy with nephrotoxic agents such as cisplatin, in order to reduce systemic toxicity. In one study, GSH was administered intravenously to patients with advanced neoplastic disease, in two divided doses of 2,500 mg, shortly before and after doses of cyclophosphamide. GSH was well tolerated and did not produce unexpected toxicity. The lack of bladder damage, including microscopic hematuria, supports the protective role of this compound. Other studies have shown that co-administration of GSH intravenously with cisplatin and/or cyclophosphamide combination therapy, reduces associated nephrotoxicity, while not unduly interfering with the desired cytotoxic effect of these drugs.

GSH has an extremely low toxicity, and oral LD50 measurements are difficult to perform due to the sheer mass of GSH, which has to be ingested by the animal in order to see any toxic effects. GSH can be toxic, especially in cases of ascorbate deficiency, and these effects may be demonstrated in, for example, ascorbate deficient guinea pigs given 3.75 mmol/kg daily (1,152 mg/kg daily) in three divided doses, whereas in non-ascorbate deficient animals, toxicity was not seen at this dose, but were seen at double this dose.
There is a need in the art for other compounds and therapeutic aspects to treat a number of diseases that are linked to oxidative stress and the presence of free oxygen radicals and associated disease pathogenesis in cells and tissues. Needed are antioxidant compounds, other than GSH, that are safe and even more potent, to overcome high oxidative stress in the pathogenesis of diseases. Ideally, such compounds should readily cross the blood-brain barrier and easily permeate the cell membrane. Antioxidants such as vitamins E and C are not completely effective at decreasing oxidative stress, particularly because, in the case of vitamin E, they do not effectively cross through the cell membrane to reach the cytoplasm so as to provide antioxidant effects.

SUMMARY OF THE INVENTION

The present invention provides the use of a potent antioxidant N-acetylcysteine amide (NAC amide) or derivatives thereof, or a physiologically acceptable derivative, salt, or ester thereof, in new applications to treat disorders, conditions, pathologies and diseases that result from, or are associated with, the adverse effects of oxidative stress and/or the production of free radicals in cells, tissues and organs of the body. NAC amide and its derivatives are provided for use in methods and compositions for improving, treating, and/or preventing such disorders, conditions, pathologies and diseases.

As used herein, a "subject" within the context of the present invention encompasses, without limitation, mammals, e.g., humans, domestic animals and livestock including cats, dogs, cattle and horses. A "subject in need thereof" is a subject having one or more manifestations of disorders, conditions, pathologies, and diseases as disclosed herein in which administration or introduction of NAC amide or its derivatives would be considered beneficial by those of ordinary skill in the art.

In an aspect of the present invention, methods and compositions comprising NAC amide provide an antioxidant to cells and tissues to reduce oxidative stress, and the adverse effects of cellular oxidation, in an organism. The invention provides a method of reducing oxidative stress associated with the conditions, diseases, pathologies as described herein, by administering a pharmaceutically acceptable formulation of NAC amide or derivatives thereof to a human or non-human mammal in an amount effective to reduce oxidative stress.

In another aspect of the present invention, NAC amide and its derivatives are provided to treat an organism having a disorder, condition, pathology, or disease that is
associated with the overproduction of oxidants and/or oxygen free radical species. According to this invention NAC amide treatment can be prophylactic or therapeutic.

"Therapeutic treatment" or "therapeutic effect" means any improvement in the condition of a subject treated by the methods of the present invention, including obtaining a preventative or prophylactic effect, or any alleviation of the severity of signs or symptoms of a disorder, condition, pathology, or disease or its sequelae, including those caused by other treatment methods (e.g., chemotherapy and radiation therapy), which can be detected by means of physical examination, laboratory, or instrumental methods and considered statistically and/or clinically significant by those skilled in the art.

"Prophylactic treatment" or "prophylactic effect" means prevention of any worsening in the condition of a subject treated by the methods of the present invention, as well as prevention of any exacerbation of the severity of signs and symptoms of a disorder, condition, pathology, or disease or its sequelae, including those caused by other treatment methods (e.g., chemotherapy and radiation therapy), which can be detected by means of physical examination, laboratory, or instrumental methods and considered statistically and/or clinically significant by those skilled in the art.

In another aspect of the present invention, NAC amide is used in the treatment and/or prevention of cosmetic conditions and dermatological disorders of the skin, hair, nails, and mucosal surfaces when applied topically. In accordance with the invention, compositions for topical administration are provided that include (a) NAC amide, or derivatives thereof, or a suitable salt or ester thereof, or a physiologically acceptable composition containing NAC amide or its derivatives; and (b) a topically acceptable vehicle or carrier. The present invention also provides a method for the treatment and/or prevention of cosmetic conditions and/or dermatological disorders that entails topical administration of NAC amide- or NAC-amide derivative-containing compositions to an affected area of a patient.

In yet another of its aspects the present invention provides methods and compositions useful for cancer and pre-cancer therapy utilizing NAC amide or a derivative thereof, or its pharmaceutically acceptable salts or esters. The present invention particularly relates to methods and compositions comprising NAC amide or a derivative thereof in which apoptosis is selectively induced in cells of cancers or precancers.

In another aspect, the present invention provides compositions and methods comprising NAC amide or a derivative thereof for the suppression of allograft rejection in recipients of allografts.
In another aspect, the present invention provides a NAC amide or a derivative thereof in a method of supporting or nurturing the growth of stem cells for stem cell transplants, particularly stem cells cultured in vitro prior to introduction into a recipient animal, including humans.

In another aspect, the present invention provides methods of inhibiting, preventing, treating, or both preventing and treating, central nervous system (CNS) injury or disease, traumatic brain injury, neurotoxicity or memory deficit in a subject, involving the administration of a therapeutically effective amount of NAC amide, or derivative thereof or a pharmaceutically acceptable composition thereof.

In another of its aspects, the present invention provides a method of killing or inhibiting the growth of microorganisms by providing NAC amide in an amount effective to increase cellular levels of HIF-1 or HIF-1α to enhance the capacity of white blood cells to kill or inhibit the growth of the microorganisms. Also in accordance with the invention, NAC amide is used as a countermeasure for biodefensive purposes, e.g., in killing or growth inhibiting microorganisms, viruses, mycoplasma, etc., and in treating and/or preventing resulting diseases and conditions, as further described herein.

In another aspect, the present invention provides a method of preventing tissue destruction resulting from the effects of metalloproteinases, such as MMP-3, which has been found to cause normal cells to express the Rac1b protein, an unusual form of Rho GTPase that has previously been found only in cancers. Rac1b stimulates the production of highly reactive oxygen species (ROS), which can promote cancer by activating major genes that elicits massive tissue disorganization. In accordance with the present invention NAC amide is used to block the effects of Rac1b-induced ROS production by administering or introducing NAC amide to cells, tissues, and/or the body of a subject in need thereof, to target molecules in the pathways leading to tissue damage and degradation. Thus, NAC amide can be used to inhibit MMP-3 and its adverse functions, to target ROS indirectly or directly via the processes by which ROS activates genes to induce the EMT.

Another aspect of the present invention provides a method of stimulating endogenous production of cytokines and hematopoietic factors, comprising administering or introducing NAC amide to cells, tissues, and/or a subject in need thereof for a period of time to stimulate the endogenous production. NAC amide can be used to stimulate production of cytokines and hematopoietic factors, such as but not limited to, TNF-cc, IFN-a, IFN-β, IFN-γ, IL-1, IL-2, IL-6, IL-10, erythropoietin, G-CSF, M-CSF, and GM-CSF, which are factors that modulate
the immune system and whose biological activities are involved in various human diseases, such as neoplastic and infectious diseases, as well as those involving hematopoiesis and immune depressions of various origin (such as, without limitation, erythroid, myeloid, or lymphoid suppression). Stimulation of endogenous production of these cytokines and hematopoietic factors by NAC amide is particularly advantageous, since exogenous administration of these cytokines and hematopoietic factors have limitations associated with the lack of acceptable formulations, their exhorbitant cost, short half-life in biological media, difficulties in dose-determination, and numerous toxic and allergic effects.

In another embodiment, the present invention encompasses methods and composition comprising NAC amide for detecting NAC-amide responsive changes in gene expression in a cell, tissue, and/or a subject, comprising administering or introducing NAC amide or derivative of NAC amide to the cell, tissue, and/or subject for a period of time to induce changes in gene expression and detecting the changes in gene expression. NAC amide and derivatives thereof can induce changes in gene expression such as genes involved in apoptosis, angiogenesis, chemotaxis, among others.

In another aspect, the present invention provides directed delivery of NAC amide to cells, such as cancer cells that express high levels of receptors for folic acid (folate) or glutathione. According to this aspect, NAC amide ("NACA") is coupled to a ligand for the receptor (e.g., folic acid or glutathione) to form a conjugate, and then this NACA-ligand conjugate is coated or adsorbed onto readily injectable nanoparticles using procedures known to those skilled in the art. According to this aspect, the nanoparticles containing NAC amide ("nano-NACA particles") may be preferentially taken up by cancer or tumor cells where the NAC amide will exert its desired effects. Accordingly, the present invention provides a method of directed delivery of NAC amide to host cells expressing high levels of surface receptor for a ligand, in which the method involves (a) coupling NAC amide to the surface receptor ligand to form a NAC amide-ligand conjugate; (b) adsorbing the NAC amide-ligand conjugate onto nanoparticles; and (c) introducing the nanoparticles of (b) into the host. The invention further provides a method of directed delivery of NAC amide to host cells expressing high levels of surface receptor for a ligand, in which the method involves (a) conjugating acetylated dendritic nanoparticles to a ligand; (b) coupling the conjugated ligand of (a) to NAC amide to form NAC amide-ligand nanoparticles; and (c) introducing the nanoparticles of (b) into the host.

Another aspect of the present invention provides a compound of the formula I:
wherein: 
\( R_i \) is OH, SH, or S-S-Z;
\( X \) is C or N;
\( Y \) is \( \text{NH}_2 \), OH, CH\(_3\)-C=0, or NH-CH\(_3\);
\( R_2 \) is absent, H, or =0

\[ \text{II} \]

wherein: 
\( R_4 \) is NH or O;
\( R_5 \) is CF\(_3\), NH\(_2\), or CH\(_3\)

and wherein: 
\( Z \) is

with the proviso that if \( R_4 \) is S-S-Z, \( X \) and \( X' \) are the same, \( Y \) and \( Y' \) are the same, \( R_2 \) and \( R_6 \) are the same, and \( R_3 \) and \( R_7 \) are the same.

The present invention also provides a NAC amide compound and NAC amide derivatives comprising the compounds disclosed herein.

In another aspect, a process for preparing an L- or D- isomer of the compounds of the present invention are provided, comprising adding a base to L- or D-cystine diamide dihydrochloride to produce a first mixture, and subsequently heating the first mixture under vacuum; adding a methanolic solution to the heated first mixture; acidifying the mixture with
alcoholic hydrogen chloride to obtain a first residue; dissolving the first residue in a first solution comprising methanol saturated with ammonia; adding a second solution to the dissolved first residue to produce a second mixture; precipitating and washing the second mixture; filtering and drying the second mixture to obtain a second residue; mixing the second residue with liquid ammonia and an ethanolic solution of ammonium chloride to produce a third mixture; and filtering and drying the third mixture, thereby preparing the L- or D-isomer compound.

In some embodiments, the process further comprises dissolving the L- or D-isomer compound in ether; adding to the dissolved L- or D-isomer compound an ethereal solution of lithium aluminum hydride, ethyl acetate, and water to produce a fourth mixture; and filtering and drying the fourth mixture, thereby preparing the L- or D-isomer compound.

Another aspect of the invention provides a process for preparing an L- or D-isomer of the compounds disclosed herein, comprising mixing S-benzyl-L- or D-cysteine methyl ester hydrochloride or O-benzyl-L- or D-serine methyl ester hydrochloride with a base to produce a first mixture; adding ether to the first mixture; filtering and concentrating the first mixture; repeating steps (c) and (d), to obtain a first residue; adding ethyl acetate and a first solution to the first residue to produce a second mixture; filtering and drying the second mixture to produce a second residue; mixing the second residue with liquid ammonia, sodium metal, and an ethanolic solution of ammonium chloride to produce a third mixture; and filtering and drying the third mixture, thereby preparing the L- or D-isomer compound.

Yet another aspect of the invention provides a process for preparing a compound as disclosed herein, comprising mixing cystamine dihydrochloride with ammonia, water, sodium acetate, and acetic anhydride to produce a first mixture; allowing the first mixture to precipitate; filtering and drying the first mixture to produce a first residue; mixing the second residue with liquid ammonia, sodium metal, and an ethanolic solution of ammonium chloride to produce a second mixture; filtering and drying the second mixture, thereby preparing the compound. In an aspect of the present invention a method is presented for preventing tissue damage in a human subject exposed to a high-energy impulse blasts. The method comprises administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to high-energy impulse blasts. The method may involve prophylactic treatment of lung injury due to exposure to a high-energy impulse blasts.
In another aspect of the present invention a method is presented for preventing pulmonary inflammation after exposure to blast overpressure or related conditions in a human subject in need thereof. The method comprises administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing pulmonary inflammation or related conditions after exposure to blast overpressure. The related conditions from exposure to blast are contusions or barotrauma-like injury to air-filled organs, wherein the air-filled organs are ears, lungs, and the gastrointestinal tract.

In another aspect of the present invention a method is presented for preventing blunt chest trauma in a human subject in need thereof. The method may comprise administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing blunt chest trauma.

In one aspect of the present invention a method is presented for preventing lung contusion in a human subject in need thereof. The method may comprise administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing lung contusion.

In another aspect of the present invention a method is presented for preventing traumatic brain injury in a human subject in need thereof. The method may comprise administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing traumatic brain injury.

In another aspect of the present invention a method is presented for preventing malaria in a human subject infected with a malarial parasite, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for prevention of malaria. The method may involve prophylactic treatment of malaria.

One aspect of the present invention provides for inhibiting replication of HIV virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication.

A method is presented, as an embodiment of the current invention, for preventing HIV/AIDS in a human subject infected with HIV virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative
thereof, to the subject in a dose effective for prevention of HIV/AIDS. The method may involve prophylactic treatment of HIV/AIDS.

Another embodiment of the present invention involves a method for inhibiting replication of dengue virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication.

Yet another embodiment, the present invention provides a method for preventing dengue fever in a human subject infected with dengue virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for prevention of dengue fever. The method may involve prophylactic treatment of dengue fever.

A method for preventing tissue damage in a human subject exposed to radiological materials is presented as an embodiment of the current invention. The method comprises administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to radiological materials. The method may be for prophylactic treatment of radiological exposure.

In another aspect, the present invention provides a method for preventing tissue damage in a human subject exposed to airborne particulate matter, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to airborne particulate matter.

In another aspect, the present invention provides a method for preventing tissue damage in a human subject exposed to toxic gas or fumes, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to toxic gas or fumes.

The invention also provides a method for inhibiting replication of an influenza virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication. A method is also presented for preventing influenza in a human subject infected with an influenza virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative
thereof, to the subject in a dose effective for prevention of influenza. The method may involve prophylactic treatment of influenza.

A method is presented for inhibiting replication of a virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication. In one embodiment the method for preventing infection by a virus in a human subject comprises administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective. The method involves prophylactic treatment of viral infection.

In one embodiment, a method is presented for improving survivability after exposure to a high energy impulse blast or blast overpressure comprising administering to a human subject in need thereof N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing pulmonary inflammation after exposure to a high energy impulse blast or blast overpressure.

In another embodiment of the present invention a method is presented for preventing pulmonary damage after exposure to blast overpressure in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing pulmonary inflammation after exposure to blast overpressure.

Another embodiment provides a method for preventing multiple organ damage or related conditions after exposure to blast overpressure in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing organ damage or related conditions after exposure to blast overpressure. The related conditions of multiple organ damage are contusions or barotrauma-like injury to air-filled organs, where the air-filled organs are ears, lungs, and the gastrointestinal tract. Additional affected organs may be lung, heart, brain, liver, kidneys, or gastrointestinal tract.

In another aspect, the present invention provides a method for preventing or treating Parkinson's disease in a mammalian subject, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing or treating neurodegenerative disease. In one of the
embodiments the neurodegenerative disease may be Parkinson's disease. The mammalian subject according to the current invention may be a human.

The current invention also embodies prevention or inhibition of neurotoxin induced cell death, where the cell may be a neuronal cell in an animal, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof. In certain embodiment of the current invention the neurotoxin may be acetaminophen.

The current invention also embodies prevention or inhibition of toxicity induced by an antibiotic in kidney cells, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof. In certain embodiment of the current invention the antibiotic may be gentamycin.

In several embodiments of the current invention 50-10,000 mg per dose, or in an equivalent amount of N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof may be administed. Alternatively, the dose may be 25-500 mg per dose, or in an equivalent amount.

In several embodiments of the current invention, NAC amide may be delivered orally via a capsule.

The present invention also provides a food additive comprising NAC amide or a NAC amide derivative as disclosed herein.

Additional aspects, features and advantages afforded by the present invention will be apparent from the detailed description and exemplification hereinbelow.

**BRIEF DESCRIPTION OF THE FIGURES**

FIG. 1A presents the structure of N acetyl cysteine. FIG. 1B presents the structure of N-acetylcysteine amide (NAC amide).

FIGS. 2A-2D show the cytotoxic response of PC12 cells to glutamate and protection by NAC amide. PC12 cells were plated at a density 25 x 10^3 cells/well in a 24 well plate and grown for 24 h in culture medium. They were treated or not (control) with 10 mM Glu with or without NAC amide, as described in Example 1. Twenty-four hours later, cells were examined and photographed. FIG. 2A: Control; FIG. 2B: NAC amide (NACA) only; FIG. 2C: Glutamate only; FIG. 2D: Glutamate and NACA.

FIG. 3 shows the protective effect of NAC amide against glutamate cytotoxicity. Cells were plated and grown for 24 hours in a culture medium; then they were treated or not
(control) with 10 mM Glu, with or without NAC amide. Twenty-four hours later, the % LDH release was determined using LDH analysis. Values represent means ± SD. Statistically different values of * P < 0.0001 and ** P < 0.05 were determined, compared to control. *** P < 0.0001 compared to glutamate-treated group.

FIG. 4 shows the effect of NAC amide on glutamate-induced cytotoxicity. Cells were exposed to 10 mM Glu, with or without NAC amide, for 24 hours; the effects were compared to the control. Cell viability was quantified by the MTS assay. Values represent means ± SD. Statistically different values of *P < 0.0005 and ** P < 0.05 were determined, compared to control. *** P < 0.05 compared to glutamate-treated group.

FIG. 5 shows the effects of NAC amide [NAC amide] on cysteine levels in PC12 cells. Cells were plated and grown for 24 hours, and then they were exposed to glutamate (10 mM) in the presence or absence of NAC amide (750 μM). Twenty-four hours later, the cells were harvested and cysteine levels were measured. Values represent means ± SD. Statistically different values of * P < 0.005 and ** P < 0.05 were determined, compared to control. *** P < 0.05 compared to glutamate-treated group.

FIG. 6 is a graph depicting a comparison of survival rates of Sprague-Dawley rats after X-ray irradiation treatment in combination with pre-treatment or post-treatment with NAC or NAC amide (TOVA).

FIG. 7. Gross pathological signs of lung injury after exposure to blast overpressure. Isolated areas of hemorrhage were observed in lungs 2 days (B) after exposure to 140-kPa level of blast. At 8 days (C), there were no detectable pathological signs of lung damage compared with control (A).

FIG. 8. Representative pictures of evolving lung injury in rats 2 days after exposure to 140-kPa blast. Hematoxylin and eosinYstained sections of lung tissue from control animal (A), placebo-treated animal (B), NACA-treated (C) animal. Adjacent to a large bronchus, there is a focally extensive area of hemorrhage and inflammation (B and C). Alveoli are filled with erythrocytes, fibrin, and cellular debris. Surrounding alveolar septa are expanded by an inflammatory infiltrate (open arrow, B). Similar, but less extensive, area of hemorrhage and inflammation in NACA-treated animal (C). Magnification: 40X.

FIG. 9. Area of hemorrhage (B) and inflammation (C) 2 days after blast at higher power. Arrows indicate alveolar septa expanded up to three times normal by erythrocytes, fibrin, inflammatory cells (mostly macrophages and lymphocytes), and fibroblasts (B). Alveoli contain moderate numbers of inflammatory cells (macrophages, lymphocytes, fewer
neutrophils), and low amounts of fibrin and edema (pink flocculent material), hemorrhage, and cellular debris (C). Magnification: 200X.

FIG. 10. Histopathology of lung injury in rats 8 days after exposure to 140-kPa blast. Arrows indicate small foci of inflammation in placebo- (A) and NACA-treated (B) animals. At higher magnification (C and D), the inflammation in both cases consists mostly of alveolar macrophages (large round cells in the alveolar space) and lymphocytes (mostly expanding the alveolar septa, C, D). Magnification: X 40 (A, B) or X 200 (C, D).

FIG. 11. Myeloperoxidase activity (A) and CD11b mRNA (B) level in rat lungs at 2 and 8 days after exposure to blast. Myeloperoxidase activity and CD11b mRNA was increased 2 days after exposure in animals injected with placebo. N-Acetylcysteine amide treatment significantly reduced MPO activity and CD11b mRNA induction compared with placebo-treated animals. No change in both groups compared with nonblasted controls was observed 8 days after blast. Data are mean ± SD from five to eight animals in each group. *P < 0.05 compared with nonblasted controls; **P < 0.05 compared with controls and placebo-injected animals.

FIG. 12. Macrophage inflammatory protein 1 (A), MCP-1 (B), and CINC-1 mRNA (C) levels in rat lungs at 2 and 8 days after exposure to blast. mRNA levels were increased 2 days after exposure. N-Acetylcysteine amide treatment completely eliminated the mRNA increases in all chemokines. No change in both groups compared with controls was observed 8 days after blast. Data are mean ± SD from five to eight animals in each group. *P < 0.05 compared with nonblasted controls.

FIG. 13. Heme oxygenase 1 mRNA (A), MnSOD mRNA (B), and GR mRNA (C) levels in rat lungs at 2 and 8 days after exposure to blast. Heme oxygenase 1 mRNA was increased in animals injected with placebo 2 days after exposure and returned to the control level 8 days after blast. N-Acetylcysteine amide treatment eliminated the HO-1 mRNA increase after blast. No change compared with nonblasted controls was observed at MnSOD and GR mRNA expression at 2 or 8 days after blast. Data are mean ± SD from five to eight animals in each group. *P < 0.05 compared with nonblasted controls.

FIG. 14. NACA increased survival following blast injury by ~3-fold.

FIG. 15. Mean fold change in inflammatory gene mRNA in lung tissue measured 8 days after BOP injury. NACA-treated animals were protected from the up regulation in mRNA caused by BOP injury.
FIG. 16. Mean fold change in MIP niRNA in lung tissue measured 2 and 8 days after BOP injury. Chemokine levels caused by BOP injury in NACA-treated animals (blue bars) were decreased approximately 50% as compared to the up regulation in mRNA in controls.

FIG. 17. Mean fold change in MCP mRNA in lung tissue measured 2 and 8 days after BOP injury. Chemokine levels in NACA-treated animals (blue bars) were significantly lower on day 2 as compared to controls.

FIG. 18. Mean fold change in IL-1β mRNA in lung tissue measured 2 and 8 days after BOP injury. No significant differences were observed in chemokine levels caused by BOP injury in NACA-treated animals (blue bars) as compared to controls.

FIG. 19. Mean fold change in HO-1 mRNA in lung tissue measured 2 and 8 days after BOP injury. HO-1 levels in NACA-treated animals (blue bars) were significantly lower on day 2 as compared to controls.

FIG. 20. Histopathology of lung injury in control and NACA-treated animals on days 2 and 8 after exposure to BOP.

FIG. 21. TOVA is neuroprotective following TBI. Tissue Sparing assessment at 7 days post TBI; TOVA increases tissue sparing following TBI. Sections shown are representative.

FIG. 22. TOVA is neuroprotective following TBI. TOVA significantly increases tissue sparing following TBI. Cortical tissue sparing was assessed 7 days following TBI using the Cavalieri method. TOVA and TOVA + CsA significantly increased tissue sparing at 7 days post injury. Administration of TOVA + CsA and TOVA alone showed no difference in tissue sparing; indicating no synergistic effect between the two compounds. N = 3/group, bars are group means, S.D., ANOVA *p < 0.01, SNK.

FIG. 23. TOVA reduces biomarkers for oxidative stress following TBI. Tissue sections were stained for the lipid peroxidation marker, 4-FINE, and for the protein nitrosylation marker, 3-NT. Sections were reacted with primary antibody (rabbit anti-FTNE polyclonal antibody, Calbiochem) (mouse anti-3-NT monoclonal antibody, Upstate). Secondary antibodies were conjugated with different IR dyes that fluoresce at different wavelengths (800 secondary goat anti-rabbit antibody, Rockland) (IR Dye 700 D conjugated goat anti-mouse IgG antibody). Representative tissue section sets from individual rats shown.

FIG. 24. TOVA reduces oxidative stress following TBI. TOVA reduced lipid peroxidation (FINE levels) following TBI. TOVA did not reduce protein nitrosylation (3-NT
levels); which was expected based on the upstream utilization of superoxide by peroxynitrite.
N = 3/group, bars are group means, S.D. T test *p < 0.01.

Fig. 26 A and B. Lack of HIV Induction and Cytotoxicity in Chronically Infected U1 Cells by TOVA. Day 4 post-stimulation.

Fig. 27 A and B. Inhibitory Effects of TOVA on Cytokine-Induced HIV Expression in Chronically Infected U1 Cells. Day 5 post-stimulation.

Fig. 28. Schematic overview of study protocol for DEP-induced inflammation and oxidative stress in the lungs of C57BL/6 mice, and the role of N-acetylcysteineamide (NACA). Inflammation and oxidative stress were induced in male C57BL/6 mice following exposure to diesel exhaust particles (1.5 h and 3 h each day) for 9 days. All of the animals were pretreated with NACA or saline (on alternate days), 30 min before exposure to DEPs. The mice were sacrificed by urethane injection 24 h after the last exposure.

Fig. 29. Representative photomicrographs of H&E stained lung section of male C57BL/6 mice exposed to DEPs or clean filtered air for 9 days and sacrificed 24 h thereafter. Large arrows indicate macrophages filled with DEPs. Small arrows indicate macrophages with little or no DEPs. Magnification 40*.

Fig. 30. Enumeration of macrophages in the lungs of mice that were pretreated with NACA or saline and then exposed to DEPs. The number of macrophages per 5 high power field (hpf) was measured in H&E stained lung sections of C57BL/6 mice. *Values were significantly different from those of the control. #Values were significantly different from the DEPs 1.5 h exposed group. ##Values were significantly different from those of the DEPs 3 h exposed group. Data are expressed as mean±SD, p < 0.05.

Fig. 31. Representative photomicrograph of H&E stained lung sections of male C57BL/6 mice exposed to DEPs for 3 h every day for 9 days and then sacrificed 24 h thereafter. Large arrows indicate mucus. Small arrows indicate macrophages filled with DEPs. Magnification 40*.

Fig. 32. Effect of NACA on thiol levels in the lungs. GSH (A) and cysteine (B) levels in the lungs of mice exposed to DEPs for 9 days, as mentioned in Section 2. *Values were significantly different from the control. #,##Values were significantly different from the respective NACA-untreated group. Data are expressed as mean±SD, p < 0.05.

Fig. 33. Effect of NACA on lipid peroxidation in the lungs. MDA levels in the lungs of mice exposed to DEPs for 9 days, as mentioned in Section 2. *Values were significantly different from the control. #Values were significantly different from the DEPs 1.5 h exposed
group. ##Values significantly different from those of the DEPs 3 h exposed group. Data are expressed as mean±SD, p < 0.05.

Fig. 34. Effect of NACA on the activity of the antioxidant enzyme catalase (CAT) in the lungs. Catalase activity in the lungs of mice exposed to DEPs for 9 days as mentioned in Section 2. *Values were significantly different from those of the control. Data are expressed as mean±SD, p < 0.05.

Fig. 35. Effect of NACA on cytotoxicity levels in the lungs. Lactate dehydrogenase levels in the lungs of mice exposed to DEPs for 9 days. *Values were significantly different from those of the control. Data are expressed as mean±SD, p < 0.05.

Fig. 36. Schematic representation of the role of N-acetylcysteineamide (NACA) in diesel particulate matter induced oxidative stress in the lungs. After the DEPs enter into the lungs, they are engulfed by macrophages. The particle-laden macrophages exert oxidative stress, which leads to lowering of the GSH and catalase levels, and upregulation of the MDA levels in the lungs. These conditions further aggravate the inflammation and allergic reactions, leading to increased toxicity and damage to the lungs. However, pretreatment with the novel antioxidant, NACA, restores the balance between the pro and antioxidant enzymes, increasing the GSH levels in the lungs thus abrogating the inflammatory reactions and toxic effects of DEPs.

FIG. 37 provides a chromatogram of a plasma sample from an animal sacrificed 30 min after administration of 500 mg/kg body weight NACA. It shows NACA conversion to NAC, GSH, and cysteine.

FIG 38 shows exposure to Acetaminophen decreased the cell viability in dose dependent manner. Around 8000 HEPG2 cells/well were seeded in a 96 well plate. After attachment of the cells overnight, the cells were exposed to different concentrations of AAP for 24 hrs.

FIG 39 shows exposure to Acetaminophen (AAP) decreased the cell viability in dose dependent manner. Around 8000 HEPG2 cells/well were seeded in a 96 well plate. After attachment of the cells overnight, the cells were exposed to different concentrations of AAP for 48 hrs.

FIG 40 shows the effect of NAC and NAC amide (NACA) on HEPG2 cell viability. Viability studies of HEPG2 cells exposed to different concentrations of NAC and NAC amide (NACA) were performed. NAC and NACA were dissolved directly in media. NAC and NACA did not have any significant toxicity on the HEPG2 cells even at 10 mM levels.
FIG 41 shows the protective effects of NAC and NACA against AAP toxicity. HEPG2 cells were exposed to 20 mM of AAP with 5 mM of NAC or NACA for 24 hrs and viability was tested using MTS assay.

FIG 42 shows the protective effects of NAC and NACA against AAP toxicity. HEPG2 cells were exposed to 30 mM of AAP with 5 mM of NAC or NACA for 24 hrs and viability was tested using MTS assay.

FIG 43 shows the effect of NAC and NAC amide (NACA) on cysteine levels in cells exposed to AAP (20 mM) for 24 hrs. Around 350,000 cells were seeded in 25 cm² and were allowed to attach overnight. The cells were pretreated with NAC and NACA for 2 hrs. and later exposed to acetaminophen. After the exposure the cells were analyzed for cysteine levels using HPLC- Flurometric method.

FIG 44 shows the ability of NACA to increase the viability by acting as thiol donor in the absence or depletion of GSH. HEPG2 cells were seeded in 96 well plates and pretreated with NAC and NACA for 2 hrs and later exposed to BSO 5 mM, AAP 20 mM each for 2 days. MTS assay was done to determine the viability.

FIG 45 provides effects of various concentrations of gentamycin on kidney cells.

FIG 46 provides gentamycin induced apoptosis in kidney cells.

FIG 47 shows increased levels of iNOS, p38MAPK, NF-κB, and Bcl-2 after gentamycin treatment.

FIG 48 shows inhibition of gentamycin induced apoptosis by NAC amide (NACA).

FIG 49 shows rescue of gentamycin mediated iNOS and p38MAPK induction by NAC amide (NACA).

FIG 50 shows rescue of gentamycin mediated NF-κB and Bcl-2 induction by NAC amide (NACA).

FIG 51 shows rescue of gentamycin mediated Bax induction by NAC amide (NACA).

FIG 52 shows rescue of gentamycin mediated Bcl-2 and Bax induction by SB.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention involves the use of an effective and potent antioxidant, glutathione N-acetylcysteine amide (NAC amide), (FIG. 1), or a physiologically or pharmaceutically acceptable derivative or salt or ester thereof, for use in a variety of disorders, conditions, pathologies and diseases in which oxidative stress and/or free radical formation cause damage, frequently systemic damage, to cells, tissues and organs of the
body. The invention encompasses a pharmaceutically acceptable composition comprising NAC amide, e.g., water-soluble NAC amide, or physiologically acceptable derivatives, salts, or esters thereof, which can be used in treatment and therapeutic methods in accordance with this invention.

Glutathione N-acetylcysteine amide (NAC amide), the amide form of N-acetylcysteine (NAC), is a novel low molecular weight thiol antioxidant and a Cu$^{2+}$ chelator. NAC amide provides protective effects against cell damage in its role as a scavenger of free radicals. In mammalian red blood cells (RBCs), NAC amide has been shown to inhibit tert.-butylhydroperoxide (BuOOH)-induced intracellular oxidation and to retard BuOOH-induced thiol depletion and hemoglobin oxidation in the RBCs. This restoration of thiol-depleted RBCs by externally applied NAC amide was significantly greater than that found using NAC. Unlike NAC, NAC amide protected hemoglobin from oxidation. (L. Grinberg et al., Free Radic Biol Med., 2005 Jan 1, 38(1): 136-45). In a cell-free system, NAC amide was shown to react with oxidized glutathione (GSSG) to generate reduced glutathione (GSH). NAC amide readily permeates cell membranes, replenishes intracellular GSH, and, by incorporating into the cell's redox machinery, protects the cell from oxidation. Because of its neutral carboxyl group, NAC amide possesses enhanced properties of lipophilicity and cell permeability. (See, e.g., U.S. Patent No. 5,874,468 to D. Atlas et al.). NAC amide is also superior to NAC and GSH in crossing the cell membrane, as well as the blood-brain barrier. NAC amide can be prepared as described in U.S. Patent No. 6,420,429 to D. Atlas et al., the contents of which are incorporated by reference herein.

NAC amide may function directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells from free-radical mediated damage. NAC amide is a potent cellular antioxidant responsible for maintaining the proper oxidation state within cells. NAC amide is synthesized by most cells and can recycle oxidized biomolecules back to their active reduced forms. As an antioxidant, NAC amide may be as effective, if not more effective, than GSH.

In one embodiment, the present invention encompasses methods and compositions comprising NAC amide for preventing, reducing, protecting, or alleviating glutamate-induced cytotoxicity in neurodegenerative diseases, particularly in neuronal cells and tissues (See, e.g., Example 1). In this embodiment, NAC amide can protect cells of the nervous system from the effects of oxidative toxicity induced by glutamate. Without wishing to be bound by
theory, NAC amide treatment can function to supply GSH as a substrate for GSH peroxidase activity in affected cells. In accordance with the present invention, NAC amide can inhibit lipid peroxidation, scavenge for reactive oxygen species (ROS) and enhance intracellular levels of GSH to combat and overcome oxidative stress. In addition, NAC amide can chelate lead and protect against lead-induced oxidative stress. NAC amide is particularly beneficial and advantageous for neurological disorders and diseases affecting the brain and associated parts thereof, because it more readily crosses the blood-brain barrier to enter the brain and provide its antioxidant effects.

Different neurodegenerative conditions and diseases that can be treated according to this embodiment include cerebral ischemia, Parkinson's disease. NAC amide can be used in the reduction of brain damage during seizures; to provide resistance to induced epileptic seizures; for protection during traumatic brain injury through the effect on mitochondrial function, reduction of inflammation and attenuation of and improvement in re-profusion with decreased re-profusion injury; for reduction of traumatic brain injury; and for treating and/or preventing prion disease, such as Creutzfeldt-Jakob disease and mad cow disease, by acting as an NMDA receptor antagonist, by enhancing intracellular levels of the anti-apoptotic protein Bcl-2; and by increasing antioxidants to glutathione. NAC amide can be used in neural protection, mitochondrial preservation and therapy potential after nerve injury, particularly to prevent primary sensory neuronal death.

In another embodiment, the invention embraces methods and compositions comprising NAC amide for protecting cells and tissues from radiation-induced oxidative stress. In accordance with this embodiment, NAC amide is superior to NAC in protecting tissues from radiation-induced oxidative stress. (Example 2). The medical crisis following the Chernobyl incident and the threat of a terrorist nuclear attack have raised awareness that high-dose total body irradiation may occur and result in death due to the induction of three potentially lethal cerebrovascular, gastrointestinal and hematopoietic clinical syndromes, which result from high dose radiation exposure. The combination of the prodromal syndrome followed by the gastrointestinal syndrome and bone marrow death induces dehydration, anemia, and infection that lead to irreversible shock. Current treatment for the subacute gastrointestinal and hematopoietic syndromes includes supportive therapy such as plasma volume expansion, platelets, and antibiotics to prevent dehydration and infection and promote bone marrow repopulation. Human total body exposure to a radiation dose above 10 Gy has
been regarded as uniformly fatal. With therapeutic intervention, survival may be possible up to 15 Gy of total body irradiation, but beyond 20 Gy the symptoms would not be manageable.

The systemic damage observed following irradiation is partially due to the overproduction of reactive oxygen species (ROS), which disrupt the delicate pro-oxidant/antioxidant balance of tissues leading to protein, lipid and DNA oxidation. For example, oxidation of the glucosamine synthetase active site sulphydryl groups is a key factor in the toxicity of the gastrointestinal syndrome. Polyunsaturated fatty acids, when exposed to ROS, can also be oxidized to hydroperoxides that decompose in the presence of metals to hydrocarbons and aldehydes such as malondialdehyde (MDA). This lipid peroxidation can cause severe impairment of membrane function through increased membrane permeability and membrane protein oxidation. DNA oxidation can lead to strand breakage and consequent mutation or cell death. GSH is the principal intracellular thiol responsible for scavenging ROS and maintaining the oxidative balance in tissues, such as plasma, brain, kidney, liver and lung. In accordance with this embodiment, NAC amide significantly improves GSH levels in these tissues after radiation exposure. (Example 2). The prevention of spinal cord damage resulting from radiation exposure is also encompassed by the use of NAC amide.

In another embodiment, the present invention encompasses methods and compositions comprising NAC amide for stimulating endogenous production of cytokines and hematopoietic factors, comprising administering or introducing NAC amide to cells, tissues, and/or a subject in need thereof for a period of time to stimulate the endogenous production. NAC amide can be used to stimulate production of cytokines and hematopoietic factors, such as but not limited to, TNF-a, IFN-a, IFN-β, IFN-γ, IL-1, IL-2, IL-6, IL-10, erythropoietin, G-CSF, M-CSF, and GM-CSF, which are factors that modulate the immune system and whose biological activities are involved in various human diseases, such as neoplastic and infectious diseases, as well as those involving hematopoiesis and immune depressions of various origin (such as, without limitation, erythroid, myeloid, or lymphoid suppression).

As used herein, "endogenous" means naturally occurring within a cell, tissue, or organism, or within a subject.

In another embodiment, the present invention encompasses methods and composition comprising NAC amide for detecting NAC-amide responsive changes in gene expression in a cell, tissue, and/or a subject, comprising administering or introducing NAC amide or derivative of NAC amide to the cell, tissue, and/or subject for a period of time to induce changes in gene expression and detecting the changes in gene expression. The cell can be an
endothelial cell, smooth muscle cell, immune cell such as erythroid, lymphoid, or myeloid cell, progenitors of erythroid, lymphoid, or myeloid cells, epithelial cell, fibroblasts, neuronal cell and the like. The tissue can be any tissue of the subject, such as hair, skin, or nail tissue, vascular tissue, brain tissue, among many others. Preferably, the changes in gene expression are detected by microarray analysis, but other detection means can encompass, without limitation, reverse-transcription polymerase chain reaction (RT-PCR), Northern Blotting, immunofluorescence, immunoblotting, or enzyme-linked immunosorbent assay, all of which are familiar techniques to those skilled in the art.

NAC amide and derivatives of NAC amide can induce changes in, for example, endothelial cells that are indicative of an anti-angiogenic effect. NAC has been shown to inhibit chemotaxis of endothelial cells in culture, and produce anti-angiogenic effects, such as modulation of genes responsible for blood vessel growth and differentiation, through its antioxidant effects and upregulation of angiostatin (Pfeffer, U. et al, (2005) Mut. Res. 591 : 198-211). Thus, NAC amide and NAC amide derivatives can be used to inhibit angiogenesis as an anti-cancer agent, for example, by preventing or inhibiting tumor growth and metastasis.

Cells, tissues, and/or a subject can be exposed to stimuli in the presence of NAC amide or derivatives of NAC amide. Stimuli include, for example, cells cultured in the presence of chemotactic or chemoattractant agents, like chemokines CXCL1-16, CCL1-27, XCL1, XCL2, RANTES, MIP 1-5 (alpha, beta, and gamma isoforms), MCP-1 through 5, and the like. Cells, tissues, and subjects can also be stimulated with pharmaceutical agents, drugs, or treatment modalities. After stimulation, DNA, RNA, or protein can be isolated from the cells, tissues, and/or subject, and changes in gene expression can be detected. For example, total RNA can be isolated from cells according to standard techniques known in the art and resultant cDNAs can be synthesized and subsequently hybridized to a solid support, such as a silicon chip for microarray analysis. Expression data and changes in the expression of genes in response to the stimuli can then be analyzed using computer software programs, such as GeneSpring (Silicon Genetics).

Non-limiting examples of such genes that exhibit changes in their expression include genes involved in or pertaining to cellular adhesion, apoptosis, chemokine and cytokine biosynthesis, synthesis of extracellular matrix components, endothelium, inflammation, MAP kinases, metalloproteinases, NF-κB, nitric oxide, transforming growth factor (TGF) signaling, and blood vessels. Pfeffer et al reported that a plurality of NAC-responsive genes
that are modulated (i.e., up- or downregulated) include HSP40 (heat shock protein 40; DnaJ homolog), SERCA2 (Ca2+ transporting ATPase in cardiac muscle), MKP2 (MAP kinase phosphatase), TIP30 (HIV-1 Tat interactive protein 2), BTG1 (B-cell translocation gene 1), TXL (thioredoxin-like), CRADD (Death receptor adaptor protein), WSX1 (Class I cytokine receptor), EMAP2 (endothelial monocyte-activating protein), Jagged 1 (ligand for Notch receptor), MEA5 (hyaluronoglucosaminidase), VRNA (Integrin αV), COL4A1 (Type IV collagen α1), uPA (urokinase plasminogen activator), CPE (carboxypeptidase E), TSPAN-6 (transmembrane 4 superfamily member 6), FGFB (basic fibroblast growth factor), I-TRAF (TRAf interacting factor), CDHH (cadherin 13), ILIORB (Interleukin-10 receptor β), MAP-1 (modulator of apoptosis 1), hCOX-2 (cyclooxygenase-2), CAS-L (Cas-like docking protein), CED-6 (CED-6 protein), CX37 (gap junction protein c4), ABCG1 (ATP-binding cassette protein, subfamily G), TRAIL (TNF ligand superfamily member 10), and ESEL (endothelial adhesion molecule 1; Selectin E), as well as CHOP (DNA-damage-inducible transcript 3), PIM2 (pim-2 oncogene, MIF-1 (homocysteine-inducible protein), PIG-A (phosphatidylinositol glycan, class A), KIAA0062, HK2 (hexokinase 2), UDPGDH (UDP-glucose dehydrogenase), ERF2 (Zinc finger protein 36, C3H type-like 2), RAMP (Zinc finger protein 198), Docl (Downregulated in ovarian cancer 1), GBP-1 (Guanylate-binding protein 1, interferon-inducible), GR (glucocorticoid receptor), ENH (LIM protein - enigma homolog), Id-2H (Inhibitor of DNA binding 2), BPGM (2,3-bisphosphoglycerate mutase), HOXA4 (Homeobox A10), EFNB2 (ephrin-B2), ART4 (Dombrock blood group), KIAA0740 (Rho-related BTB domain containing protein 1).

In another embodiment, the present invention encompasses methods and compositions comprising NAC amide for stimulating macrophages and neutrophils to phagocytize infectious agents and other foreign bodies and to eliminate microorganisms, mediated by reactive oxygen species and proteases. NAC amide can be used to improve macrophage function by increasing glutathione availability, which, in turn, will improve alveolar function in fetal alcohol syndrome and to augment premature alveolar macrophage function.

In another embodiment, the invention encompasses methods and compositions comprising NAC amide to increase levels of intracellular reduced glutathione levels, which blocks the formation of irreversibly sickled cell red blood cells. Methods involving the administration of NAC amide to prevent and treat sickle cell anemia and thalassemia are provided.
In another embodiment, the invention encompasses methods and compositions comprising NAC amide to treat leishmania through the mechanism of histopathological modulation, in which cytokine pattern is modified as demonstrated by a sustained higher frequency of interferon-γ (IFN-γ) and tumor necrosis factor alpha producing cells. NAC amide is used in the modulation of effector responses in animals, in conjunction with bi-glutathione.

In an embodiment, NAC amide is used to down-regulate cytokine synthesis, activation and downstream processes and/or to exert an antagonistic effect on pro-inflammatory signals. Such an effect is beneficial in the treatment of many diseases in which cytokines participate in the pathophysiology of the disease. For example, cytokines, which are mediators of oxidative stress, can alter the redox equilibrium by affecting GSH/oxidized glutathione disulfide (GSSG) shuttling and recycling. (For a review of the glutathione-mediated regulation of cytokines and the role of antioxidants, see, J.J. Haddad, 2005, Mol. Immunol, 42(9):987-1014; and J.J. Haddad, 2002, Cellular Signalling, 14(11):879-897).

Additionally, liver injury related to the administration of certain drugs can be initiated or intensified by inflammation states that stimulate unregulated production of proinflammatory cytokines or growth factors, such as interferon γ, which leads to the down-regulation of enzymes and proteins involved in drug metabolism and elimination. NAC amide, or derivative thereof as an agent that can decrease proinflammatory cytokine levels, is thus useful for preventing and/or managing drug-induced hepatocytotoxicity.

In another embodiment, the invention encompasses methods and compositions comprising NAC amide or a derivative thereof for use as a chemoprotectant against bone marrow toxicity after or during chemotherapy, including alkylators with or without glutathione depletion.

In another embodiment, the invention encompasses methods and compositions comprising NAC amide or a derivative thereof to treat various aspects of sepsis, particularly bacterial sepsis and septic shock, including gram-negative septic shock. NAC amide and its derivatives can act as an inhibitor of the nuclear factor NF-κB, which prevents staphylococcal enterotoxin A (SCC) fever by acting through the human peripheral blood mononuclear cells to block the stimulation and synthesis or release pyrogenic cytokines and to block inflammatory sponsors through the regulation of genes in coding for proinflammatory cytokines. In accordance with this embodiment, NAC amide or a derivative thereof is used to block lipid peroxidation and to improve the disease status in children with acute purulent
meningitis and encephalitis. NAC amide and its derivatives can be used to block pertussis
toxin secretion by Bordetella pertussis and for the treatment of lethal sepsis by limiting
inflammation and potentiating host defense. Because decreased bacterial colonies improve
survival, migration of neutrophils to the site of infection and to a distant site is upregulated
and optimal GSH levels are important for an efficient response to sepsis. In addition, ROS
release by immune cells is important mediators in sepsis and septic shock. During a normal
immune response antioxidant serves to down-regulate the ongoing immune response mostly
through modulation of proinflammatory mediators.

In another embodiment, methods and compositions comprising NAC amide or a
derivative thereof can be used in the treatment of infection and disease caused by
microorganisms and the like, such as bacteria, parasites, nematodes, yeast, fungi, plasmodia,
mycoplasma, spores, and the like, e.g., malarial infections and tuberculosis and rickettsia
infection. In a related aspect, it has recently been found that infection by a number of types of
bacteria, such as Streptococcus, Staphylococcus, Salmonella, Bacillus (Tubercule bacillus)
etc., which cause diseases in humans, induce a direct response by leukocytes (i.e., white
blood cells) in the body, to increase their levels of hypoxia inducible transcription factor-1, or
HIF-1. The HIF-1 protein binds to cellular DNA and activates specific genes to help cells
function in a low oxygen environment. HIF-1, in turn, stimulates the white blood cells to
produce and release antimicrobial compounds, e.g., small proteins, enzymes and nitric oxide,
that work together to kill bacteria. In addition, it has been found that low oxygen levels,
which occur at the site of an infection, activate HIF-1 in macrophages and neutrophils, which
typically ingest and destroy invading microorganisms. The greater the increase in HIF-1
levels in the white blood cells, the greater their anti-bacterial activity. In accordance with this
aspect of the invention and in view of the influence of HIF-1 in regulating the killing
functions of white blood cells, an alternative to the direct killing of bacteria, etc. is to use
agents, e.g., small molecules, that promote HIF-1 activity in white blood cells to boost their
bacterial killing ability, thereby promoting a resolution to infection through the actions of the
immune system's natural defense mechanisms. One such agent is NAC amide, which can be
used in a method of killing or inhibiting the growth of microorganisms by increasing cellular
levels of HIF-1, i.e., HIF-1α, thereby enhancing the capacity of white blood cells, such as
macrophages, to kill the microorganisms. Because N-acetyl-L-cysteine, NAC, a glutathione
(GSH) precursor and a ROS scavenger, which does not possess the enhanced properties of
lipophilicity and cell permeability of NAC amide, has been shown to induce HIF-1 α in
epithelial cells (J.J.E. Haddad et al., 2000, J. Biol. Chem., 275:21 130-21 139), the use of NAC amide to modify HIF-1α production in white blood cells in order to activate the bacterial killing potential of these cells is embraced as an improved antioxidant treatment provided by the present invention. The present invention is further directed to the use of NAC amide or a derivative thereof as a bacteriostatic agent when used as a treatment for bacterial infection, particularly antibiotic resistant, or multi-antibiotic resistant bacteria such as tuberculosis-causing microorganisms.

In a related embodiment, the present invention is directed to the use of NAC amide or a derivative thereof as a biodefensive agent for inducing the killing of infecting or contaminating microorganisms. These types of microorganisms may pose a severe health threat if they should be disseminated to the public and/or genetically altered so as to be antibiotic resistant. The following lists set forth categories of microorganisms, viruses, diseases and agents for which NAC amide or its derivative is provided as a suitable countermeasure, used alone, or in combination with other active compounds, agents and substances to treat affected organisms and/or cells thereof:

Infectious Diseases: Aflatoxins, Alphavirus Eastern equine encephalitis virus, Alphavirus Venezuelan equine encephalitis virus, Antibiotic-resistant Mycobacterium tuberculosis, Arenavirus Junin Virus, Arenavirus Lassa Virus, Ascaris lumbricoides (roundworm), Avian influenza, Bacillus anthracis (anthrax), Borrelia, Brucella, Burkholderia mallei (glanders), Chlamydia psittaci (parrot fever), Chlamydia trachomatis (Trachoma), Clostridium botulinum (botulism), Clostridium perfringens (gas gangrene), Coccidiodomycosis immitis, Coxiella burnetti (Q fever), Cryptosporidium parvum, Dinoflagellate neurotoxin (Paralytic Shellfish Toxin), Drancunculus medianensis (guinea worm), Ebola virus, Entamoeba histolytica (amoebiasis), Epsilon toxin of Clostridium perfringens, Escherichia coli, Flavivirus Yellow Fever virus (e.g., West Nile virus, Dengue), Francisella tularensis (tularemia), Giardia lamblia (giardiasis), Hantavirus, Henipavirus Nipah virus (Nipa encephalitis), HIV and AIDS, Influenza, Leishmania donovane, Marburg virus, Methicillin-resistant staphylococcus aureus (MRSA), Mycobacterium lepra (leprosy), Mycobacterium ulcerans (Burulu ulcer), Nairobi virus Crimean-Congo hemorrhagic fever virus, Necator Americanus / Ancylostoma duodenale (hookworm), Onchocerca volvulus (river blindness), Orthopox virus, Pathogenic Haemophilus, Pathogenic Salmonella, Pathogenic Shigella, Pathogenic Streptococcus, Phlebovirus Rift Valley fever virus, Plasmodium falciparum, P. ovale, P. vivax, P. malariae (malaria), Ricin toxin (castor bean
oil), Rickettsia rickettsii (Rocky Mountain Spotted Fever), Rickettsia typhi (typhus), Salmonella typhi (typhoid fever), Schistosoma mansoni, S. haematobium, S. japonicum, Shigella dysenteriae, Smallpox, Staphylococcus enterotoxin B, Tickborne encephalitis virus, Tickborne hemorrhagic fever viruses, Toxoplasma gondii, Treponema, Trichothecene Mycotoxins, Trichuris trichiura (whipworm), Trypanosoma brucei, T. gambiense or T. rhodesiense, Vibrio species (cholera), Wuchereria bancrofti and Brugia malayi, Yersinia pestis (black death).

Other Threats: Blister agents, including Lewisite, nitrogen and sulfur mustards; Blood agents, including hydrogen cyanide and cyanogens chloride; Exotic agents, including hybrid organisms, genetically modified organisms, antibiotic-induced toxins, autoimmune peptides, immune mimicry agents, binary bioweapons, stealth viruses and bioregulators and biomodulators; Heavy metals, including arsenic, lead and mercury; incapacitating agents, including BZ; nerve agents, including Tabun, Sarin, Soman, GF, VX, V-gas, third generation nerve agents, organophosphate pesticides and carbamate insecticides; nuclear and radiological materials, pulmonary agents, including phosgene and chorine vinyl chloride; volatile toxins, including benzene, chloroform and trihalomethanes. In accordance with the present invention, NAC amide or derivatives thereof can serve as an innovative treatment for known and emerging natural infectious disease threats, as well as trauma, e.g., excessive bleeding and other events, associated with and/or resulting from an act of bioterrorism.

Illustratively, Rickettsia, which causes the pathogenesis of typhus and spotted fever rickettsioses, results in serious adverse vascular and hemorrhagic conditions, (e.g., increased vascular permeability and edema) notably in the brain and lung, following its entry into vascular endothelial cells. R. rickettsii-infected endothelial cells produce ROS causing peroxidative damage to cell membranes. (D.J. Silverman et al., 1990, Ann. N.Y. Acad. Sci., 590:1 11-1 17; D.H. Walker et al., 2003, Ann. N.Y. Acad. Sci., 990:1-1 1). Because the oxidative-stress mediated damage to R. rickettsii-infected endothelial cells is associated with the depletion of host components such as GSH and levels of catalase that act as host defenses against ROS-induced damage, the concentration of hydrogen peroxide and ROS increase in the cells to cause ROS-induced cellular damage. In a similar manner, cells, e.g., fibroblasts that are infected with Mycoplasma (e.g., Mycoplasma pneumoniae) also produce increased intracellular levels of hydrogen peroxide and decreased levels of catalase, resulting in oxidative stress that can lead to death of the infected cells. (M. Almagor et al., 1986, Infect. Immun., 52(1):240-244). To provide an ameliorating effect of oxidative stress induced in
cells by infecting microorganisms such as Rickettsia, Mycoplasma, etc., NAC amide or a
derivative thereof is provided to an infected host as an antioxidant therapeutic. NAC amide
administration to cells and/or organisms (e.g., infected host mammals) in accordance with the
present invention, alone or in combination with other agents and/or antioxidants, can limit the
amount and/or extent of oxidative damage that is induced by microbial infection.

In another embodiment, the invention encompasses methods and compositions
comprising NAC amide or a derivative thereof for use in preventing periventricular
leukomalacia (PVL). NAC amide or a derivative thereof may provide neural protection and
attenuate the degeneration of OPCs against LPS evoked inflammatory response in white
matter injury in developing brain. Moreover, NAC amide or a derivative thereof may be used
as a treatment for placental infection as a means of minimizing the risk of PVL and cerebral
palsy (CP).

In another embodiment, the invention encompasses methods and compositions
comprising NAC amide or a derivative thereof for the treatment of osteoporosis. The tumor
tumor necrosis factor member RANKL regulates the differentiation, activation and survival of
osteoclasts through binding of its cognate receptor, RANK. RANK can interact with several
TNF-receptor-associated factors (TRAFs) and activate signaling molecules including Akt,
NF-κB and MAPKs. Although the transient elevation of reactive oxygen species by receptor
activation has been shown to act as a cellular secondary messenger, the involvement of ROS
in RANK signal pathways has not been characterized. RANKL can stimulate ROS
generation and osteoclasts. According to this embodiment, NAC amide can be used to
pretreat or treat osteoclasts so as to achieve a reduction in RANKL-induced Akt, NF-κB, and
ERK activation. The reduced NF-κB activity by NAC amide may be associated with
decreased IKK activity and IκBα phosphorylation. Pretreatment with NAC amide or a
derivative thereof can be used to reduce RANKL-induced actin ring formation required for
bone resorbing activity and osteoclast survival. The methods and compositions comprising
NAC amide or a derivative thereof can be used for the improvement of osteoporosis through
blockage and interference with osteoclasts, and to lower reactive oxidative stress levels so as
to have beneficial effects on preventing bone loss by reducing RANKL-induced cellular
function.

In a related embodiment, NAC amide or a derivative thereof is used in the treatment
of osteoporosis by blockage of thiol thioredoxin-1, which mediates osteoclast stimulation by
reactive oxidation species (ROS), as well as blockage of TNF-α, which causes loss of bone, particularly in circumstances of estrogen deficiency.

In another embodiment, the invention embraces methods and compositions comprising NAC amide or a derivative thereof are used for the treatment of polycystic ovary syndrome. NAC amide or a derivative thereof may also be used as a therapeutic agent to ameliorate the homocysteine and lipid profiles in PCOS-polycystic ovary syndrome.

In another embodiment, the invention encompasses the use of NAC amide or a derivative thereof in treatments and therapies for toxin exposure and conditions related thereto, e.g., sulfur mustard (HD-induced lung injury). Treatment of individuals having been exposed to toxins or suffering from toxin exposure with NAC amide or a derivative thereof may reduce neutrophil counts to achieve a decreased inflammatory response. NAC amide and its derivatives may be useful as a treatment compound for patients having sulfur mustard vapor exposure induced lung injury. Administration of NAC amide or a derivative thereof can be either orally or as a bronchoalveolar lavage. As an agent having anti-glutamate toxin activity, NAC amide and its derivatives are useful in methods and compositions for the blockage of brain and/or lung damage and cognitive dysfunction in mechanical warfare agents including CW, vesicants, sulfur mustard, nitrogen mustards, chloroethyl amine, lewisite, nerve agents O-ethyl S-(2-[di-isopropylamino] ethyl) methyl phosphorothioate (VX), tabun (GA) and sarin (GB) and soman DG and the blood agents cuianogenchloride, and in the prevention of organophosphate induced convulsions and neuropsychopathological damage.

In another embodiment, the present invention encompasses methods and compositions comprising NAC amide for use in the treatment of burn trauma. NAC amide or a derivative thereof can block NF-κB, which has been shown to reduce burn and burn sepsis. NAC amide or a derivative thereof can be used to protect microvascular circulation, reduce tissue lipid peroxidation, improve cardiac output and reduce volume of required fluid resuscitation. NAC amide or a derivative thereof can be used in the prevention of burn related cardiac NF-κB nuclear migration, and improve cardiomyocyte secretion of TNF-α, IL-1β, and IL-6 and to improve cardiac malfunction. An association between cellular oxidative stress and burn-mediated injury provides an avenue for administering NAC amide or a derivative thereof as an antioxidant that can inhibit free radical formation and/or scavenge free radicals to protect tissues and organs in patients with burn injury.
In another embodiment, the present invention encompasses methods and compositions comprising NAC amide or a derivative of NAC amide for use in the prevention of lung injury due to the adverse effects of air pollution, smoke inhalation, poison gas or diesel exhaust particles.

In another embodiment, the present invention encompasses methods and compositions comprising NAC amide or a derivative thereof for use in the treatment and therapy of cardiovascular disease and conditions. NAC amide and its derivatives can be used as a blocker of angiotensin-converting enzyme. In acute myocardial infarction, NAC amide or a derivative thereof can be used to decrease oxidative stress, and to cause more rapid reperfusion, better left ventricular preservation, reduced infarct size, better preservation of global and regional left ventricular function and modification of QSR complex morphology and ECG. NAC amide or a derivative thereof can also be used in the treatment of focal cerebral ischemia with protection of the brain and reduction of inflammation in experimental stroke. NAC amide can be used in the treatment of reperfusion injuries, as well as apoptosis of myocardial endothelial cells and interstitial tissue. As a nutriceutical, NAC amide or a derivative thereof may assist in the elevation of nitric oxide levels, play an important role in the management of cardiovascular disease, reduce chronic inflammation in cardiovascular disease and prevent restenosis of cardiovascular stents placed in coronary arteries and carotid arteries. NAC amide and its derivatives can be used in the prevention of cardiac failure following MI and cardiomyopathy due to prevention of oxidative stress and improvement of left ventricular remodeling. Use of NAC amide or a derivatives of NAC amide in this capacity supports the involvement of oxidative stress in myocardial vascular dysfunction and hypertension and provides a role for antioxidant strategies to preserve the myocardial microvasculature. NAC amide or a derivative thereof can also be used in the prevention of oxidized proteins in muscles.

In another embodiment of the present invention, method and compositions comprising NAC amide or a derivative thereof can be used to treat arterial sclerosis and to increase high density lipoprotein (HDL)-cholesterol serum levels in hyperlipidemic and normal lipidemic individuals with documented coronary stenosis. NAC amide or a derivative thereof can also be used to decrease coronary and alpha-beta stress; to prevent further myocardial infarctions; and to cause a reduction in body fat thereby improving glucose tolerance, particularly in overweight or obese individuals. NAC amide or a derivative thereof be used to improve muscular performance and decrease levels of tumor necrosis factor in old age.
In other embodiments, the present invention is directed to the use of method and compositions comprising NAC amide or a derivative thereof in the treatment of thalassemic blood by ameliorating oxidative stress in platelets. The activation of platelets causes thromboembolic consequences and produces a hypercoagulable state that is amenable to treatment by the antioxidant NAC amide or a derivative thereof. In an embodiment, NAC amide or a derivative thereof is useful as a wound dressing to permit enhancement of neutrophil function. In an embodiment, NAC amide or a derivative thereof is used to block the effects of leptin, which is a cardiovascular risk factor in diabetic patients. In an embodiment, NAC amide or a derivative thereof is used in the treatment of total plasma homocysteine and cysteine levels with increased urinary excretion, as well as in the treatment for hyperhomocysteinemic conditions, to improve oxidative stress. It has been found that elevated levels of homocysteine pose a significant risk in vascular disease, such as atherosclerosis, venous thrombosis, heart attack and stroke, as well as neural tube defects and neoplasia. Homocysteine promotes free radical reactions. In patients with defective homocysteine metabolism, relatively high levels of homocysteine are present in the blood. Thus, in accordance with this invention, NAC amide or a derivative thereof is administered to patients with elevated homocysteine levels. In an embodiment, NAC amide or a derivative thereof is used as a chemoprotectant against bone marrow toxicity after or during chemotherapy, e.g., alkylators, with or without accompanying glutathione depletion. In an embodiment, NAC amide or a derivative thereof is used in the treatment of lithium induced renal failure. In an embodiment, NAC amide or a derivative thereof is used in the treatment of prostatic inflammation, which may contribute to prostatic carcinogenesis and inflammation.

In another embodiment, NAC amide or a derivative thereof is used in pulmonary disease medicine, particularly in oxygen-mediated lung disease. NAC amide or a derivative thereof can improve oxygenation in cardiopulmonary bypass during coronary artery surgery and is useful in the treatment of chronic obstructive pulmonary disease and pulmonary hypertension. In an embodiment, NAC amide or a derivative thereof is used in the treatment of injury in the lung due to high-energy impulse noise-blasts, which can induce antioxidant depletion. Thus, the administration of NAC amide or its derivatives provide an advantageous antioxidant source. NAC amide or a derivative thereof is particularly useful if provided as a supplement prior to noise blast exposure. NAC amide or a derivative thereof is useful in the treatment of asthma with increased oxidative stress. NAC amide or a derivative thereof is
useful for the treatment of adult respiratory distress syndrome; in the treatment of pulmonary fibrosis, in the treatment of idiopathic pulmonary fibrosis and asbestos exposure; and in the treatment of chronic lung rejection. Further, NAC amide or a derivative thereof is contemplated for use in occupational isocyanate exposure and the development of isocyanate allergy, which is believed to develop by two processes, namely, isocyanate-protein conjugation and airway epithelial cell toxicity. More specifically, NAC amide or a derivative thereof can serve to protect against hexamethylene diisocyanate (HDI) conjugation to cellular proteins and to reduce HDI toxicity to human airway epithelial cells following isocyanate exposure. Thus, NAC amide or a derivative thereof can help to prevent the development of allergic sensitization and asthma that are associated with this occupational hazard.

In another embodiment, the present invention encompasses the use of NAC amide or a derivative thereof to inhibit HIV replication in chronically and acutely infected cells. NAC amide can be used in GSH replacement therapy, as NAC amide and its derivatives may interfere with the expression of the integrated HIV genome, thus, attacking the virus in a manner that is different from that of the currently employed anti-retrovirals, e.g., AZT, ddI, ddC or D4T. NAC amide or a derivative thereof can also be beneficial in countering the excess free radical reactions in HIV infection, which may be attributable to: 1) the hypersecretion of TNF-a by B-lymphocytes in HIV infection, and 2) the catalysis of arachidonic acid metabolism by the gp 120 protein of HIV. The physiologic requirements for antioxidants by key cell types of the immune system, and the ability of macrophages to take up intercellular antioxidants, as well as to metabolically interact with T-lymphocytes to indirectly cause their antioxidant levels to increase, offer additional reasons that NAC amide or a derivative thereof is useful for correcting antioxidant deficiency in patients with HIV/AIDS. NAC amide and its derivatives can serve as a suppressant of viral and bacterial species in vaginal tissues by the use of intravaginal placement of gel induced thiol.

Because HIV is known to start pathologic free radical reactions which lead to the destruction of antioxidant molecules, as well as the exhaustion of GSH and destruction of cellular organelles and macromolecules, NAC amide and its derivatives can be used to restore antioxidant levels in a mammal in need thereof, to arrest the replication of the virus at a unique point, and specifically prevent the production of toxic free radicals, prostaglandins, TNF-a, interleukins, and a spectrum of oxidized lipids and proteins that are immunosuppressive and cause muscle wasting and neurological symptoms. The
administration of NAC amide or a derivative thereof to elevate or replace antioxidant levels could slow or stop the diseases progression safely and economically.

Because certain viral infections, such as infection by HIV, are associated with reduced antioxidant levels, an aspect of this invention is to increase intracellular levels of antioxidant in infected cells, as well as to increase extracellular of antioxidant, by introducing or administering AD3 so as to interfere with the replication of HIV and to prevent, delay, reduce or alleviate the cascade of events that are associated with HIV infection. Because AIDS may also be associated with reduced GSSG levels, providing an amount of NAC amide to cells and/or to an individual in need thereof, can overcome any interference with de novo synthesis of antioxidant such as GSH, as well as the oxidation of existing GSH, which may occur in HIV infected cells. In accordance with the present invention NAC amide or a derivative thereof is used to inhibit cytokine-stimulated HIV expression and replication in acutely infected cells, chronically infected cells, and in normal peripheral blood mononuclear cells. NAC amide or derivatives thereof can be used to effect concentration-dependent inhibition of HIV expression induced by TNF-α or IL-6 in chronically infected cells. Due to NAC amide’s superior ability to cross cellular membranes and enhanced lipophilic properties, NAC amide and derivatives thereof can be used at lower concentrations as compared to NAC or GSH, such as 2-fold, 5-fold, 10-fold, 100-fold, 1000-fold, 10,000-fold or lower, concentrations.

Further, the depletion of antioxidants by HIV in infected cells is also associated with a process known as apoptosis, or programmed cell death. By providing NAC amide or a derivative thereof to HIV infected individuals and/or cells, the intercellular processes, which artificially deplete GSH and which may lead to cell death can be prevented, interrupted, or reduced. Similarly, the NAC amide thiol can be used as a blocker of bio-replication from West Nile Virus and protection of cells from the cytopathic effect after infection of West Nile Virus, as well as other RNA and DNA virus infections.

In accordance with the invention, NAC amide or a derivative thereof may be administered by several routes that are suited to the treatment or therapy method, as will be appreciated by the skilled practitioner. Nonlimiting examples of routes and modes of administration for NAC amide and its derivatives include parenteral routes of injection, including subcutaneous, intravenous, intramuscular, and intrasternal. Other modes of administration include, but are not limited to, oral, inhalation, topical, intranasal, intrathecal, intracutaneous, ophthalmic, vaginal, rectal, percutaneous, enteral, injection cannula, timed
release and sublingual routes. Administration of NAC amide and its derivatives may also be achieved through continuous infusion. In one embodiment of the present invention, administration of NAC amide and its derivatives may be mediated by endoscopic surgery. For the treatment of various neurological diseases or disorders that affect the brain, NAC amide or a derivative thereof can be introduced into the tissues lining the ventricles of the brain. The ventricular system of nearly all brain regions permits easier access to different areas of the brain that are affected by the disease or disorder. For example, for treatment, a device, such as a cannula and osmotic pump, can be implanted so as to administer a therapeutic compound, such as NAC amide, or derivative thereof as a component of a pharmaceutically acceptable composition. Direct injection of NAC amide and its derivatives are also encompassed. For example, the close proximity of the ventricles to many brain regions is conducive to the diffusion of a secreted or introduced neurological substance in and around the site of treatment by NAC amide.

For administration to a recipient, for example, injectable administration, a composition or preparation formulated to contain water-soluble NAC amide or a derivative thereof is typically in a sterile solution or suspension. Alternatively, NAC amide or a derivative thereof can be resuspended in pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives, stabilizers, and material for rendering the solution or suspension isotonic with body fluids (i.e. blood) of the recipient. Non-limiting examples of excipients suitable for use include water, phosphate buffered saline (pH 7.4), 0.15M aqueous sodium chloride solution, dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inorganic acids, and organic acids, which may be used either on their own or as admixtures.

Formulations comprising NAC amide or a derivative thereof for topical administration may include but are not limited to lotions, ointments, gels, creams, suppositories, drops, liquids, sprays and powders. NAC amide or a derivative thereof may be administered to mucous membranes in the form of a liquid, gel, cream, and jelly, absorbed into a pad or sponge. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Compositions comprising NAC amide or a derivative thereof for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, sachets, capsules or tablets. Thickeners, diluents, flavorings, dispersing aids, emulsifiers or binders may be desirable. Formulations for
parenteral administration may include, but are not limited to, sterile solutions, which may also contain buffers, diluents and other suitable additives.

The present invention also provides a food additive comprising NAC amide or a derivative thereof for mammalian, preferably human, consumption. NAC amide and other cysteine derivatives have been detected in many different food products, including but not limited to, garlic, peppers, turmeric, asparagus, and onions. See, for example, Hsu, C.C., et al, (2004) J. Nutr. 134:149-152 and Demirkol, O. et al, (2004) J. Agric. Food Chem. 52. The food additive can comprise NAC amide or its derivative in a liquid or solid material intended to be added to a foodstuff. The food additives can be added to "food compositions" including any products—raw, prepared or processed—which are intended for human consumption in particular by eating or drinking and which may contain nutrients or stimulants in the form of minerals, carbohydrates (including sugars), proteins and/or fats, and which have been modified by the incorporation of a food additive comprising NAC amide or a derivative of NAC amide as provided herein. The present modified food compositions can also be characterized as "functional foodstuffs or food compositions". "Foodstuffs" can also be understood to mean pure drinking water.

The term "food additive" is understood to mean any a liquid or solid material intended to be added to a foodstuff. This material can, for example, have a distinct taste and/or flavor, such as a salt or any other taste or flavor potentiator or modifier. It is to be noted, however, that the food additive comprising NAC amide or a NAC amide derivative does not necessarily have to be an agent having a distinct taste and/or flavor.

Other food additives that can be added in combination with NAC amide, or in food additive formulations of NAC amide include, but are not limited to, acids which are added to make flavours "sharper", and also act as preservatives and antioxidants, such as vinegar, citric acid, tartaric acid, malic acid, fumaric acid, lactic acid, acidity regulators, anti-caking agents, antifoaming agents, antioxidants such as vitamin C and tocopherols such as vitamin E, bulking agents, such as starch are additives, food coloring, color retention agents, emulsifiers flavors, flavor enhancers, humectants, preservatives, propellants, stabilizers, thickeners and gelling agents, like agar or pectin, and sweeteners.

Doses, amounts or quantities of NAC amide, or derivative thereof as well as the routes of administration used, are determined on an individual basis, and correspond to the amounts used in similar types of applications or indications known to those having skill in the art. As is appreciated by the skilled practitioner in the art, dosing is dependent on the severity
and responsiveness of the condition to be treated, but will normally be one or more doses per day, with course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Persons ordinarily skilled in the art can easily determine optimum dosages, dosing methodologies and repetition rates. For example, a pharmaceutical formulation for orally administrable dosage form can comprise NAC amide, or a pharmaceutically acceptable salt, ester, or derivative thereof in an amount equivalent to at least 25-500 mg per dose, or in an amount equivalent to at least 50-350 mg per dose, or in an amount equivalent to at least 50-150 mg per dose, or in an amount equivalent to at least 25-250 mg per dose, or in an amount equivalent to at least 50 mg per dose. NAC amide or a derivative thereof can be administered to both human and non-human mammals. It therefore has application in both human and veterinary medicine.

Examples of suitable esters of NAC amide include alkyl and aryl esters, selected from the group consisting of methyl ester, ethyl ester, hydroxyethyl ester, t-butyl ester, cholesteryl ester, isopropyl ester and glyceryl ester.

As described herein, a number of conditions, diseases and pathologies are believed to be associated with reduced intracellular antioxidant levels, including AIDS, diabetes, macular degeneration, congestive heart failure, cardiovascular disease and coronary artery restenosis, lung disease, asthma, virus infections, e.g., toxic and infectious hepatitis, rabies, HIV; sepsis, osteoporosis, toxin exposure, radiation exposure, burn trauma, prion disease, neurological diseases, blood diseases, arterial disease, muscle disease, tumors and cancers. Many of these diseases and conditions may be due to insufficient glutathione levels. Further, exposure to toxins, radiation, medications, etc., may result in free radical reactions, including types of cancer chemotherapy. Accordingly, the present invention provides NAC amide or a derivative thereof as an agent that can treat these diseases and conditions in a convenient and effective formulation, particularly for oral administration. The administration of exogenous NAC amide or a derivative thereof can serve to supplement or replace the hepatic output of GSH and to assist in the maintenance of reduced conditions within the organism. The failure to alleviate free radical reactions allows an undesirable cascade that can cause serious damage to macromolecules, as well as lipid peroxidation and the generation of toxic compounds. Maintaining adequate levels of GSH is necessary to block these free radical reactions. When natural GSH levels are debilitated or jeopardized, NAC amide or a derivative thereof is able to provide efficient and effective remedial action.
NAC amide can form chelation complexes with copper and lead. NAC amide may also form circulating complexes with copper in the plasma. Thus, NAC amide or a derivative thereof can be administered to treat metal toxicity. NAC amide-metal complexes will be excreted, thus reducing the metal load. Thus, NAC amide or a derivative thereof may be administered for the treatment of toxicity associated with various metals, e.g., iron, copper, nickel, lead, cadmium, mercury, vanadium, manganese, cobalt, transuranic metals, such as plutonium, uranium, polonium, and the like. It is noted that the chelation properties of NAC amide are independent from its antioxidant properties. However, because some metal toxicities are free radical mediated, e.g., iron, NAC amide administration may be particularly advantageous for such conditions.

In order to provide high bioavailability, NAC amide or a derivative thereof can be provided in a relatively high concentration in proximity to the mucous membrane, e.g., the duodenum for oral administration. Thus, NAC amide or a derivative thereof can be administered as a single bolus on an empty stomach. The preferred dosage is between about 100-10,000 mg NAC amide or between about 250-3,000 mg NAC amide. Further, the NAC amide or NAC amide derivative formulation can be stabilized with a reducing agent, e.g., ascorbic acid, to reduce oxidation both during storage and in the digestive tract prior to absorption. The use of crystalline ascorbic acid has the added benefit of providing improved encapsulation and serving as a lubricant for the encapsulation apparatus. Capsules, e.g., a two-part gelatin capsule, are dosage forms that protect NAC amide from air and moisture, while dissolving quickly in the stomach. The capsule is preferably a standard two-part hard gelatin capsule of double-0 (OO) size, which may be obtained from a number of sources. After filling, the capsules are preferably stored under nitrogen to reduce oxidation during storage. The capsules are preferably filled according to the method of U.S. Patent No. 5,204,141 incorporated herein by reference in its entirety, using crystalline ascorbic acid as both an antistatic agent and stabilizer. Further, each capsule preferably contains 500 mg of NAC amide and 250 mg of crystalline ascorbic acid. A preferred composition includes no other excipients or fillers; however, other compatible fillers or excipients may be added. While differing amounts and ratios of NAC amide and stabilizer may be used, these amounts are preferable because they fill a standard double-0 capsule, and provide an effective stabilization and high dose. Further, the addition of calcium carbonate is avoided as it may contain impurities and may accelerate the degradation of NAC amide in the small intestine due to its action as a base, which neutralizes stomach acid.
NAC amide or a derivative thereof is advantageously administered over extended periods. Therefore, useful combinations include NAC amide or NAC amide derivatives and drugs intended to treat chronic conditions. Such drugs are well absorbed on an empty stomach and do not have adverse interactions or reduced or variable combined absorption.

One particular class of drugs includes central or peripheral adrenergic or catecholenergic agonists, or reuptake blockers, which may produce a number of toxic effects, including neurotoxicity, cardiomyopathy and other organ damage. These drugs are used, for example, as cardiac, circulatory and pulmonary medications, anesthetics and psychotropic / antipsychotic agents. Some of these drugs also have abuse potential, as stimulants, hallucinogens, and other types of psychomimetics. Other free radical initiation associated drugs include thorazine, tricyclic antidepressants, quinolone antibiotics, benzodiazepines, acetaminophen and alcohol. Accordingly, NAC amide or a derivative thereof can advantageously be provided in an oral pharmaceutical formulation in an amount of between about 50-10,000 mg, along with an effective amount of a pharmacological agent that is capable of initiating free radical reactions in a mammal. The pharmacological agent is, for example, an adrenergic, dopaminergic, serotonergic, histaminergic, cholinergic, gabaergic, psychomimetic, quinone, quinolone, tricyclic, and/or steroid agent.

In the following aspects of the invention, formulations of NAC amide or a derivative thereof provide an advantageous alternative to GSH administration. NAC amide or a derivative thereof offers beneficial properties of lipophilicity and cell-permeability, allowing it to more readily enter cells and infiltrate the blood-brain barrier more readily than GSH, NAC or other compounds. The properties of NAC amide or a derivative thereof may increase its bioavailability following administration to provide an improved treatment for the various diseases, disorders, pathologies and conditions as described herein.

Hepatic glutathione is consumed in the metabolism, catabolism and/or excretion of a number of agents, including aminoglycoside antibiotics, acetaminophen, morphine and other opiates. The depletion of hepatic glutathione may result in hepatic damage or a toxic hepatitis. High dose niacin, used to treat hypercholesterolemia, has also been associated with a toxic hepatitis. The present invention therefore encompasses an oral pharmaceutical formulation comprising NAC amide or a derivative thereof in an amount between about 50-10,000 mg, administered in conjunction with an effective amount of a pharmacological agent that consumes hepatic glutathione reserves.
A number of pathological conditions result in hepatic damage. This damage, in turn, reduces the hepatic reserves of glutathione and the ability of the liver to convert oxidized glutathione to its reduced form. Other pathological conditions are associated with impaired glutathione metabolism. These conditions include both infectious and toxic hepatitis, cirrhosis, hepatic primary and metastatic carcinomas, traumatic and iatrogenic hepatic damage, or resection. The present invention encompasses a pharmaceutical formulation comprising NAC amide or a derivative of NAC amide and an antiviral or antineoplastic agent. The antiviral or antineoplastic agent is, for example, a nucleoside analog.

Glutathione is degraded, and cysteine is excreted, possibly in the urine. Very high doses of glutathione may therefore result in cysteinuria, which may result in cysteine stones. Other long term toxicity or adverse actions may result. Therefore, a daily intake of greater than about 10 gm for extended period should be medically monitored. On the other hand, individual doses below about 50 mg are insufficient to raise the concentration of the duodenal lumen to high levels to produce high levels of absorption, and to provide clinical benefit. Therefore, the formulations according to the present invention have an NAC amide or NAC amide derivative content greater than 50 mg, and are provided in one or more doses totaling up to about 10,000 mg per day.

In the treatment of HIV infection, it is believed that the oral administration of a relatively high dose bolus of glutathione, i.e., 1-3 grams per day, on an empty stomach, will have two beneficial effects. First, HIV infection is associated with a reduction in intracellular glutathione levels in PBMs, lung, and other tissues. It is further believed that by increasing the intracellular glutathione levels, the functioning of these cells may be returned to normal. Therefore, the administration of NAC amide or a derivative thereof according to the present invention will treat the effects of HIV infection. Oral administration of NAC amide, or derivative thereof, optionally in combination with ascorbic acid and/or with an antiretroviral agent. It is noted that the transcription mechanisms and control involved in retroviral infection is believed to be relatively conserved among the different virus types. Therefore, late stage retroviral suppression is expected for the various types of human retroviruses and analogous animal retroviruses. It has also been found in in vitro tests that by increasing the intracellular levels of glutathione in infected monocytes to the high end of the normal range, the production of HIV from these cells may be suppressed for about 35 days. This is believed to be related to the interference in activation of cellular transcription of cytokines, including NF-KB and TNF-α. Therefore, the infectivity of HIV infected persons may be reduced,
helping to prevent transmission. This reduction in viral load may also allow the continued existence of uninfected but susceptible cells in the body.

NAC amide, or derivative thereof administered according to the present method, can be use in the treatment of congestive heart failure (CHF). In CHF, there are believed to be two defects. First, the heart muscle is weakened, causing enlargement of the heart. Second, peripheral vasospasm is believed to be present, causing increased peripheral resistance. NAC amide or a derivative thereof can be effective in enhancing the effects of nitric oxide, and therefore can be of benefit to these patients by decreasing vasoconstriction and peripheral vascular resistance, while increasing blood flow to the tissues. The present invention thus encompasses the oral administration of NAC amide or a derivative thereof in conjunction with a congestive heart failure medication, for example, digitalis glycosides, dopamine, methyldopa, phenoxybenzamine, dobutamine, terbutaline, amrinone, isoproterenol, beta blockers, calcium channel blockers, such as verapamil, propranolol, nadolol, timolol, pindolol, alprenolol, oxprenolol, sotalol, metoprolol, atenolol, acebutolol, bevantolol, tolamolol, labelalol, diltiazem, dipyridamole, bretylium, phenytoin, quinidine, clonidine, procainamide, acecaainide, amiodarone, disopyramide, encainide, flecaainide, lorcaainide, mexiletine, tocainide, captopril, minoxidil, nifedipine, albuterol, parergine, vasodilators, including niopropusside, nitroglycerin, phenolamine, phenoxybenzamine, hydrazazine, prazosin, trimazosin, tolazoline, trimazosin, isosorbide dinitrate, ephythityl tetranitrate, aspirin, papaverine, cyclandelate, isoxsuprine, niacin, nicotinyl alcohol, nyldrin, diuretics, including furosemide, ethacrynic acid, spironolactone, triamterine, amiloride, thiazides, bumetanide, caffeine, theophylline, nicotine, captopril, salalasin, and potassium salts.

In another of its embodiments, the present invention embraces NAC amide or a derivative thereof to treat hepatitis of various types by oral administration. For example, both alcohol and acetaminophen are hepatotoxic and result in reduced hepatocyte glutathione levels. Therefore, these toxicities may be treated according to the present invention with the use of NAC amide or a derivative thereof. NAC amide and its derivatives may also be effective in the treatment of toxicities to other types of cells or organs, which result in free radical damage to cells or reduced glutathione levels.

Diabetes, especially uncontrolled diabetes, results in glycosylation of various enzymes and proteins, which may impair their function or control. In particular, the enzymes which produce reduced glutathione (e.g., glutathione reductase) become glycosylated and non-functional. Therefore, diabetes is associated with reduced glutathione levels, and in fact,
many of the secondary symptoms of diabetes may be attributed to glutathione metabolism defects. According to this invention, NAC amide or a derivative thereof can be used to supplement diabetic patients in order to prevent a major secondary pathology. The present invention also encompasses an oral pharmaceutical formulation comprising NAC amide and an antihyperglycemic agent.

High normal levels of glutathione deactivate opiate receptors. Thus, the administration of NAC amide or a derivative thereof may be of benefit for treating and/or preventing obesity and/or eating disorders, other addictive or compulsive disorders, including tobacco (nicotine) and opiate additions. This invention also encompasses administering NAC amide or a derivative thereof in conjunction with nicotine. The physiologic effects of nicotine are well known. NAC amide or a derivative thereof may cause vasodilation and improve cerebral blood flow, thereby resulting in a synergistic cerebral function-enhancing effect.

In mammals, the levels of glutathione in the plasma are relatively low, in the micromolar range, while intracellular levels are typically in the millimolar range. Therefore, intracellular cytosol proteins are subjected to vastly higher concentrations of glutathione than extracellular proteins. The endoplasmic reticulum, a cellular organelle, is involved in processing proteins for export from the cell. It has been found that the endoplasmic reticulum forms a separate cellular compartment from the cytosol, having a relatively oxidized state as compared to the cytosol, and thereby promoting the formation of disulfide links in proteins, which are often necessary for normal activity. In a number of pathological states, cells may be induced to produce proteins for export from the cells, and the progression of the pathology is interrupted by interference with the production and export of these proteins. For example, many viral infections rely on cellular production of viral proteins for infectivity. The interruption of the production of these proteins will interfere with infectivity. Likewise, certain conditions involve specific cell-surface receptors, which must be present and functional. In both cases, cells that are induced to produce these proteins will deplete reduced glutathione in the endoplasmic reticulum. It is noted that cells that consume glutathione will tend to absorb glutathione from the plasma, and may be limited by the amounts present. Therefore, by increasing plasma glutathione levels, even transiently, the reducing conditions in the endoplasmic reticulum may be interfered with, and the protein production blocked. Normal cells may also be subjected to some interference; however, in viral infected cells, or cells otherwise abnormally stimulated, the normal regulatory
mechanisms may not be intact, and the redox conditions in the endoplasmic reticulum will not be controlled by the availability of extracellular glutathione. The administration of NAC amide or its derivatives may serve to replenish GSH or the effects of GSH and provide significant effects for such conditions.

Reproduction of herpes viruses, which are DNA viruses, is inhibited or reduced in cell culture by the administration of extracellular glutathione. Examples of DNA viruses include Herpes Simplex Virus I, Herpes Simplex Virus II, Herpes zoster, cytomegalovirus, Epstein Barr virus and others. Therefore, according to the present invention, DNA virus and herpes virus infections may be treated by administering NAC amide or a derivative thereof. In addition, infection by the rabies virus, an RNA virus, may be treated by the administration of glutathione. While standard treatments are available, and indeed effective when timely administered, glutathione may be useful in certain circumstances. Therefore, rabies virus infection may be treated, at least in part, by administering NAC amide or a derivative thereof according to the present invention. One available treatment for rabies is an immune serum. The present invention encompasses the parenteral administration of NAC amide, or derivative thereof separately, or in combination with one or more immunoglobulins.

Coronary heart disease risk is increased by the consumption of a high-fat diet and is reduced by the intake of antioxidant vitamins, including vitamin E and vitamin C, as well as flavonoids. High fat meals impair the endothelial function through oxidative stress, resulting in impaired nitric oxide availability. It has been found that vitamin C and vitamin E restore the vasoconstriction resulting from nitric oxide production by endothelium after a high fat meal. Accordingly, their rates of formation will differ, as will the different types of inciting agents that may have to be simultaneously controlled. For example, for those with macular degeneration, continued, unprotected exposure of the eyes to strong sunlight and to tobacco smoke would limit the benefits from an antioxidant used as a therapeutic agent for control of this disease. Therefore, one aspect of the invention provides synergistic therapies to patients by increasing antioxidant levels systemically or in specific organs as well as reducing oxidative, free radical generating and ionizing influences. In this case, NAC amide therapy would be complemented with ultraviolet blocking sunglasses, and a tobacco smoking cessation plan, as necessary. NAC amide or a derivative thereof can be used in combination
with alpha tocopherol succinate, if necessary. Free radicals occur in different parts or subparts of tissues and cells, with different inciting agents. For example, in trauma to the brain or spinal cord, the injurious free radicals are in the fatty (lipid) coverings that insulate nerve fibers, i.e., the myelin sheaths. Extremely high doses of a synthetic corticosteroid, 5 to 10 grams of methyl prednisolone sodium succinate (MPSS), given for just 24 hours, rapidly reach the brain and spinal cord and diffuse rapidly into the myelin, neutralizing the trauma-induced radicals. The present invention therefore provides a pharmaceutical composition comprising a combination of NAC amide or a NAC amide derivative and a glucocorticoid agent.

According to the present invention, orally administered NAC amide or a derivative thereof can raise cell levels of glutathione to inhibit a number of pathologic processes. For example, NAC amide can be used to curtail the virtually self-perpetuating, powerful biochemical cycles producing corrosive free radicals and toxic cytokines that are largely responsible for the signs and symptoms of AIDS. These biochemical cycles destroy considerable quantities of glutathione but they can eventually be brought under control, and normalized with sufficient, ongoing NAC amide therapy. A typical example is the over production of a substance, 15 HPETE (15-hydroperoxy eicosatetraenoic acid), from activated macrophages. 15 HPETE is a destructive, immunosuppressing substance and requires glutathione for conversion into a non-destructive, benign molecule. The problem is that once macrophages are activated, they are difficult to normalize. Once inside cells, GSH curtails the production of free radicals and cytokines, corrects the dysfunctions of lymphocytes and of macrophages, reinforces defender cells in the lungs and other organs and halts HIV replication in all major infected cell types, by preventing the activation of the viral DNA by precluding the activation of NF-κB, inhibiting the TAT gene product of HIV that drives viral replication and dismantling the gp120 proteins of the virus coat. NAC amide can be provided to disrupt the gp120 protein, thereby offering a potential mode of preventing transmission of virus not only to other cells in the patient, but perhaps to others.

Besides classic antiviral or antiretroviral agents (reverse transcriptase inhibitors, protease inhibitors), a number of other therapies may be of benefit for AIDS patients, and the present invention provides combinations of NAC amide or a derivative thereof with the following drugs: cycloporin A, thalidomide, pentoxifylline, selenium, desferroxamine, 2L-oxothiazolidine, 2L-oxothiazolidine-4-carboxylate, diethylthiocarbamate (DDTC), BHA, nordihydroguaiaretic acid (NDGA), glucarate, EDTA, R-PIA, alpha-lipoic acid, quercetin,
tannic acid, 2'-hydroxychalcone, 2-hydroxychalcone, flavones, alpha-angelicalactone, fraxetin, curcurmin, probucol, and arcanut (areca catechul).

Inflammatory responses are accompanied by large oxidative bursts, resulting in large numbers of free radicals. Therefore, NAC amide and its derivatives may have application in the therapy for inflammatory diseases. NAC amide or a derivative thereof may advantageously reduce the primary insult, as well as undesired aspects of the secondary response. According to the present invention, NAC amide or a derivative thereof may be administered to patients suffering from an inflammatory disease, such as arthritis of various types, inflammatory bowel disease, etc. The present invention also provides combination pharmaceutical therapy including NAC amide or NAC amide derivative and an analgesic or anti-inflammatory agent, for example, opiate agonists, glucocorticoids or non-steroidal anti-inflammatory drugs (NSAIDS), including opium narcotics, meperidine, propoxyphene, nalbuphine, pentazocine, buprenorphine, aspirin, indomethacin, diflunisal, acetaminophen, ibuprofen, naproxen, fenoprofen, piroxicam, sulindac, tolmetin, meclofenamate, zomepirac, penicillamine, phenylbutazone, oxyphenbutazone, chloroquine, hydroxychloroquine, azathiaprine, cyclophosphamide, levamisole, prednisone, prednisolone, betamethasone, triamcinolone, and methylprednisolone. NAC amide and its derivatives may also be beneficial for the treatment of parotitis, cervical dysplasia, Alzheimer's disease, Parkinson's disease, aminoquinoline toxicity, gentamycin toxicity, puromycin toxicity, aminoglycoside nephrotoxicity, paracetamol, acetaminophen and phenacetin toxicity.

NAC amide or a derivative thereof may be added to a virus-contaminated fluid or potentially contaminated fluid to inactivate the virus. This occurs, for example, by reduction of critical viral proteins. According to an embodiment, NAC amide or a derivative thereof is added to blood or blood components prior to transfusion. The added NAC amide or derivative of NAC amide is added in a concentration of between about 100 micromolar to about 500 millimolar or to a solubility limit, whichever is lower, and more preferably in a concentration of about 10-50 millimolar. Additionally, the addition of NAC amide or a derivative thereof to whole blood, packed red blood cells, or other formed blood components (white blood cells, platelets) may be used to increase the shelf life and/or quality of the cells or formed components.

In another embodiment, the present invention encompasses the use of NAC amide, or derivative thereof or a pharmaceutically acceptable salt or ester thereof, in the treatment and/or prevention of cosmetic conditions and dermatological disorders of the skin, hair, nails,
and mucosal surfaces when applied topically. In accordance with the invention, compositions for topical administration are provided that include (a) NAC amide, or derivative thereof or a suitable salt or ester thereof, or a physiologically acceptable composition containing NAC amide; and (b) a topically acceptable vehicle or carrier. The present invention also provides a method for the treatment and/or prevention of cosmetic conditions and/or dermatological disorders that entails topical administration of NAC amide- or NAC amide-derivative containing compositions to an affected area of a patient. Such compositions and methods are useful in anti-aging treatments and therapies, as well as for the treatment of wrinkles, facial lines and depressions, particularly around the eyes and mouth, creases in the skin, age spots and discolorations, and the like.

In another embodiment, the present invention provides methods and compositions useful for cancer and pre-cancer therapy utilizing NAC amide, or derivative thereof or its pharmaceutically acceptable salts or esters. The present invention particularly relates to methods and compositions comprising NAC amide or a derivative thereof in which apoptosis is selectively induced in cells of cancers or precancers. In another embodiment, the present invention relates to a method of selectively inducing apoptosis of precancer cells by administering an effective amount of NAC amide or a derivative thereof to a subject. In this embodiment, NAC amide or a derivative thereof can be topically administered to the subject. In another embodiment, the present invention relates to a method of selectively inducing apoptosis in cancer cells by administering an effective amount of NAC amide or a derivative thereof to a subject. NAC amide or its derivative can be topically administered to the subject in this embodiment. Selective apoptosis refers to a situation in which corresponding normal, non-transformed cells do not undergo NAC amide-induced cell death. In yet another embodiment, the present invention relates to a method comprising reducing the number of cancer cells present in a subject by administering NAC amide or a derivative thereof to the subject as an adjunct to chemotherapy or radiation therapies such that the susceptibility of the cancer cells to apoptosis is enhanced relative to the non-cancer cells of the subject. In a further embodiment, the present invention relates to a method comprising administering an effective amount of NAC amide or a derivative thereof as an adjunct to p53 therapy, including p53 gene therapy. The cancer or precancer cells in which apoptosis is induced are generally those which exhibit at least one functional p53 allele. In certain instances, administration of NAC amide results in restoration of mutant p53 protein conformation
and/or activity to a functional state. It is to be understood that an endogenous functional p53 allele is not necessary for methods comprising p53 therapy, including p53 gene therapy.

In another embodiment of the invention, methods are provided which comprise administering NAC amide or a derivative thereof to selectively induce cells which arise in hyperproliferative or benign dysproliferative disorders. Another embodiment of the present invention encompasses the use of NAC amide or a derivative thereof in methods for selective cell cycle arrest comprising contacting the cell with an amount of NAC amide or a derivative thereof to selectively arrest cells at a particular stage of the cell cycle. For example, administration of NAC amide can lead to prolonged transition through G1 phase. This cell cycle arrest may be influenced by an increase in p21 expression. The methods of the present invention can also be utilized to reduce or inhibit tumor vascularization, or to induce differentiation in cancer cells.

In another of its aspects, the present invention is directed to the use of NAC amide or a derivative thereof to treat cancers and tumors that may be induced by faulty signals from the microenvironment that result in loss of tissue organization in cancerous organs and loss of genomic stability in individual cancer cells. Loss of tissue structure may lead to certain cancers. Involved in this process are matrix metalloproteinases (MMPs), which are enzymes that are important not only during an organism's development and during wound healing, but also in promoting tumorigenesis or carcinogenesis. In particular, MMPs contribute prominently to microenvironmental signals because these proteolytic enzymes degrade structural components of the basement membrane and extracellular matrix (ECM) and digest the contacts that bind epithelial cells into sheets, thereby permitting the invasion of tumor cells and metastasis. MMPs can also release cell-bound inactive precursor forms of growth factors; degrade cell-cell and cell-ECM adhesion molecules; activate precursor zymogen forms of other MMPs; and inactivate inhibitors of MMPs and other proteases. Further, these enzymes induce the epithelial-mesenchymal transition, or EMT, a transition of one cell state to another that causes epithelial cells to disassociate from their neighbors, break free and acquire the ability to move through the body. While this process is essential for normal development in the embryo, in cancers, such as breast cancer, EMT provides mobility for tumor cells and assists tumor cells in penetrating barriers, such as wall of lymph and blood vessels, thus facilitating metastasis.

MMP-3 is a particular type of metalloproteinase that has been observed to induce transformation in mammary epithelial cells in culture and in transgenic mice. MMP-3 has
been found to cause normal cells to express the Raclb protein, an unusual form of Rho GTPase that has previously been found only in cancers. Raclb dramatically alters the cell skeleton, which facilitates the separation and movement of epithelial cells from surrounding cells. (D.C. Radisky et al., 2005, Nature, 436: 123-127). Changes in the cell skeleton induced by Raclb stimulate the production of highly reactive oxygen molecules, called reactive oxygen species (ROS), which can promote cancer by leading to tissue disorganization and by damaging genomic DNA. The increased amounts of ROS induced by Raclb activate major genes that control the EMT, which then begins a cascade of massive tissue disorganization and stimulates the development of cancer by directly affecting genomic DNA, for example, causing deletion or duplication of large regions of the DNA. By altering the tissue structure, MMPs can also activate oncogenes and comprising the integrity of the DNA in an organism's genome.

For treating and/or preventing cancers, e.g., breast cancer, especially those involving the above-described mechanisms leading to abnormal cell structure and function and loss of tissue integrity, NAC amide in accordance with the present invention can be used to block the effects of ROS. This can be achieved, for example, by administering or introducing NAC amide or a derivative thereof to cells, tissues, and/or the body of a subject in need thereof, to affect or target molecules in the pathways leading to epithelial-mesenchymal transition. Accordingly, NAC amide or a derivative thereof can be used to inhibit MMP-3 and its functions, such as MMP-3-induced downregulation of epithelial cytokeratins and upregulation of mesenchymal vimentin, as well as MMP3-induced cell motility, invasion and morphological alterations. NAC amide or a derivative thereof can also be used to target ROS indirectly or directly, and/or the processes by which ROS activate genes that induce the EMT.

In another embodiment, the present invention encompasses compositions and methods comprising NAC amide or a derivative thereof for the suppression of allograft rejection in recipients of allografts.

In another embodiment, the present invention provides a NAC amide or derivative of NAC amide in a method of supporting or nurturing the growth of stem cells for stem cell transplants, particularly stem cells cultured in vitro prior to introduction into a recipient animal, including humans.

In another embodiment, the present invention provides methods of inhibiting, preventing, treating, or both preventing and treating, central nervous system (CNS) injury or disease, neurotoxicity or memory deficit in a subject, involving the administration of a
therapeutically effective amount of NAC amide, or derivative thereof or a pharmaceutically acceptable composition thereof. Examples of CNS injuries or disease include traumatic brain injury (TBI), posttraumatic epilepsy (PTE), stroke, cerebral ischemia, neurodegenerative diseases of the brain such as Parkinson’s disease, Dementia Pugilistica, Huntington’s disease, Alzheimer’s disease, brain injuries secondary to seizures which are induced by radiation, exposure to ionizing or iron plasma, nerve agents, cyanide, toxic concentrations of oxygen, neurotoxicity due to CNS malaria or treatment with anti-malaria agents, and other CNS traumas. In other related embodiments, the present invention embraces a method of treating and/or preventing a subject suffering from a CNS injury or disease comprising administering to the subject a composition comprising a therapeutically effective amount of NAC amide or a derivative thereof. In another embodiment, the present invention relates to a method of preventing or inhibiting a CNS injury or disease in a subject comprising administering to the subject a composition comprising a therapeutically effective amount of NAC amide or a derivative thereof. In other embodiments, the present invention embraces a method of preventing, inhibiting or treating neurotoxicity or memory deficit in a subject comprising administering to the subject a composition comprising a therapeutically effective amount of NAC amide or a derivative thereof. Where the memory deficit may be induced by electroconvulsive shock therapy for treating and/or preventing diseases and disorders such as depression and schizophrenia, the composition may be administered before the electroconvulsive shock therapy to mitigate memory loss. In related embodiments, the CNS injury or disease may be traumatic brain injury (TBI), posttraumatic epilepsy (PTE), stroke, cerebral ischemia, or a neurodegenerative disease. In related embodiments, CNS injury may be induced by fluid percussion, by trauma imparted by a blunt object, for example on the head of the subject, by trauma imparted by an object which penetrates the head of the subject, by exposure to radiation, ionizing or iron plasma, a nerve agent, cyanide, toxic concentrations of oxygen, CNS malaria, or an anti-malaria agent. In the embodiments of the present invention, the therapeutically effective amount of NAC amide or a derivative thereof administered to the subject is the amount required to obtain the appropriate therapeutic effect, for example, about 0.001 mg to about 20 mg per kg of the subject, preferably about 1 mg to about 10 mg per kg of the subject, more preferably about 3 mg to about 10 mg per kg of the subject. In additional embodiments, the total daily amount of NAC amide or a derivative thereof administered to the subject is about 50 mg to about 1200 mg, or about 100 mg to about 1000 mg, or about 200 mg to about 800 mg, or about 300 mg to about 600 mg.
In other embodiments, the invention encompasses a method of treating a subject (e.g., an animal, including humans) before the subject is exposed or likely to be exposed to a risk of CNS injury or damage, or before the subject is exposed to conditions likely to cause neurotoxicity or memory deficit or both, by administering NAC amide or a derivative thereof to a subject in a period of time prior to the exposure of the subject to the risk of CNS injury or damage, etc. Illustratively, conditions that may cause CNS injury or damage, neurotoxicity or memory deficit include electroconvulsive shock therapy, traumatic brain injury (TBI), posttraumatic epilepsy (PTE), stroke, cerebral ischemia, neurodegenerative diseases, fluid percussion, a blunt object impacting the head of the subject, an object penetrating the head of the subject, radiation, ionizing or iron plasma, nerve agents, cyanide, toxic concentrations of oxygen, CNS malaria, and anti-malaria agents. Other conditions that may cause CNS injury or damage, neurotoxicity or memory deficit include, without limitation, certain medical procedures or conditions associated with risk for CNS ischemia, hypoxia or embolism such as brain tumor, brain surgery, other brain-related disorders, open heart surgery, carotid endarterectomy, repair of aortic aneurysm, atrial fibrillation, cardiac arrest, cardiac or other catheterization, phlebitis, thrombosis, prolonged bed rest, prolonged stasis (such as during space travel or long trips via airplane, rail, car or other transportation), CNS injury secondary to air/gas embolism or decompression sickness. The period of time may be about 72 hours prior to the time of expected exposure, or about 48 hours prior to the time of expected exposure, or about 12 hours prior to the time of expected exposure, or about 4 hours prior to the time of expected exposure, or about 30 minutes-2 hours prior to the time of expected exposure. The administration of NAC amide may be continuous from the initial time of treatment to the end of treatment. For example, a transdermal patch or a slow-release formulation may be used to continually administer NAC amide or a derivative thereof to the subject for a given period of time. Alternatively, NAC amide or a derivative thereof may be administered to the subject periodically. For example, NAC amide or a derivative thereof may first be administered at about 24 hours before the time of expected exposure and then administered at about every 2 hours thereafter. For these embodiments of the invention, the NAC amide- or NAC amide derivative-containing composition may further comprise a pharmaceutically acceptable excipient and the composition may be administered intravenously, intradermally, subcutaneously, orally, transdermally, transmucosally or rectally.
In other embodiments, the present invention encompasses a pharmaceutical composition for treating or preventing CNS injury, disease or neurotoxicity in a subject comprising a therapeutically effective amount of NAC amide or a derivative thereof and a pharmaceutically acceptable excipient. In a further embodiment, the invention embraces a kit comprising a composition comprising a therapeutically effective amount of NAC amide or a derivative thereof. The kit may further comprise a device for administering the composition to a subject such as an injection needle, an inhaler, a transdermal patch, as well as instructions for use.

In another embodiment of the present invention, anti-cancer treatments involving NAC amide or a derivative thereof are designed to specifically target cancer and tumor cells. This embodiment is directed to the use of nano-sized particles for the in vivo and ex vivo administration of NAC amide or a derivative thereof to cancer and tumor cells. According to this embodiment, cancer cells, which display more receptors for the vitamin folic acid (or folate) and absorb more folic acid than do normal, healthy cells, are able to be preferentially targeted. To this end, core or shell nanogels, or nanoparticles, can be functionalized with folic acid or folate conjugated or linked to NAC amide or a derivative thereof without disrupting or destroying the folic acid binding site to its cell receptor. Such functionalized nanoparticles can be introduced into a subject, particularly a folate-deprived subject, with a cancer, e.g., epithelial cancer, in whom the cancer cells have excess folic acid receptors which will preferentially bind the folic acid-NAC amide (or folic acid-NAC amide derivative) nanoparticles and endocytose them. Once inside the cancer cell, NAC amide or a derivative thereof exert its therapeutic effects, for example, by inhibiting ROS and/or other target molecules that play a role in initiating, fueling, and/or maintaining cancer cells, and/or ultimately killing the cancer cells.

Illustratively, PAMAM dendritic polymers <5 nm in diameter can be used as carriers of NAC amide, as described in J.F. Kukowska-Latallo et al., 2005, Cancer Res., Jun 15:65(12):53 17-24, to target folic acid receptor-expressing (overexpressing) tumor and cancer cells. Acetylated dendrimers can be conjugated to folic acid as a targeting agent and then coupled to NAC amide or a derivative thereof and either fluorescein or 6-carboxytetramethylrhodamine. Alternatively, NAC amide or a derivative thereof can be coupled to folic acid to form a conjugate and the conjugate can be coupled to the nanoparticles. These conjugates can be injected i.v. into a tumor-bearing patient or mammal, especially those tumors that overexpress the folic acid receptor. The folate-conjugated
nanoparticles can then concentrate in the tumor and tissue following administration, where the delivered NAC amide or NAC derivative can interact with ROS in the cells, and/or target other molecules to kill the cancer or tumor cells. The tumor tissue localization of the folate-targeted polymer may be attenuated by prior i.v. injection of free folic acid.

In a similar embodiment, polymers or nanoparticles can be functionalized to display glutathione-NAC amide or glutathione-NAC amide derivative conjugates, which can then be used to deliver NAC amide or a derivative thereof to cancer cells which display increased numbers of glutathione receptors on their cell surfaces. The NAC amide-glutathione nanoparticles can then be targeted to those cancer cells having glutathione receptors and preferentially endocytosed by the cells. In these embodiments, the present invention provides directed delivery of NAC amide or a derivative thereof to cells, such as cancer cells that express high levels of receptors for folic acid (folate) or glutathione. In accordance with these embodiments, NAC amide ("NACA") or a derivative thereof is coupled to a ligand for a cell surface receptor (e.g., folic acid or glutathione) to form a conjugate. This NACA-ligand conjugate is coated or adsorbed onto readily injectable nanoparticles using procedures known to those skilled in the art. Accordingly, the nanoparticles containing NAC amide or a derivative thereof ("nano-NACA particles") may be preferentially taken up by cancer or tumor cells where the NAC amide will exert its desired effects.

In an embodiment, the present invention is drawn to a method of directed delivery of NAC amide or a derivative thereof to host cells expressing high levels of surface receptor for a ligand, comprising: a) conjugating acetylated dendritic nanoparticles to ligand; b) coupling the conjugated ligand of step (a) to NAC amide or a derivative thereof to form NAC amide-ligand nanoparticles; and c) injecting the nanoparticles of (b) into the host. In another embodiment, the present invention is drawn to a method of directed delivery of NAC amide or a derivative thereof to host cells expressing high levels of surface receptor for a ligand, comprising: a) coupling NAC amide or a derivative thereof to the surface receptor ligand to form a NAC amide-ligand conjugate; b) adsorbing the NAC amide-ligand conjugate onto nanoparticles; and c) injecting the nanoparticles of (b) into the host.

Another embodiment of the present invention provides a compound of the formula 1:
wherein:

- $R_i$ is OH, SH, or S-S-Z;
- $X$ is C or N;
- $Y$ is NH$_2$, OH, CH$_3$-C=0, or NH-CH$_3$;
- $R_2$ is absent, H, or =0

$R_3$ is absent or

wherein:

- $R_4$ is NH or O;
- $R_5$ is CF$_3$, NH$_2$, or CH$_3$

and wherein: $Z$ is

with the proviso that if $R_i$ is S-S-Z, $X$ and $X'$ are the same, $Y$ and $Y'$ are the same, $R_2$ and $R_6$ are the same, and $R_3$ and $R_7$ are the same.

In one embodiment, $R_i$ is S, $X$ is C, $Y$ is NH-CH$_3$, $R_2$ is H, $R_3$ is O, and $R_5$ is CH$_3$. In another embodiment, $R_1$ is S, $X$ is N, $Y$ is CH$_3$-C=0, $R_2$ is H, and $R_3$ is absent.
The present invention also provides compounds of the formula I above, wherein R_i is S, X is C, Y is NH_2, R_2 is =0, R_3 is HN, R_4 is O, and R_5 is CF_3. Compounds of the present invention also include compounds of formula I wherein R_1 is 0, X is C, Y is NH_2, R_2 is =0, R_3 is HN, R_4 is O, and R_5 is CH_3. Also provided by the present invention are compounds of formula I wherein R_i is S, X is C, Y is OH, R_2 is absent, R_3 is HN, R_4 is O, and R_5 is CH_3. Another embodiment of the present invention provides compounds of formula I wherein R_i is O, X is C, Y is OH, R_2 is absent, R_3 is R_4 is O, and R_5 is CH_3; or wherein R_i is S, X is C, Y is NH_2, R_2 is =0, R_3 is R_4 is O, and R_5 is CH_3; or wherein R_i is S, X is C, Y is NH_2, R_2 is =0, R_3 is R_4 is O, and R_5 is CH_3. In a further embodiment, the present invention provides compounds of formula I wherein R_i is S-S-Z, X is C, Y is NH_2, R_2 is =0, R_3 is , R_4 is O and R_5 is CH_3.

The compounds disclosed herein can be chiral, i.e., enantiomers, such as L- and D-isomers, or can be racemic mixtures of D- and L-isomers. Preferred compounds include, but are not limited to, the following:
In one embodiment, Compounds I through XVIII comprise NAC amide or NAC amide derivatives.

In another embodiment, a process for preparing an L- or D- isomer of the compounds of the present invention are provided, comprising adding a base to L- or D-cystine diamide dihydrochloride to produce a first mixture, and subsequently heating the first mixture under vacuum; adding a methanolic solution to the heated first mixture; acidifying the mixture with alcoholic hydrogen chloride to obtain a first residue; dissolving the first residue in a first solution comprising methanol saturated with ammonia; adding a second solution to the dissolved first residue to produce a second mixture; precipitating and washing the second mixture; filtering and drying the second mixture to obtain a second residue; mixing the second residue with liquid ammonia, and an ethanolic solution of ammonium chloride to produce a third mixture; and filtering and drying the third mixture, thereby preparing the L- or D-isomer compound.

The base can comprise liquid ammonia or methylamine. The second solution comprises water, an acetate salt, and an anhydride, wherein the acetate salt can comprise...
sodium acetate or sodium trifluoroacetate, and the anhydride can comprise acetic anhydride or trifluoroacetic anhydride. Alternatively, the second solution can comprise dichloromethane, triethylamine, and 1,3-bis(benzylxycarbonyl)-2-methyl-2-thiopseudourea. In addition to liquid ammonia and an ethanolic solution of ammonium chloride, the second residue can be further mixed with sodium metal.

In some embodiments, the process further comprises dissolving the L- or D-isomer compound in ether; adding to the dissolved L- or D-isomer compound an ethereal solution of lithium aluminum hydride, ethyl acetate, and water to produce a fourth mixture; and filtering and drying the fourth mixture, thereby preparing the L- or D-isomer compound.

The compounds of formula II and III are prepared by mixing L- or D-cystine diamide dihydrochloride with liquid ammonia; warming the mixture to remove volatiles; warming mixture in vacuo to 50°C; adding a warm methanolic solution; filtering the solution; acidifying the filtrate with alcoholic hydrogen chloride for obtaining a first residue, dissolving the first residue in a solution of methanol saturated with ammonia; concentrating to dryness; adding water, sodium acetate and acetic anhydride; raising the temperature to 50°C; precipitating the mixture and washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue, mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula IV and V are prepared by mixing L- or D-cystine diamide dihydrochloride with methylamine; warming the mixture to remove volatiles; warming mixture in vacuo to 50°C; adding a warm methanolic solution; filtering the solution; acidifying the filtrate with alcoholic hydrogen chloride for obtaining a first residue, dissolving the first residue in a solution of methanol saturated with ammonia; concentrating to dryness; adding water, sodium acetate and acetic anhydride; raising the temperature to 50°C; precipitating the mixture and washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue, mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.
The compounds of formula VII and VIII are prepared by mixing L- or D-cystine diamide dihydrochloride with ammonia; warming the mixture to remove volatiles; warming mixture \textit{in vacuo} to 50°C; adding a warm methanolic solution; filtering the solution; acidifying the filtrate with alcoholic hydrogen chloride for obtaining a first residue, dissolving the first residue in a solution of methanol saturated with ammonia; concentrating to dryness; adding water, sodium trifluoroacetate and trifluoroacetic anhydride; raising the temperature to 50°C; precipitating the mixture and washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue, mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula XIII and XIV are prepared by mixing L- or D-cystine diamide dihydrochloride with ammonia; warming the mixture to remove volatiles; warming mixture \textit{in vacuo} to 50°C; adding a warm methanolic solution; filtering the solution; acidifying the filtrate with alcoholic hydrogen chloride for obtaining a first residue, dissolving the first residue in a solution of methanol saturated with ammonia; concentrating to dryness; adding dichloromethane, triethylamine, and 1,3-bis(benzyloxy carbonyl)-2-methyl-2-thiopseudourea; lowering the temperature to 0°C; precipitating the mixture and washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue, mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula XI and XII are prepared by mixing L- or D-cystine diamide dihydrochloride with liquid ammonia; warming the mixture to remove volatiles; warming mixture \textit{in vacuo} to 50°C; adding a warm methanolic solution; filtering the solution; acidifying the filtrate with alcoholic hydrogen chloride for obtaining a first residue; dissolving the first residue in a solution of methanol saturated with ammonia; concentrating to dryness; adding water, sodium acetate and acetic anhydride; raising the temperature to 50°C; precipitating the mixture; washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue; mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic
solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; dissolving the third residue in ether; slowly adding an ethereal solution of lithium aluminum hydride; slowly adding ethyl acetate; slowly adding water; filtering and separating the inorganic salts; concentrating and cooling the filtrate to obtain a fourth residue; and crystallizing the fourth residue from isopropanol.

The compounds of formula XVII and XVIII are prepared by mixing L-or D-cystine diamide dihydrochloride with liquid ammonia; warming the mixture to remove volatiles; warming mixture in vacuo to 50°C; adding a warm methanolic solution; filtering the solution; acidifying the filtrate with alcoholic hydrogen chloride for obtaining a first residue; dissolving the first residue in a solution of methanol saturated with ammonia; concentrating to dryness; adding of water, sodium acetate and acetic anhydride; raising the temperature to 50°C; precipitation of the mixture; washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue; and crystallizing the second residue from isopropanol.

Another embodiment of the invention provides a process for preparing an L- or D-isomer of the compounds disclosed herein, comprising mixing S-benzyl-L- or D-cysteine methyl ester hydrochloride or O-benzyl-L- or D-serine methyl ester hydrochloride with a base to produce a first mixture; adding ether to the first mixture; filtering and concentrating the first mixture; repeating steps (c) and (d), to obtain a first residue; adding ethyl acetate and a first solution to the first residue to produce a second mixture; filtering and drying the second mixture to produce a second residue; mixing the second residue with liquid ammonia, sodium metal, and an ethanolic solution of ammonium chloride to produce a third mixture; and filtering and drying the third mixture, thereby preparing the L- or D-isomer compound.

The base can comprise liquid ammonia or methylamine. The second solution comprises water, an acetate salt, and an anhydride, wherein the acetate salt can comprise sodium acetate or sodium trifluoroacetate, and the anhydride can comprise acetic anhydride or trifluoroacetic anhydride. Alternatively, the second solution can comprise dichloromethane, triethylamine, and 1,3-bis(benzyloxy carbonyl)-2-methyl-2-thiopseudourea.

In some embodiments, the process further comprises dissolving the L- or D-isomer compound in ether; adding to the dissolved L- or D-isomer compound an ethereal solution of lithium aluminum hydride, ethyl acetate, and water to produce a fourth mixture; and filtering and drying the fourth mixture, thereby preparing the L- or D-isomer compound.
The compounds of formula II and III are prepared by mixing S-benzyl-L- or D-
cysteine methyl ester hydrochloride with a cold methanolic solution of ammonia; passing a
stream of ammonia over the mixture; sealing the flask securely; concentrating the mixture;
adding ether; filtering the solution; concentrating the filtrate; adding ether and filtering again,
to obtain a residue; suspending the residue with ethyl acetate; adding acetic anhydride to this
suspension; adding water, sodium acetate and acetic anhydride; raising the temperature to
65°C; cooling the mixture; filtering the crude solid; washing with ethyl acetate; drying the
precipitate for obtaining a second residue; mixing the second residue with liquid ammonia;
slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of
ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the
filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula IV and V are prepared by mixing S-benzyl-L- or D-
cysteine methyl ester hydrochloride with a cold methanolic solution of methylamine; passing
a stream of methylamine over the mixture; sealing the flask securely; concentrating the
mixture; adding ether; filtering the solution; concentrating the filtrate; adding ether and
filtering again, to obtain a residue; suspending the residue with ethyl acetate; adding acetic
anhydride to this suspension; adding water, sodium acetate and acetic anhydride; raising the
temperature to 65°C; cooling the mixture; filtering the crude solid; washing with ethyl
acetate; drying the precipitate for obtaining a second residue; mixing the second residue with
liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an
ethanolic solution of ammonium chloride; filtering and separating the inorganic salt;
concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third
residue from isopropanol.

The compounds of formula VII and VIII are prepared by mixing S-benzyl-L- or D-
cysteine methyl ester hydrochloride with a cold methanolic solution of ammonia; passing a
stream of methylamine over the mixture; sealing the flask securely; concentrating the
mixture; adding ether; filtering the solution; concentrating the filtrate; adding ether and
filtering again, to obtain a residue; suspending the residue with ethyl acetate; adding
trifluoroacetic anhydride to this suspension; adding water, sodium trifluoroacetate and
trifluoroacetic anhydride; raising the temperature to 65°C; cooling the mixture; filtering the
crude solid; washing with ethyl acetate; drying the precipitate for obtaining a second residue;
mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the
solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating
the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula IX and X are prepared by mixing O-benzyl-L- or D-serine methyl ester hydrochloride with a cold methanolic solution of ammonia; passing a stream of methylamine over the mixture; sealing the flask securely; concentrating the mixture; adding ether; filtering the solution; concentrating the filtrate; adding ether and filtering again, to obtain a residue; suspending the residue with ethyl acetate; adding acetic anhydride to this suspension; adding water, sodium acetate and acetic anhydride; raising the temperature to 65°C; cooling the mixture; filtering the crude solid; washing with ethyl acetate; drying the precipitate for obtaining a second residue; mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula XIII and XIV are prepared by mixing S-benzyl-L- or D-cysteine methyl ester hydrochloride with a cold methanolic solution of ammonia; passing a stream of ammonia over the mixture; sealing the flask securely; concentrating the mixture; adding ether; filtering the solution; concentrating the filtrate; adding ether and filtering again, to obtain a residue; suspending the residue with ethyl acetate; adding acetic anhydride to this suspension; adding dichloromethane, triethylamine, and 1,3-bis(benzyloxycarbonyl)-2-methyl-2-thiopseudourea; lowering the temperature to 0°C; precipitating the mixture; washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue; mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

The compounds of formula XI and XII are prepared by (a) mixing S-benzyl-L- or D-cysteine methyl ester hydrochloride with a cold methanolic solution of ammonia; passing a stream of ammonia over the mixture; sealing the flask securely; concentrating the mixture; adding ether; filtering the solution; concentrating the filtrate; adding ether and filtering again, to obtain a residue; suspending the residue with ethyl acetate; adding acetic anhydride to this suspension; adding of water, sodium acetate and acetic anhydride; raising the temperature to 65°C; cooling the mixture; filtering the crude solid; washing with ethyl acetate; drying the precipitate for obtaining a second residue; mixing the second residue with liquid ammonia;
slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; dissolving the third residue in ether; slowly adding an ethereal solution of lithium aluminum hydride; slowly adding ethyl acetate; slowly adding water; filtering and separating the inorganic salts; concentrating and cooling the filtrate to obtain a fourth residue; and crystallizing the fourth residue from isopropanol.

The compounds of formula XV and XVI are prepared by (a) mixing O-benzyl-L- or D-serine methyl ester hydrochloride with a cold methanolic solution of ammonia; passing a stream of ammonia over the mixture; sealing the flask securely; concentrating the mixture; adding ether; filtering the solution; concentrating the filtrate; adding ether and filtering again, to obtain a residue; suspending the residue with ethyl acetate; adding acetic anhydride to this suspension; adding of water, sodium acetate and acetic anhydride; raising the temperature to 65°C; cooling the mixture; filtering the crude solid; washing with ethyl acetate; drying the precipitate for obtaining a second residue; mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and cooling the filtrate to obtain a third residue; dissolving the third residue in ether; slowly adding an ethereal solution of lithium aluminum hydride; slowly adding ethyl acetate; slowly adding water; filtering and separating the inorganic salts; concentrating and cooling the filtrate to obtain a fourth residue; and crystallizing the fourth residue from isopropanol.

Yet another embodiment of the invention provides a process for preparing a compound as disclosed herein, comprising mixing cystamine dihydrochloride with ammonia, water, sodium acetate, and acetic anhydride to produce a first mixture; allowing the first mixture to precipitate; filtering and drying the first mixture to produce a first residue; mixing the second residue with liquid ammonia, sodium metal, and an ethanolic solution of ammonium chloride to produce a second mixture; filtering and drying the second mixture, thereby preparing the compound.

The compound of formula VI is prepared by mixing cystamine dihydrochloride with ammonia; adding water, sodium acetate and acetic anhydride; raising the temperature to 50°C; precipitating the mixture; washing the mixture with water; filtering the crude solid; drying the mixture for obtaining a second residue; mixing the second residue with liquid ammonia; slowly adding sodium metal; removal of the solvent; slowly adding an ethanolic solution of ammonium chloride; filtering and separating the inorganic salt; concentrating and
cooling the filtrate to obtain a third residue; and crystallizing the third residue from isopropanol.

In some embodiments NAC Amide (NACA) shows better protection that NAC in acetaminophen induced cell death in HEPG2 cells.

5

EXAMPLES

Example 1

In this Example, NAC amide was assessed for its protective effects against oxidative toxicity induced by glutamate in PC12 cells.

Materials and methods: N-(I-pyrenyl)-maleimide (NPM) was purchased from Aldrich (Milwaukee, WI, USA). N-acetylcysteine amide was obtained from Novia Pharmaceuticals, (Israel). High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell culture and toxicity studies: Stock culture of PC12 cells, purchased from ATCC, were grown in 75 cm² tissue culture flasks in RPMI 1640, supplemented with 10% (v/v) heat-inactivated horse serum, and 5% (v/v) fetal bovine serum, to which 1% (v/v) penicillin and streptomycin were added. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were passaged twice a week. Unless specified, all of the experiments were performed using Dulbecco's modified Eagle's medium (DMEM) as differentiation medium, supplemented with 0.5% (v/v) fetal bovine serum, 1% (v/v) penicillin and streptomycin. PC12 cells were plated at a density of 25 x 10³ cells/well in a 24-well, collagen-coated plate for morphological assessment. The plate was divided into five groups in triplicate: 1) control: no glutamate, no NAC amide; 2) Nerve Growth Factor (NGF) control: NGF (100 ng/ml), no glutamate, no NAC amide; 3) NAC amide only: NGF (100 ng/ml), no glutamate, NAC amide (750 μM); 4) glutamate only: NGF (100 ng/ml), glutamate (10 mM), no NAC amide; and 5) Glu + NAC amide: NGF (100 ng/ml), glutamate (10 mM), NAC amide (750 μM). All wells received 100ng/ml NGF every other day, except Group 1.

After one week, cells were treated or not (control) with 10 mM glutamate, with or without NAC amide, for 24 hours. Twenty-four hours later, the cells were fixed with 0.5% (v/v) glutaraldehyde in PBS and micropictures were taken.
LDH assay: For the lactate dehydrogenase (LDH) assay, cells were plated at a density of 2.5 x 10^5 cells/well in a 24 well collagen-coated culture plate and, after 24 h; the medium was replaced with fresh DMEM medium containing the desired concentration of glutamate and NAC amide. After the desired incubation period, the LDH activity released was determined using the kit as described below. For the MTS assay, cells were plated at a density of 10^5 cells/well in a 24 well collagen-coated plate. At the end of the experiments, cell viability was assayed using the kit as described. The LDH activity assay was performed with the CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA), which quantitatively measured the activity of LDH, a stable cytosolic enzyme that is released upon cell lysis [Technical Bulletin No. 163, Promega]. LDH in culture supernatants was measured with a 30-minute coupled enzymatic assay, which resulted in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed was proportional to the degree of damage to the cell membranes. Absorbance data were collected using a BMG microplate reader (BMG Labtechnologies, Inc., Durham, NC, USA) at 490 nm. LDH leakage was expressed as the percentage (%) of the maximum LDH release in the cells treated with glutamate alone (100%), according to the formula:

Experimental LDH release

\[
\text{% LDH released} = \frac{\text{Absorbance}_{490\text{ nm}}}{\text{Absorbance}_{490\text{ nm}}^{\text{ Max}}} \times 100
\]

MTS assay: The MTS assay (Cell Titer 96® Aqueous One solution cell proliferation Assay, Promega) is a cell proliferation assay in which the administered (3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS) [21] is bioreduced by viable cells to a colored formazan product that is soluble in media. Absorbance at 490 nm is proportional to the number of living cells in the culture.

GSH measurement: Cellular levels of GSH were determined by using the method as described in Winters R.A. et al., Anal Biochem., 227(1):14-21, 1995. Cells were seeded at a density of 80,000 cells/cm^2 on poly-D-lysine coated (0.05 mg/ml) 75 cm^2 flasks (5 ml/flask) for GSH measurement. After 24 hours, the flasks were incubated with fresh medium containing glutamate (10 mM), or BSO (0.2 mM) or Glu + BSO + NAC amide (750 μM) at 370 C for another 24 h. After the incubation period, cells were removed from the cultures and homogenized in serine borate buffer (100 mM Tris-HCl, 10 mM boric acid, 5 mM L-
serine, 1 mM DETAPAC, pH 7.4). Twenty (20) µl of the diluted cell homogenate were added to 230 µl of serine borate buffer and 750 µl of NPM (1 mM in acetonitrile). The resulting solutions were incubated at room temperature for 5 min. The reaction was stopped by the addition of 5 µl of 2N HCl. The samples were then filtered through a 0.2 µμ Acrodisc filter and injected onto the HPLC system.

MDA measurement: To prepare the solution, 350 µl of straight cell homogenate, 100 µl of 500 ppm BHT (butylated hydroxytoluene), and 550 µl of 10% TCA (trichloroacetic acid) were combined and boiled for 30 min. The tubes were cooled on ice and centrifuged for 10 min at 2500 rpm. Five hundred (500) µl of the supernatant were removed and 500 µl of TBA (thiobarbituric acid) were added. The tubes were boiled again for 30 min, and then cooled on ice. From this solution, 500 µl were removed, added to 1.0 ml of n-butanol, vortexed, and centrifuged for 5 min at 60 g to facilitate a phase separation. The top layer was then filtered through 0.45 µμ filters and injected onto a 5 µμ C18 column (250 x 4.6 mm) on reverse phase HPLC system. The mobile phase consisted of 69.4% 5mM sodium phosphate buffer (pH = 7.0), 30% acetonitrile, and 0.6% THF (tetrahydrofuran). The excitation wavelength was 515 nm; the emission wavelength was 550 nm (Draper H.H. et al., Free Radic Biol Med., 15(4):353-63, 1993).

Protein determination and statistical analysis: Protein levels were determined by the Bradford method with Coomassie Blue (Bio-Rad) (Bradford M.M., Anal Biochem., 72:248-54, 1976). The data were given as the mean ± SD. The one-way analysis of variance test was used to analyze the significance of the differences between the control and experimental groups.

This Example shows that NAC amide protects cells against glutamate toxicity. Glutamate toxicity was evaluated by 1) morphological assessment of PC12 cells in the presence of glutamate; 2) measuring the amount of LDH released in the media 24 h after glutamate exposure; and 3) measuring cell viability using the MTS assay. As shown in FIGS. 2A-D, cells completely lost the normal morphology of their neurites in the presence of 10 mM glutamate, as compared to the control cells. To determine whether NAC amide could protect the cells from glutamate toxicity, PC12 cells were exposed to 10 mM glutamate for 24 hours in the presence of 750 µM NAC amide, and cell viability was examined by light microscopy. The addition of NAC amide protected the PC12 cells from glutamate toxicity by slightly decreasing the bleb formation on neurites.
To quantify the protection provided by NAC amide, PC12 cells were exposed to 10 mM glutamate in the presence of NAC amide for 24 hours, and then the amount of LDH released was measured using the LDH assay. As shown in FIG. 3, inclusion of 750 μM NAC amide in the assay completely protected the cells from cell damage, even in the presence of 10 mM glutamate (the % LDH released was 28.9 ± 3.7%). Similar results were obtained when cells were exposed to 10 mM glutamate in the presence of NAC amide for 24 hours, and the cell viability was assessed by the MTS assay.

The results of Example 1 demonstrate that NAC amide treatment significantly increased PC12 cell GSH levels. When cells were exposed to 10 mM glutamate, a significant reduction in GSH levels was observed (Table 1).

**Table 1: Effect of NAC amide on intracellular GSH levels in the presence of BSO and Glutamate**

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH Levels (nM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54 ± 13.4</td>
</tr>
<tr>
<td>GLU (10 mM) *</td>
<td>23 ± 4.2</td>
</tr>
<tr>
<td>BSO (0.2 mM)</td>
<td>ND</td>
</tr>
<tr>
<td>NAC amide (750 μM) *</td>
<td>112 ± 17.8</td>
</tr>
<tr>
<td>GLU + NAC amide **</td>
<td>88 ± 11.0</td>
</tr>
<tr>
<td>GLU + BSO + NAC amide ***</td>
<td>30 ± 4.3</td>
</tr>
</tbody>
</table>

PC12 cells were seeded and grown for 24 hours, then they were treated with either GLU (10 mM); NAC amide (750 μM); GLU (10 mM) + NAC amide (750 μM); GLU (10 mM) + BSO (0.2 mM) + NAC amide (750 μM); or BSO (0.2 mM). Twenty hours later, cells were removed and analyzed for GSH levels, as described in the text. Values represent means ± SD. Statistically different values of * P < 0.05 were determined, compared to control. ** P < 0.001 compared to glutamate-treated group. *** P < 0.05 compared to glutamate-treated group. At a 750 μM concentration and 24 hour treatment time, NAC amide increased the PC12 cell GSH level two fold, compared to the control group. Interestingly, similar results were obtained when Chinese hamster ovary (CHO) cells were incubated with NAC amide (data not shown).

The intracellular levels of GSH were determined in PC12 cells incubated with 10 mM glutamate for 24 hours, and the effects of NAC amide were analyzed. Treatment of cells with NAC amide prevented the marked decline of cellular GSH levels that normally occurs after glutamate treatment (Table 1). Glutamate inhibits cystine uptake, resulting in the loss of cellular GSH, while buthionine-sulfoximine (BSO) inhibits γ-GCS activity and thereby causes the depletion of intracellular GSH. To determine whether the increase in intracellular
GSH by NAC amide was γ-GCS-dependent, cells were treated with 0.2 mM BSO. The simultaneous treatment of glutamate and BSO, depleted the cell GSH to almost undetectable levels (Table 1). Interestingly, in GSH synthesis-arrested cells, NAC amide treatment was effective and maintained 56% of the cells' GSH levels. NAC amide further protected cells against intracellular peroxide accumulation. Malondialdehyde (MDA) is a by-product of a free radical attack on lipids. Marked increase in MDA levels was observed in glutamate-exposed cells, as compared with the corresponding control cells (Table 2). Treatment with NAC amide completely protected cells against glutamate toxicity by lowering MDA levels.

Table 2: Effects of NAC amide on MDA levels in Glutamate-exposed PC12 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA Levels (nM/100 mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>GLU (10 mM)</td>
<td>247 ± 26</td>
</tr>
<tr>
<td>NAC amide (750 µM)</td>
<td>81 ± 22</td>
</tr>
<tr>
<td>GLU + NAC amide</td>
<td>88 ± 11</td>
</tr>
</tbody>
</table>

Cells were plated and grown for 24 hours, and then they were exposed to glutamate (10 mM) in the presence or absence of NAC amide (750 µM). Twenty-four hours later, the cells were harvested and malondialdehyde levels were measured. Values represent means ± SD. Statistically different values of *P < 0.002 and **P < 0.05 were determined, compared to control. ***P < 0.05 compared to glutamate-treated group.

In this Example, it was determined that a high concentration of glutamate-induced oxidative toxicity was characterized by various potentially detrimental changes in intracellular GSH levels, MDA levels, and LDH activity, resulting in a reduction of PC12 cell viability. Treatment with NAC amide increased intracellular GSH, and reduced MDA levels, thereby attenuating glutamate-induced cytotoxicity. Evaluation was done by LDH and MTS assay. Glutamate cytotoxicity has been attributed to either excitatory action through the activation of glutamate receptors or inhibition of cystine uptake that leads to the decreased GSH levels. Although PC12 cells express NMDA receptors, toxicity exhibited by glutamate does not solely relate to the presence of these receptors, as NMDA has no effect on PC12 cell death. The disruption of intracellular redox homeostasis by high concentrations of glutamate is thought to be a major contributing mechanism of cellular damage in vivo. Under conditions such as cerebral ischemia, extracellular glutamate levels increase 800%, as compared to control, which would decrease brain GSH levels by blocking cystine uptake. GSH plays an important role in antioxidant defense, and redox regulation. GSH deficiency has been associated with various neurodegenerative diseases. Intracellular GSH levels were
determined by the Xc — and ASC systems. The Xc — system transports cystine intracellularly in exchange for glutamate, whereas the ASC system is a Na+-dependent neutral amino acid transporter that mediates the cellular transport of cysteine. Following uptake, cystine is reduced to cysteine for intracellular glutathione synthesis. However, elevated levels of glutamate inhibit cystine uptake, and subsequent restriction of cysteine availability for the cell, leading to GSH depletion.

In this Example, incubation of PC12 cells with glutamate resulted in reduction of GSH (Table 1) and cysteine levels (Figure 4), when compared to the control group. Reduced levels of cysteine indicate that the presence of excess glutamate inhibited cystine uptake, which led to decreased GSH levels. NAC amide treatment was able to increase GSH (Table 1) and cysteine levels (FIG. 5), compared to the control group, and effectively reversed the inhibitory action of glutamate. Increases in GSH and cysteine levels were also observed 30 minutes after NAC amide was administered to mice. The possible mechanism for NAC amide to facilitate the supply of cysteine may be by readily reaching the cell's interior, and becoming deacetylated to form cysteine. To understand whether NAC amide could restore the GSH levels in GSH synthesis-arrested cells, PC12 cells were incubated with glutamate (10 mM) plus BSO (0.2 mM) in the presence of NAC amide (750 µM). Results showed that NAC amide elevated intracellular GSH levels in the presence of BSO, suggesting that the effect is γ-GCS-independent. Therefore, NAC amide itself may act as a sulfhydryl group donor for GSH synthesis.

In summary, Example 1 shows that NAC amide protects PC12 cells against glutamate-induced cytotoxicity by preventing glutamate-induced loss of cellular GSH and inhibiting lipid peroxides. These studies also show that the restoration of GSH synthesis by NAC amide in GSH synthesis-arrested cells is γ-GCS-independent. Without wishing to be bound by theory, the possible mechanisms by which NAC amide can enhance GSH are 1) supplying the rate-limiting substrate cysteine to the cells and 2) reducing GSSG to GSH by a nonenzymatic thiol-disulfide exchange. Considering the protective effects of NAC amide against glutamate-induced cytotoxicity, in which oxidative stress seems to be involved, NAC amide can play a role in the treatment of neurodegenerative disorders such as cerebral ischemia and Parkinson's disease in which GSH levels are depleted in certain regions of the brain. **Example 2**

This Example examines the radioprotective effects of NAC amide. To evaluate the protective effects of NAC amide against radiation exposure, the radioprotective role of NAC
amide was compared with that of NAC with respect to increasing the levels of GSH and returning oxidative stress parameters to their control values.

Animal studies: The irradiation of rats was performed at the Radiation Oncology Department of the Phelps County Regional Medical Center in Rolla, Missouri, using a 16 MeV beam generated by a Varian linear accelerator, model Clinac 1800, and in accordance with the standards of humane laboratory animal protocols. A 20 x 20 or 25 x 25 cm field was used and output factors were checked once a week. Twelve animals were divided into 4 groups each containing 3 animals (Control, XRT, NAC amide+XRT and NAC+XRT). The radiation (XRT) control received whole body irradiation by 6 Gy of 16 MeV electrons. The NAC amide+XRT group received 500 mg/kg/day NAC amide immediately before irradiation and for three days after until sacrifice. The rats were anesthetized and heparinized blood was collected via cardiopuncture. Following sacrifice, liver, lung, brain and spleen were removed and stored at -70°C until homogenization.

All experiments were performed using adult Albino SASCO Sprague Dawley female rats weighing about 250 g, which were purchased from Charles River Laboratories Inc. (Portage, MI). Twelve rats were shipped in paper crates (4 in each crate). Rats were delivered with a certificate including serological, bacteriological, pathological parasitological information. They were divided into 4 cages (3 rats in each cage) and kept in a temperature controlled (20°C) room equipped to maintain a 12h light-dark cycle. Standard rat chow (Purina rat chow) and tap water were supplied in individual glass bottle and given ad libitum. Water was changed daily. Weights of the animal were taken before giving the NAC amide treatment solution and amount of food eaten and water consumed was not measured because NAC amide was given orally but not in the drinking water or food.

NAC amide was provided by Novetide Ltd (Haifa Bay, Israel) including certificate of analysis and MSDS (lot# 40233-64). NAC amide feeding solution was prepared freshly each day right before the administration by weighing 1.25g NAC amide solid sample (Type HR-120 electronic balance, A&D Company limited, Japan. S/N: 12202464) and adding into 10ml PBS solution and put on ice. One ml of this solution was administrated (gavaged) per rat orally by using animal feeding biomedical needles and 3ml BD Luer-Lok Tip syringes. Rats received one-dose total-body 6Gy/ 16MeV x-ray radiation and 3 rats in each group were held in a covered bucket and received radiation at the same time. Each day at the same time, 500 mg/Kg body weight of NAC amide was administrated to the animals.
All the results are normalized into values per unit (mg) of protein content for all the tissue samples.

Typical standard curves:
- GSH: y=8.57544x−425.092, R^2=0.9997
- CYS: y=7.53294x+84.35, R^2=0.9995

For GSH and CYS levels, 250 µL tissue homogenate was used to react with 750 µL NPM solution, therefore, the total volume was 1000 µL.

As an example:
The peak area for GSH in the sample is 90860.25. The GSH concentration (nM) is calculated from the standard curve. After determining the protein content (mg/ml) of the sample, for example: 16.5 mg/ml, the calculation is as follows:

\[
\text{GSH concentration (nM)} = \left( \frac{90860.25+425.092}{8.57544 \text{ nmol/L}} \times \frac{1 \text{ mL}}{1000 \text{ mL}} \times 250 \text{ µL} \right) / 16.5 \text{ mg/ml} = 2.58 \text{ nmol GSH/mg protein}
\]

For MDA levels, 350 µL tissue homogenate was used to react with 100 µL of 500 ppm BHT solution and 550 µL solution of 10% TCA solution, therefore, the total volume here was 1000 µL. After boiling the whole solution, 500 µL was taken out and react with 500 µL TBA and the total volume here was 1000 µL also.

As an example:
The peak area for MDA in the sample as 65289.23, The MDA concentration (nM) is calculated from the standard curve. After determining the protein content (mg/ml) of the sample, for example: 16.5 mg/ml, the resulting calculation is as follows:

\[
\text{MDA concentration (nM)} = \left( \frac{65289.23-370.488}{26.6869 \text{ nmol/L}} \times \frac{1 \text{ mL}}{1000 \text{ mL}} \times 350 \text{ µL} \right) \times \frac{1000 \text{ µL}}{350 \text{ µL}} = 84.3 \text{ nmol MDA /100 mg protein}
\]

-Catalase:
Calculation for specific activity:
In assay solution,
\[
k(\text{enzyme activity}) = \frac{1}{60} \times \ln(A0/A60) \times (\text{Total Volume of reaction/volume of sample})
\]
AO- Absorbance at 0 second
A60-Absorbance at 60 second
In sample, \(K(\text{specific activity}) = \frac{k}{\text{protein concentration}}\).

Oxidative Stress Parameters in Animals: After the blood samples were drawn, the animals were perfused by a cold antioxidant buffer first and then liver, brain and kidney...
samples were collected aseptically, rinsed in ice-cold saline and placed in petri dishes maintained on ice. The tissue samples kept at -70°C for the GSH, GSSG, and MDA determinations were made.

Glutathione (GSH) and Glutathione Disulfide (GSSG) Determination: Cells or tissue samples were homogenized on ice and derivatized with N-(1-pyrenyl)-maleimide (NPM). The derivatized samples were injected onto a 3 μη C18 column (Column Engineering) in a reverse phase HPLC system with a mobile phase of 35% water, 65% acetonitrile containing 1 mL/L of acetic acid and phosphoric acid (R. Winters, et al., Anal. Biochem., 227:14-21 (1995) and H.H. Draper et al, Free Rad. Biol. Med., 15:353-363 (1993)). Malondialdehyde (MDA) determinations were made as described in J. Gutteridge, Anal. Biochem., 69: 518-526 (1975).

Enzyme Activity Assays: Catalase (CAT) activity was determined spectrophotometrically and was expressed in units/mg protein and *units/10^6 cells as described by M. Bradford, Anal. Biochem., 72:248-256 (1976).

Statistical Analysis: Tabulated values represent means ± standard deviations. InStat® by GraphPad Software, San Diego, CA will use One-way Analysis of Variance (ANOVA) and the Student-Newman-Keuls Multiple Comparisons Test to analyze data from experimental and control groups. The p values < 0.05 is considered significant.

The results of the studies described in this Example are provided in the tables below.

In these tables, AD4 is synonymous with NAC amide.

Table 3. GSH and CYS levels in BRAIN after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
<tr>
<th>(n=3)</th>
<th>GSH (nmol/mg)</th>
<th>CYS (nmol/mg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>level Mean SD</td>
<td>level Mean SD</td>
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<tr>
<td>CTR-1</td>
<td>8.19 7.5 0.7</td>
<td>3.61 4.1 0.5</td>
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<tr>
<td>CTR-2</td>
<td>6.75</td>
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<td>CTR-3</td>
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<td>4.79</td>
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<tr>
<td>XRT-1</td>
<td>6.42 6.6 0.3</td>
<td>3.48 3.8 0.5</td>
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<tr>
<td>XRT-2</td>
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<td>3.76</td>
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<tr>
<td>XRT-3</td>
<td>6.89</td>
<td>4.36</td>
</tr>
<tr>
<td>XRT+AD4-1</td>
<td>7.93 7.6** 0.5</td>
<td>4.47 4.4 0.1</td>
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<tr>
<td>XRT+AD4-2</td>
<td>7.84</td>
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<td>XRT+NAC-1</td>
<td>7.32 7.0 0.3</td>
<td>4.16 4.1 0.4</td>
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<td>XRT+NAC-2</td>
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<td>XRT+NAC-3</td>
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<td>4.47</td>
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</table>
Table 4. GSH and CYS levels in LIVER after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
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<th></th>
<th>GSH (nmol/mg)</th>
<th>CYS (nmol/mg)</th>
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<tbody>
<tr>
<td></td>
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<td>CTR-1</td>
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<tr>
<td>CTR-3</td>
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</tr>
<tr>
<td>XRT-1</td>
<td>14.54</td>
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</tr>
<tr>
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<td>XRT-3</td>
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<td>XRT+NAC-3</td>
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Table 5. GSH and CYS levels in KIDNEY after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
<tr>
<th></th>
<th>GSH (nmol/mg)</th>
<th>CYS (nmol/mg)</th>
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<tbody>
<tr>
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<td>CTR-1</td>
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<td>XRT+NAC-2</td>
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<tr>
<td>XRT+NAC-3</td>
<td>6.33</td>
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</table>

Table 6. GSH and CYS levels in LUNG after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
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<th></th>
<th>GSH (nmol/mg)</th>
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</tr>
</thead>
<tbody>
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<td>GSH (nmol/mg)</td>
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<tr>
<td></td>
<td>(n=3) level</td>
<td>Mean</td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>6.28</td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>5.19</td>
<td>5.8</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>7.24</td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>4.95</td>
<td></td>
</tr>
</tbody>
</table>

Table 7. GSH and CYS levels in PLASMA after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
<tr>
<th></th>
<th>GSH (nmol/mg)</th>
<th></th>
<th>CYS (nmol/mg)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=3) level</td>
<td>Mean</td>
<td>SD</td>
<td>level</td>
</tr>
<tr>
<td>CTR-1</td>
<td>7.65</td>
<td>7.4</td>
<td>0.4</td>
<td>16.03</td>
</tr>
<tr>
<td>CTR-2</td>
<td>7.49</td>
<td></td>
<td></td>
<td>15.20</td>
</tr>
<tr>
<td>CTR-3</td>
<td>6.92</td>
<td></td>
<td></td>
<td>15.39</td>
</tr>
<tr>
<td>XRT-1</td>
<td>5.27</td>
<td>5.3*</td>
<td>0.1</td>
<td>12.68</td>
</tr>
<tr>
<td>XRT-2</td>
<td>5.39</td>
<td></td>
<td></td>
<td>13.63</td>
</tr>
<tr>
<td>XRT-3</td>
<td>5.31</td>
<td></td>
<td></td>
<td>14.45</td>
</tr>
<tr>
<td>XRT+AD4-1</td>
<td>7.10</td>
<td>7.6**</td>
<td>0.4</td>
<td>16.00</td>
</tr>
<tr>
<td>XRT+AD4-2</td>
<td>7.44</td>
<td></td>
<td></td>
<td>15.45</td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>7.94</td>
<td></td>
<td></td>
<td>15.40</td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>7.08</td>
<td>6.5**/***</td>
<td>0.5</td>
<td>14.64</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>6.18</td>
<td></td>
<td></td>
<td>13.75</td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>6.27</td>
<td></td>
<td></td>
<td>14.36</td>
</tr>
</tbody>
</table>

* P<0.05 compared to the CTR group; ** P<0.05 compared to the XRT only group
*** P<0.05 compared to the XRT+AD4-treated group

Table 8. MDA levels in BRAIN after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=3) level</td>
</tr>
<tr>
<td>CTR-1</td>
<td>4.93</td>
</tr>
<tr>
<td>CTR-2</td>
<td>3.33</td>
</tr>
<tr>
<td>CTR-3</td>
<td>4.02</td>
</tr>
<tr>
<td>XRT-1</td>
<td>5.64</td>
</tr>
<tr>
<td>XRT-2</td>
<td>6.76</td>
</tr>
<tr>
<td>XRT-3</td>
<td>5.55</td>
</tr>
<tr>
<td>XRT+AD4-1</td>
<td>5.79</td>
</tr>
<tr>
<td>XRT+AD4-2</td>
<td>5.53</td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>5.13</td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>6.42</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>6.69</td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>5.33</td>
</tr>
</tbody>
</table>
Table 9. MDA levels in LIVER after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
<tr>
<th>(n=3)</th>
<th>level</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR-1</td>
<td>4.36</td>
<td>4.62</td>
<td>0.39</td>
</tr>
<tr>
<td>CTR-2</td>
<td>4.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR-3</td>
<td>5.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-1</td>
<td>8.9</td>
<td>8.36*</td>
<td>0.53</td>
</tr>
<tr>
<td>XRT-2</td>
<td>8.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-3</td>
<td>7.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-1</td>
<td>4.14</td>
<td>4.38**</td>
<td>0.26</td>
</tr>
<tr>
<td>XRT+AD4-2</td>
<td>4.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>4.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>5.1</td>
<td>5.07**/***</td>
<td>0.04</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>5.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 10. MDA levels in KIDNEY after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally)

<table>
<thead>
<tr>
<th>(n=3)</th>
<th>level</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR-1</td>
<td>1.61</td>
<td>1.69</td>
<td>0.09</td>
</tr>
<tr>
<td>CTR-2</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR-3</td>
<td>1.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-1</td>
<td>2.48</td>
<td>2.28*</td>
<td>0.17</td>
</tr>
<tr>
<td>XRT-2</td>
<td>2.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-3</td>
<td>2.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-1</td>
<td>1.5</td>
<td>1.64**</td>
<td>0.28</td>
</tr>
<tr>
<td>XRT+AD4-2</td>
<td>1.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>1.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>1.76</td>
<td>1.65**</td>
<td>0.21</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>1.78</td>
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<td></td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>1.41</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11. MDA levels in LUNG after 6Gy total-body x-ray radiation with AD4 or NAC Administration (500mg/kg orally)

<table>
<thead>
<tr>
<th>(n=3)</th>
<th>level</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR-1</td>
<td>1.47</td>
<td>1.54</td>
<td>0.07</td>
</tr>
<tr>
<td>CTR-2</td>
<td>1.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR-3</td>
<td>1.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-1</td>
<td>2.3</td>
<td>2.80*</td>
<td>0.45</td>
</tr>
<tr>
<td>XRT-2</td>
<td>2.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-3</td>
<td>3.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 12. Catalase activities in KIDNEY after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally):

<table>
<thead>
<tr>
<th>(n=3)</th>
<th>level</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRT+AD4-1</td>
<td>1.72</td>
<td>1.53**</td>
<td>0.22</td>
</tr>
<tr>
<td>XRT+AD4-2</td>
<td>1.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>2.58</td>
<td>2.52**</td>
<td>0.15</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>2.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>2.63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P<0.05 compared to the CTR group
** P<0.005 compared to the XRT only group
*** P<0.05 compared to the XRT+AD4-treated group

Table 13. Catalase activities in LUNG after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally):

<table>
<thead>
<tr>
<th>(n=3)</th>
<th>level</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR-1</td>
<td>2.75</td>
<td>2.34</td>
<td>0.78</td>
</tr>
<tr>
<td>CTR-2</td>
<td>2.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR-3</td>
<td>1.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-1</td>
<td>8.73</td>
<td>8.69*</td>
<td>1.05</td>
</tr>
<tr>
<td>XRT-2</td>
<td>7.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT-3</td>
<td>9.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-1</td>
<td>3.89</td>
<td>3.97**</td>
<td>0.56</td>
</tr>
<tr>
<td>XRT+AD4-2</td>
<td>3.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+AD4-3</td>
<td>4.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-1</td>
<td>5.85</td>
<td>4.41</td>
<td>1.48</td>
</tr>
<tr>
<td>XRT+NAC-2</td>
<td>3.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC-3</td>
<td>4.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P<0.05 compared to the CTR group
** P<0.005 compared to the XRT only group
*** P<0.05 compared to the XRT+AD4-treated group
Table 14. Catalase activities in LIVER after 6Gy total-body x-ray radiation with AD4 or NAC administration (500mg/kg orally).

<table>
<thead>
<tr>
<th></th>
<th>(n=3)</th>
<th>level</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRT+NAC-3</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data presented support the finding that NAC amide functions as a strong thiol antioxidant in radiation-induced oxidative stress. NAC does not increase GSH levels in tissues, presumably because it does not cross the cell membranes. Although plasma Cys level increased significantly, this was not reflected in the liver. NAC generally provides GSH only during increased demand on the GSH pool.

Upon irradiation, reactive oxygen species are formed through oxygen's acceptance of electrons, which are involved in free radical chain reactions and are highly damaging to the cell through disruption of the cellular pro-oxidant/antioxidant balance. Normal tissue damage limits the radiation dose and treatment volume in radiotherapy. Radioprotection of normal tissue by thiols offers one way in which radiation dosage can be increased. The focus in this Example was to examine the radioprotective effects of NAC amide using a whole body radiation dose of 6 Gy, sufficient to insure that all animals should progress with lethal gastrointestinal and hematopoietic syndromes. The time point chosen for analyses, 4 days, approximates the time that the animals would begin to succumb to the gastrointestinal syndrome, but would be expected to show only early changes in the hematopoietic syndrome.
GSH, a tripeptide consisting of γ-glutamyl-cysteinyl-glycine, is the principle water-soluble intracellular free thiol and acts as a radioprotector. Several distinct mechanisms of radioprotection by GSH can be identified and include radical scavenging, hydrogen donation to damaged molecules, reduction of peroxides, and protection of protein thiol oxidative status. GSH has been shown to decrease in tissues following irradiation. Since GSH is an endogenous radioprotector, modification of GSH concentration may be useful as radiation protection. Cysteine provides the rate-limiting step in GSH synthesis since its apparent Km value for γ-glutamyl-cysteine synthetase is close to the intracellular concentration of the amino acid. However, administration of cysteine is not the ideal way to increase intracellular GSH, since it auto-oxidizes rapidly and can lead to the production of hydroxy land thyl radicals.

NAC, a cysteine analogue that is a mucolytic agent and a treatment for paracetamol intoxication, promotes hepatic GSH synthesis. It penetrates the cell membrane and is rapidly deacetylated to L-cysteine, while also stimulating GSSG reductase. NAC can rapidly increase the hepatic GSH levels and maintain these levels for at least 6 hours (B. Wong et al., J. Pharm. Sci., 75:878-880 (1986)). NAC has also been shown to protect Chinese hamster ovary cells from lead and δ-aminolevulinic acid-induced toxicity through restoration of the oxidative status of the cells by GSH replenishment. It has been demonstrated that NAC protects liver and brain of C57BL/6 mice from GSH depletion as a result of lead poisoning.

Radioprotective effects of select thiols such as indomethacin, WR-2721, cysteamine, and diethyldithiocarbamate have been reported, though at higher concentrations these induce cellular toxicity. The radioprotective effect of NAC has been demonstrated in human granulocyte/macrophage-colony forming cells. However, it has also been shown that the more radioresistant SW-1573 human squamous lung carcinoma cell line was not protected from X-ray induced cell death by NAC. NAC amide is more lipophilic and able to more easily cross cell membranes than NAC. In this Example, the radioprotective function of NAC amide was compared with that of NAC in terms of increasing GSH levels and returning oxidative stress parameters to their control values.

The exposure of membrane lipids to reactive oxygen species such as the hydroxyl radical can initiate a chain reaction in polyunsaturated fatty acid moieties, which results in peroxidation and causes degradation of membrane function. MDA is a degradation product of the highly unstable lipid peroxides. As observed in this Example, irradiation of Sprague Dawley rats resulted in increased MDA levels in liver and lung. Upon treatment with NAC
amide concurrent with irradiation, lung MDA levels were significantly lowered, while treatment with NAC did not change the MDA levels significantly.

It is generally accepted in the field of radiobiology that the mechanism of individual cell killing by radiation exposure is due to direct and indirect ionizing effects specifically upon DNA in the cell nucleus, although it becomes apparent that in a complex organism there are ROS effects of some potential importance on membrane lipids and proteins as well as on nucleic acids. Furthermore, acute whole body irradiation of the intact animal under conditions modeling the so called "gastrointestinal syndrome" causes changes in several tissues apart from gastrointestinal tract, and some of these effects can be ameliorated by the use of NAC amide. A given syndrome such as the "gastrointestinal syndrome" can actually involve a complex of changes in multiple tissues and organs. Radiation pneumonitis can be a serious hazard in the therapeutic irradiation of patients with lung cancer. NAC amide may be considered for use as a thiol radioprotectant to protect against such a complication. Thus, in accordance with the invention, NAC amide significantly increases thiol levels in plasma and liver and performs better than NAC as a radioprotecting agent.

**Example 3**

This Example describes a treatment regimen suitable for humans. NAC amide is administered between 1 and three grams per day, in two divided doses, between meals (on an empty stomach). Encapsulated NAC amide (a formulation of NAC amide comprising 500 mg NAC amide and optionally, 250 mg USP grade crystalline ascorbic acid, and not more than 0.9 mg magnesium stearate, NF grade in an OO-type gelatin capsule) is suitable for administration. The administration of exogenous NAC amide is expected to provide a dose response effect in patients, despite the production of large quantities of glutathione in the human body.

**Example 4**

This Example describes a combination pharmaceutical composition to ameliorate the detrimental effects of acetaminophen, a drug that consumes glutathione in the liver during metabolism and, in excess doses, causes liver damage due to oxidative damage. The composition includes 500 mg NAC amide, 250 mg crystalline ascorbic acid and 350 mg acetaminophen. NACA did not induce any cell death by itself even at higher concentrations (10μM). Cysteine levels in cells were restored to control after exposed to AAP with NACA treatment. Although HEPG2 cells showed a dose dependent decrease in viability change
when exposed to acetaminophen, the cells were exposed to concentrations 4-5 times more than the concentrations observed in AAP poisoning.

**Experiment I.**

Preliminary studies were done using HEPG2 cells (modified liver cells) as *invitro* model. Stock solutions of AAP were prepared by dissolving the drug in water and later diluted with media for the required concentration for all the experiments. The solutions were prepared fresh every time. Around 8000 cells/well were seeded in a 96 well plate. After attachment of the cells overnight, the cells were exposed to different concentrations of AAP from 24 hrs and 48 hrs. The media was removed after required exposure time and cell viability was measure using MTS reagent.

**Statistical Analysis:** All the results are expressed Mean +/- standard deviation. One way ANOVA followed by Tukeys multiple comparison tests were performed. Figures 38-39 shows exposure to Acetaminophen decreased the cell viability in dose dependent manner after 24hrs and 48hrs.

**Experiment II.**

To determine the non toxic level of NAC and NACA to be used for protective action, viability studies of HEPG2 cells exposed to different concentrations of NAC and NACA was performed. NAC and NACA were dissolved directly in media. Figure 40 shows that NAC and NACA did not have any significant toxicity on the HEPG2 cells even at 10mM levels.

**Experiment III:**

To compare the protective effects of NAC and NACA against AAP toxicity, two different concentrations of AAP were chosen (20mM and 30mM) and HEPG2 cells were exposed to 20mM and 30mM of AAP with 5mM of NAC or NACA for 24hrs and viability was tested using MTS assay. Figures 41 and 42 show that NACA improved viability at both doses of AAP.

**Experiment IV:** Effect of NAC and NACA on cysteine levels in cells exposed to AAP (20Mm) for 24 hrs were tested in this experiment. Around 350,000 cells were seeded in 25cm² and were allowed to attach overnight. The cells were pretreated with NAC and NACA for 2 hrs and later exposed to acetaminophen. After the exposure the cells were analyzed for cysteine levels using HPLC -Flurometric method. Figure 43 shows exposure to Acetaminophen decreased the cysteine levels and pretreatment with NAC and NACA restored the levels to that of control.
Experiment V: The ability of NACA to increase the viability by acting as thiol donor in the absence or depletion of GSH was tested in this experiment. HEPG2 cells were seeded in 96 well plates and pretreated with NAC and NACA for 2 hrs and later exposed to BSO 5mM, AAP 20mM each for 2 days. MTS assay was done to determine the viability. Figure 44 shows that exposure of HEPG2 cells to BSO and AAP reduced the viability to 35%. Incubation with NAC increased the viability to 66% whereas the incubation with NACA increased the viability to 74%.

Summary of Results: Preliminary experiments done with NACA show that NACA provides a better protection than NAC in acetaminophen induced cell death in HEPG2 cells. NACA did not induce any cell death by itself even at higher concentrations (10mM). Cysteine levels in cells were restored to control after exposed to AAP with NACA treatment. Although HEPG2 cells showed a dose dependent decrease in viability change when exposed to acetaminophen, the cells were exposed to concentrations 4-5 times more than the concentrations observed in AAP poisoning. Experiments have to be performed to determine the efficacy of NACA in increasing glutathione levels, decreasing ROS in cells when exposed to acetaminophen.

Example 5

This Example describes a combination pharmaceutical composition to ameliorate the detrimental effects of chlorpromazine, a phenothiazine drug that causes side effects, including tardive dyskinesia, which may be associated with excess free radical reactions. The composition includes 500 mg NAC amide, 250 mg crystalline ascorbic acid and 200 mg chlorpromazine.

Example 6

This Example describes a combination pharmaceutical composition to ameliorate the detrimental effects of aminoglycoside drugs (antibiotics), nonlimiting examples of which include neomycin, kanamycin, amikacin, streptomycin, gentamycin, sisomicin, netilmicin and tobramycin, a drug class which may be associated with various toxicities. This damage may be related to oxidative damage or consumption of glutathione during metabolism. The composition according to the present invention is an intravenous formulation, including the aminoglycoside in an effective amount, and NAC amide in an amount of about 10-20 mg/kg.
Ascorbic acid in an amount of 5-10 mg/kg may be added as a stabilizer. FIGs 45-52 show that NACA block kidney cell toxicity from the antibiotic gentamycin.

Example 7

This Example describes a urethral insert comprising NAC amide. A composition containing 200 mg NAC amide, 50 mg ascorbic acid per unit dosage is mixed with carageenam and/or agarose and water in a quick-gelling composition, and permitted to gel in a cylindrical form having a diameter of about 3 mm and a length of about 30 mm. The composition is subjected to nitric oxide to cause between 0.1-10% of the NAC amide to be converted to nitroso-NAC amide. The gelled agarose is then freeze dried under conditions that allow shrinkage. The freeze-dried gel is than packaged in a gas barrier package, such as a foil pouch or foil "bubble-pack". The freeze-dried gel may then be used as a source of nitroso-NAC amide for administration transmucosally. The cylindrical freeze-dried gel may be inserted into the male urethra for treatment of impotence, or administered sublingually for systemic vasodilation.

Example 8

This Example describes an oral formulation for prophylaxis of vascular disease, e.g., in men over 40. The composition includes 500 mg NAC amide, 250 mg USP grade crystalline ascorbic acid and 50 mg USP acetyl salicylic acid (aspirin) in an OO-type gelatin capsule. Typical administration is twice per day. The acetyl salicylic acid may be provided in enteric release pellets within the capsule to retard release.

Example 9

This Example describes an oral formulation for prophylaxis of vascular disease. The composition contains 500 mg NAC amide, 200 mg USP grade crystalline ascorbic acid, and 200 mg arginine in an OO-type gelatin capsule. Arginine is the normal starting substrate for the production of nitric oxide. Because arginine is normally in limited supply, a relative deficiency of arginine may result in impaired vascular endothelial function.

Example 10

This Example describes an oral formulation for prophylaxis of vascular disease. The composition includes 500 mg NAC amide, 200 mg USP grade crystalline ascorbic acid, and 200 mg vitamin E succinate in an OO-type gelatin capsule. Vitamin E consumption reduces the risk of heart attack and other vascular disease. Vitamin E succinate (alpha-tocopherol succinate) is a dry powder.
Example 11

This Example describes an oral formulation for prophylaxis of vascular disease. Nonspecific esterases having broad substrate specificity are present in the plasma. According to the present invention, esters are formed between agents that are useful combination therapies in order to provide for efficient administration, high bioavailability, and pharmaceutical stability. Preferred esters include alpha tocopherol-ascorbate, alpha tocopherol-salicylate, and ascorbyl-salicylate. The tocopherol ester maintains the molecule in a reduced state, allowing full antioxidant potential after ester cleavage. These esters may be administered alone or in combination with other agents, for example NAC amide. Typically, the esters are administered to deliver an effective dose of salicylate equivalent of 100 mg per day for prophylaxis, or 750-1000 mg per dose for treatment of inflammatory diseases. Tocopherol is administered in an amount of 100-500 IU equivalent. Ascorbate is administered in an amount of up to 1000 mg equivalent. In order to enhance availability, a non-specific esterase may be provided in the formulation to cleave the ester after dissolution of the capsule. Therefore, a non-specific esterase, such as a bacterial or saccharomyces (yeast) enzyme, or an enriched enzyme preparation, may be included in the formulation as a powder or as pellets in the capsule.

Example 12

This Example describes an oral formulation for prophylaxis of vascular disease. The composition includes 500 mg reduced NAC amide, 200 mg USP grade crystalline ascorbic acid, and 100 mg nordihydroguaretic acid, in an OO-type gelatin capsule. Typical administration is twice per day. Nordihydroguaretic acid is a known lipoxygenase inhibitor. Thus, this composition may be used to treat inflammatory processes or as prophylaxis against vascular disease.

Example 13

This Example describes a study observing the survival of rats receiving whole body, single-dose irradiation by X-rays (XRT) in the presence or absence of NAC or NAC amide (TOVA). In this experiment, thirty-nine female Sprague-Dawley rats ranging from about 150-200 g were subjected to total body, single-dose X-ray irradiation (9Gy, 16Mev). The same groups were designated to receive either NAC or TOVA. For the pre-treatment groups (n=6 in each group), the first treatment of NAC or TOVA was administered 30 minutes to 1 hour before irradiation. For the post-pretreatment groups (n=6 in each group), the first treatment of NAC or TOVA was administered 30 minutes to 1 hour after the irradiation. For
groups receiving NAC or TOVA, the same amount (500mg/kg NAC or TOVA daily) was administered for 4 or 5 consecutive days.

Group 1 was a control group (n=3), where rats received the same amount of saline solution daily for 5 consecutive days without XRT. Group 2 rats received NAC only (n=3) at an amount of 500mg/kg body weight NAC daily for 5 consecutive days without XRT. Group 3 rats received TOVA only (n=3) at an amount of 500mg/kg body weight TOVA daily for 5 consecutive days without XRT. Group 4 rats received radiation (XRT) only (n=6) and received the same amount of saline solution daily for 5 consecutive days after single dose total-body XRT irradiation.

Group 5 rats received one treatment of NAC at 500 mg/kg body weight before XRT (XRT+NAC pre-treated), which was then followed by 500 mg/kg body weight NAC daily for 4 consecutive days after XRT. Group 6 rats received XRT, followed by daily doses of NAC at 500 mg/kg body weight for 5 consecutive days after XRT (XRT+NAC post-treated). Group 7 rats received one treatment of NAC at 500 mg/kg body weight before XRT (XRT+TOVA pre-treated), which was then followed by 500 mg/kg body weight TOVA daily for 4 consecutive days after XRT. Group 8 rats received XRT, followed by daily doses of TOVA at 500 mg/kg body weight for 5 consecutive days after XRT (XRT+TOVA post-treated). All rats were then given a normal diet post-treatment.

The rats were observed twice a day, and the survival status of rats in each group will be recorded. The mean survival days were calculated for each group and compared to the survival differences of the three groups of rats at the end of the experiment. The radioprotective effects of NAC and TOVA treatment on the survival of those irradiated rats were then evaluated, as shown in the following tables.

Table 15 shows the number of animals that survived under conditions where NAC or TOVA was administered pre- or post-XRT treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th># of animals</th>
<th># of animals dead</th>
<th># of animals survived</th>
<th>survival rate</th>
<th>percentage survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRT only</td>
<td>(n=6)-1st time</td>
<td>2</td>
<td>4</td>
<td>(4+2)/(6+6)</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>(n=6)-2nd time</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+NAC(pre-treated)</td>
<td>(n=6)-1st time</td>
<td>1</td>
<td>5</td>
<td>(5+5)/(6+6)</td>
<td>83.3%</td>
</tr>
<tr>
<td></td>
<td>(n=6)-2nd time</td>
<td>1</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRT+TOVA(pre-treated)</td>
<td>(n=6)-1st time</td>
<td>0</td>
<td>6</td>
<td>(6+6)/(6+6)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(n=6)-2nd time</td>
<td>0</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups</td>
<td># of animals</td>
<td># of animals</td>
<td>survival rate</td>
<td>percentage survival rate</td>
<td></td>
</tr>
<tr>
<td>------------------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>---------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td>Control (no XRT and any treatment)</td>
<td>(n=3)-1st</td>
<td>0</td>
<td>3</td>
<td>(3+3)/(3+3) 100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=3)-2nd</td>
<td>0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAC only</td>
<td>(n=2)-2nd</td>
<td>0</td>
<td>2</td>
<td>(2)/(2) 100%</td>
<td></td>
</tr>
<tr>
<td>TOVA only</td>
<td>(n=3)-2nd</td>
<td>0</td>
<td>3</td>
<td>(3)/(3) 100%</td>
<td></td>
</tr>
<tr>
<td>XRT+NAC(post-treated)</td>
<td>(n=6)-2nd</td>
<td>4</td>
<td>2</td>
<td>(2)/(6) 33.3%</td>
<td></td>
</tr>
<tr>
<td>XRT+TOVA(post-treated)</td>
<td>(n=6)-2nd</td>
<td>2</td>
<td>4</td>
<td>(4)/(6) 66.7%</td>
<td></td>
</tr>
</tbody>
</table>

Table 16 shows the survival rate percentage of rats receiving NAC or TOVA pre- or post-XRT treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>percentage survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRT only</td>
<td>50%</td>
</tr>
<tr>
<td>XRT+NAC(pre-treated)</td>
<td>83.3%</td>
</tr>
<tr>
<td>XRT+TOVA(pre-treated)</td>
<td>100%</td>
</tr>
<tr>
<td>Control (no XRT and any treatment)</td>
<td>100%</td>
</tr>
<tr>
<td>NAC only</td>
<td>100%</td>
</tr>
<tr>
<td>TOVA only</td>
<td>100%</td>
</tr>
<tr>
<td>XRT+NAC(post-treated)</td>
<td>33.3%</td>
</tr>
<tr>
<td>XRT+TOVA(post-treated)</td>
<td>66.7%</td>
</tr>
</tbody>
</table>

FIG. 6 is a graphical representation comparing the percentage survival rates as presented in Table 16. These results show that rats pre-treated with NAC or TOVA before XRT have a higher survival rate than those receiving XRT alone.

As various changes can be made in the above methods and compositions without departing from the scope and spirit of the invention as described, it is intended that all subject matter contained in the above description, shown in the accompanying drawings, or defined in the appended claims be interpreted as illustrative, and not in a limiting sense.

Example 14: Attenuation Of Pulmonary Inflammation After Exposure To Blast Overpressure By N-Acetylcysteine Amide

Lung contusion is a common problem from blunt chest trauma caused by mechanical forces and by exposure to blast overpressure, often with fatal consequences. Lung contusion is also a risk factor for the development of pneumonia, severe clinical acute lung injury (ALI), and acute respiratory distress syndrome (ARDS). Infiltrating neutrophils are
considered to be central mediators of lung injuries after blunt trauma. Recent studies have demonstrated that antioxidants reduced pulmonary inflammation in different models of lung damage. This study examined the effect of antioxidant N-acetylcysteine amide (NACA) on the progression of lung inflammation after exposure to a moderate level of blast overpressure (140 kPa). Rats were administered with NACA (i.p. 100 mg/kg) or placebo (PBS) 30, 60 min and 24 h after exposure. Nonblasted sham-injected animals served as controls. Neutrophil infiltration measured by myeloperoxidase (MPO) activity in the lung was significantly increased at 2 days after blast and returned to controls at 8 days. This increase corresponded with activation of integrin CD11b mRNA and lung inflammatory chemokine mRNA expression; macrophage inflammatory protein-1 (MIP-1), monocyte chemotactic peptide-1 (MCP-1), and cytokine-induced neutrophil chemoattractant-1 (CINC-1). At 8 days, all inflammatory mediators returned to control levels. In addition, expression of heme oxygenase-1 (HO-1) mRNA increased at 2 days after exposure. No changes were detected in the lung manganase superoxide dismutase (MnSOD) or glutathione reductase (GR) mRNA expression after blast. N-Acetylcysteine amide significantly reduced infiltration of neutrophils and CD11b mRNA activation in lungs, and completely blocked activation of MIP-1, MCP-1 and CINC-1 mRNA. The relatively higher inhibition of chemokine mRNAs compared with reduction in MPO activity and CD11b is in accordance with an antioxidant effect of NACA on reactive oxygen species (ROS) accumulation, rather than by an effect on neutrophil sequestration. The inhibition of HO-1 mRNA activation after blast was likely also related to the drug antioxidant effect.

Blast resulting from explosive detonation or firing of large caliber weapons results in an abrupt, rapid rise of atmospheric pressure that is referred to as blast overpressure (BOP). Exposure to BOP results in contusion or barotrauma-like injury (primary injury) mainly to air-filled organs such as ears, lungs, and the gastrointestinal tract (1, 2). Recently, brain damage appeared as another serious consequence of exposure to BOP with long-term neurological and behavioral impairment (3). The lungs often sustain a greater degree of damage, with potentially fatal results. Body armor has been shown to protect military personnel from ballistic projectiles. However, it does not protect completely against the barotrauma of primary blast injury (4). Pulmonary barotrauma is the most critical and immediately life-threatening injury after blast in nonprotected civilian population. Pressures within the lung parenchyma and air spaces can match or greatly exceed the BOP because the lung tissue and blood vessels are compressed more slowly than air in the respiratory tract,
forcing air out through alveolar septa and blood through capillary walls, causing them to rupture (1). The resulting alveolar hemorrhage and edema greatly reduce the gas exchange capacity of the lung, contributing to bradycardia, hypotension, and apnea (5, 6). The extravasated blood initiates a cascade of reactions that involve activation and release of various vasoactive and proinflammatory factors. After such trauma and hemorrhage, free hemoglobin induces severe vasoconstriction that is associated with hypoperfusion in the cerebral, pulmonary, and coronary microcirculation (7, 8). Furthermore, auto-oxidation of oxyhemoglobin releases free iron and heme that, in turn, may cause oxidative injury (9). The sequela of pulmonary contusion varies widely, ranging from mild dyspnea to prolonged mechanical ventilation, infection, and organ failure (10, 11). There is often little correlation between the anatomic extent of contused lungs and the degree of hypoxemia (10). Specific cellular inflammatory and oxidative mechanisms that contribute to the severity of blast injury and determine its resolution or progression to acute respiratory failure remain poorly understood (12).

Oxidative stress caused by excessive accumulation of reactive oxygen species (ROS) is a common mechanism of damage in many experimental models of acute lung injury (13, 14). Excessive ROS accumulation results in depletion of antioxidants and oxidative damage to major cellular components such as lipids, proteins, and DNA. Evidence for oxidative stress in blast includes a decrease in endogenous antioxidant capacity and increase in lipid peroxidation and protein nitration in lungs after blast exposure (15, 16). In addition to released hemoglobin, another ROS-mediated mechanism of damage after blast is activation of inflammation, with leucocytes, macrophages, and neutrophils being the most prodigious source of ROS. Decrease in antioxidant defense capacity is widely recognized as a central feature of many inflammatory lung diseases. Therefore, attempts to attenuate lung injury include the restoration of the oxidant/antioxidant balance and limiting the degree of oxidative cell damage by augmenting the intracellular pool of antioxidants. Some antioxidants such as N-acetylcysteine (NAC) have been shown to protect pulmonary cells from oxidative injury and ameliorate inflammation (17, 18). In a small clinical trial, treatment with intravenous NAC was associated with improved cardiorespiratory functions in patients with ARDS (19). However, bioavailability of NAC is very low because of its low ability to cross biological membranes. A newly designed amide form of NAC, N-acetylcysteine amide (NACA), was shown to be more hydrophobic and membrane permeable (20). Recent studies demonstrated that treatment with NACA results in restoration of intracellular thiols, protection against
hemoglobin oxidation and more efficient reduction of intracellular oxidation compared with NAC (21 Y23). N-Acetylcysteine amide was also shown to ameliorate lung inflammatory injury in the mouse model of asthma (24).

In the present study, we administered NACA to rats after exposure to a moderate, nonlethal blast level to evaluate its effect on recovery from inflammatory lung injury.

MATERIALS AND METHODS

Exposure to BOP and drug administration

Adult virus-free male Sprague-Dawley rats weighing 300 to 350 g were randomly divided into two groups; one exposed to blast and one control, nonexposed group. Blast-exposed animals were anesthetized with ketamine/ xylazine (60/5 mg/kg, i.p.), placed into a compressed air-driven shock tube, and exposed to blast wave with mean peak overpressure of 140 kPa. One group was injected intraperitoneally with 100 mg/kg NACA in phosphate-buffered saline (n = 16; pH 7.4), whereas the second group was injected with phosphate-buffered saline alone (n = 16). The drug or placebo was injected 30 min, 60 min, and 24 h after exposure to blast. Half of the NACA-treated (n = 8) and placebo-treated (n = 8) animals were sacrificed at 2 days after blast exposure; the other half were killed at 8 days after blast exposure. A control group of animals (n = 8), in which half received NACA and the other half received placebo, underwent the same treatment (anesthesia, suspension, time delays) except they were not exposed to blast. Immediately after sacrifice, lungs were excised, washed in cold saline (4-C), inspected for gross pathology, and frozen at -70°C until further analysis. In this study, NACA was provided by Glenn Goldstein, M.D. (ThiolTech, New York, NY). The experiments were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals and the study was approved by our Institutional Animal Care and Use Committee.

Evaluation of gross lung injury and histopathological analysis

The extent of injury was defined by the severity type elements score, which classifies pulmonary lesions in a 1 to 5 range: 1 indicates no damage; 2, trace; scattered surface petechiation, 3, slight; extensive petechiation to ecchymosis; 4, moderate; parenchymal contusions; and 5, extensive; confluent hepatized regions. In a parallel experiment, 2 or 8 days after exposures, rats were euthanized with an intraperitoneal injection of pentobarbital, and the trachea was isolated and cannulated with an 18-gauge catheter. The lungs were inflated and en block fixed with 4% paraformaldehyde at 20 cm H2O fixative pressure. After fixation and dehydration, lungs were embedded in paraffin and cut into 10-2m sections.
Sections were stained with hematoxylin and eosin and graded on a 1 to 5 scale as percent of tissue involved in the inflammation and hemorrhage (grade 1 0 5%; grade 2, 5%Y15%; grade 3, 15%Y35%; grade 4, 36% to 70%; grade 5, 970%) by two blinded observers. Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity quantification in lung samples was carried out using colorimetric assay kit (CytoStore, Calgary, Canada). In brief, lung tissue was homogenized, sonicated, and centrifuged. The supernatant was mixed with 50 mmol/L potassium phosphate (pH 6) containing O-dianisidine and hydrogen peroxide and analyzed in duplicate on a spectrophotometric plate reader (1420 Multilabel Counter, Perkin-Elmer, Waltham, Mass) at 450 nm. Change in absorbance was measured over 1 min, and results are expressed as optical density per minute per mg lung wet weight.

Real-time polymerase chain reaction

A portion of each lung was excised and stored in RNAlater (Ambion, Austin, Tex) at 20-C and homogenized by a vortex. Total RNA from six to eight animals was extracted with Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif). First-strand cDNA was prepared from 12g of total RNA by using iScript RTYpolymerase chain reaction kit (BioRad) and oligo(dT) primer in a 20 2L reaction. For real-time PCR, 50 ng of first strand cDNA was used in a total volume 25 2L of the iQ SYBR Green Super Mix (BioRad) containing 200 n mol of each mRNA-specific primer (Sigma, St. Louis, Mo). Polymerase chain reactions consisted of initial 10 min at 90-C and then 40 cycles of 15 s at 95-C and 60 s 60-C. The ABI Prism 7700 Sequence Detection System was used to measure and quantify signals by the comparative threshold cycle method with "-actin as a housekeeping gene reference. Statistics All results were expressed as mean T SD. For multiple group comparisons, ANOVA followed by Fisher least significant difference test was used. The significance level was set at P < 0.05.

RESULTS

Survival rate and gross pathological signs of lung injury

The most consistent lesions after exposure to 140-kPa BOP observed at 2 days after blast were isolated petechial spots and ecchymoses grades 2 to 3, located on one side of the lung (Fig. 7). The severity of gross pathological injury varied between 2 and 3 for most animals; no animal had a score of 4 or 5. The average severity score was approximately the same in the drug- and placebo-treated animals (median scores, 2). At 8 days, lung gross pathology showed no or minimal damage grades 1 and 2.
All animals had immediate apnea after exposure to blast. No animals died later than 30 min after exposure (after drug or placebo administration). The survival rate after exposure to 140-kPa level of blast was approximately 60%.

Histopathologic examination showed focally extensive areas of alveolar hemorrhage with chronic-active inflammation 2 days after exposure to BOP (Fig. 8, A and B). Hemorrhage areas contained moderate numbers of macrophages that were often laden with erythrocytes and cell debris (Fig. 9B). Inflammation consisted of moderate numbers of lymphocytes, macrophages, and neutrophils (Fig. 9C). Affected areas were often lined by type II pneumocyte hyperplasia (indicative of epithelial cell damage in the alveolus) and the alveolar septa expanded by inflammation and mild fibrosis (Fig. 9B). Occasionally affected areas contained brightly eosinophilic, crystalline material located only in areas of hemorrhage. On a scale of 1 to 5, the pulmonary damage for animals administered with placebo was grade 3, and for drugtreated animals, a grade of 2. There was no difference in the grade of pathological damage between drug-treated and placebo-treated animals at 8 days postexposure (Fig. 10, A and B). Small foci of chronic inflammation consisted of lymphocytes and macrophages (Fig. 10, C and D). Occasionally, macrophages seemed to contain hemosiderin or erythrocytes as in animals 2 days postblast. However, there was no sign of hemorrhage (free erythrocytes) or any other changes such as necrosis, type II pneumocyte hyperplasia, or eosinophilic crystalline material.

Effect of BOP on lung inflammatory markers

Pulmonary neutrophil sequestration is a characteristic component of chest blunt injury and its progression/resolution. Myeloperoxidase activity in lung samples was used to assess neutrophil infiltration into lung parenchyma. Results show a massive increase in MPO activity in lungs 2 days after exposure (Fig. 11A). At 8 days, MPO activity returned to baseline, indicating resolution of inflammatory reaction. To investigate whether transmigrated inflammatory cells are activated in response to blast, the expression of CD11b mRNA was also assessed. CD11b is a member of the "2 integrin receptors and mediates neutrophil adhesion to the endothelial cells. As shown in Figure 11B, a significant increase in neutrophil CD11b was observed at 2 days after blast exposure.

To further assess the impact of blast exposure on activation of lung inflammation, the mRNA levels of CC chemokines, macrophage inflammatory protein-1 (MIP-1), monocyte chemotactic peptide-1 (MCP-1) and CXC chemokine, cytokineinduced neutrophil chemoattractant-1 (CINC-1) were assayed in the lung 2 and 8 days after exposure to 140 kPa.
blast intensity. Levels of all inflammatory markers were significantly increased 2 days after exposure in an animal injected with placebo (Fig. 12, AYC). The highest relative increase was observed in the level of MCP-1 (five to six times greater than controls), followed by all other inflammatory markers (2- to 3-fold increase). By 8 days after exposure, all inflammatory mediators in lungs returned to baseline levels (Fig. 12, AYC).

Effect of NACA on antioxidant enzyme induction in lungs after exposure to blast Systemic administration of NACA may lead to an insufficient concentration in the target site for the airway inflammation because of its inability to reach the lung. To ensure high local concentration at the time of the highest accumulation of ROS, blast-exposed animals were treated with the drug two times in a 30-min interval immediately after exposure and again 24 h after blast. Myeloperoxidase activity in lungs was significantly reduced 2 days after blast; however, it remained increased compared with not-blasted controls (Fig. 11A). To investigate whether the priming of sequestered inflammatory cells is functionally inhibited by NACA, expression of surface CD11b was evaluated in lung tissue. As shown in Figure 11B, NACA decreased expression of CD11b in lungs after exposure to blast similar to that in MPO. On the other hand, the increased mRNA levels of inflammatory chemokines MIP-1, MCP-1, and CINC-1 in lungs after exposure to blast were completely eliminated 2 days later by treatment with antioxidant (Fig. 12, AYC).

Effect of exposure to blast on antioxidant enzyme induction in lungs

The effects of ROS with the lung are counterbalanced by a complex system of enzymatic and nonenzymatic antioxidants. Some antioxidant enzymes such as heme oxygenase-1 (HO-1) and manganese superoxide dismutase (MnSOD) are induced by ROS and inflammatory cytokines and may provide inducible mechanisms of protection against ROS and recovery from oxidative cell damage. Of the two major inducible antioxidative enzymes, HO-1 mRNA expression in lungs was induced more than 6-fold 2 days after exposure. By 8 days, the HO-1 mRNA level returned to controls (Fig. 13A). The MnSOD mRNA expression did not reach statistical significance compared with controls at 2 days, and there was no change in the mRNA expression 8 days after exposure to blast (Fig. 13B). Alterations in glutathione metabolism are recognized as a central feature of many inflammatory lung diseases. In cells, physiological regulation of reduced and oxidized form of glutathione, GSH-GSSG ratio is maintained in reaction catalyzed by glutathione reductase (GR). The GR mRNA expression in lungs was not affected by blast at 2 or 8 days after exposures (Fig. 13C).
We also sought to determine whether the inhibition of blastinduced inflammatory response by NACA treatment is related to changes in induction of antioxidant enzymes. Treatment with NACA completely inhibited induction in HO-1 mRNA expression 2 days after blast (Fig. 13A) that remained at the baseline level by 8 days after exposure. No change in MnSOD mRNA was detected in lungs of the drug-treated animals compared with the placebo group (Fig. 13B). The GR mRNA expression was not affected by NACA treatment and remained unchanged from controls or placebo-treated blastexposed animals (Fig. 13C).

DISCUSSION

Pulmonary contusion is a common injury seen after blunt chest trauma and blast exposure and is often associated with increased morbidity and mortality (10, 11). Blunt chest trauma is a common problem in the critical care of trauma patients and accounts for 10% to 30% of adult deaths caused by trauma (10). Lung contusions are an important risk for the development of other conditions such as pneumonia and ALI/ARDS. Mechanical factors relevant for lung contusion include the spalling effect where alveoli and small airways are disrupted by shearing forces, as well as an inertial effect where alveolar tissue is stripped from hilar structures. In addition to spalling, an implosion effect can occur as alveolar and airway tissues rebound from overexpansion of gas after passing of a pressure wave (25).

Pulmonary contusion in our study likely involved contribution of all these mechanisms. Areas of contusion were relatively evenly distributed over all lobes, similar to other models of blast lung injury (12, 26) with mostly unilateral localization reflecting animal orientation at the time of blast. Histological analysis showed characteristic landmarks of lung contusion such as intra-alveolar and subpleural hemorrhage, massive infiltration of neutrophils, and activation of macrophages in the lung parenchyma.

It was previously demonstrated that the acute phase of lung damage reached maximum at 24 to 48 h after exposure to BOP and that was followed by gradual dissolution of inflammation and oxidation by 192 h (16). Based on these results, the time with maximal inflammatory response at 48 h was selected at this study. We confirmed this results showing early temporal neutrophil infiltration in lungs after exposure to blast measured by MPO activity and expression of neutrophil surface integrin CD11b mRNA. In addition, we extended these findings by showing that the chemokine MIP-1, MCP-1, and CINC-1 mRNA levels were also elevated after blast, and that the temporal expression of chemokines correlated with neutrophil influx to the lung. The CC chemokines MIP-1 and MCP-1 are chemotactic for and stimulate mostly monocytes and T lymphocytes (27). Macrophage
inflammatory protein-1 is produced by a variety of immune cells such as macrophages, lymphocytes, and neutrophils and has been shown to stimulate secretion of cytokines from peritoneal macrophages (28). Macrophage inflammatory protein-1, in addition to its leukocyte chemoattractant properties, can also operate as autocrine activators of alveolar macrophages, facilitating the acute inflammatory process (29). It was shown that treatment with MIP-1 antibody significantly reduced both lung recruitments of neutrophils and the increase in vascular permeability in endotoxemia (29). Monocyte chemotactic peptide 1, in addition to its monocyte chemotactic activity, has also been shown to induce a respiratory burst and upregulate expression of "2 integrin (30). Cytokine-induced neutrophil chemoattractant is a rat CXC chemokine, an important neutrophil chemotactic factor produced by rat alveolar macrophages involved in neutrophilic lung inflammation (31). Cytokine-induced neutrophil chemoattractant was activated in lungs at the same pattern observed in MPO, CD1 lb, MIP-1, and MCP-1. The early temporal expression of CC and CXC chemokines together with MPO activity correlated with the neutrophil and macrophage influx to the lung and suggests a role for these mediators in coordinating the influx of immune cells to the site of injury in the blast model of lung trauma.

We previously reported that isolated lung contusions have a relatively acute course of evolution, and resolution by 8 days was consistent with the decrease of inflammatory markers to baseline and histopathological assessment, indicating significant resolution of lung damage (16). In the present study, we used higher-intensity blast exposures 140 kPa (compared with 115 kPa previously), and we observed relatively more severe lung contusions and quantitatively higher inflammatory responses. However, there were only moderate signs of inflammation with no progressive exacerbation of lung inflammatory damage observed by both quantitative mRNA determination and histopathological observations at 8 days postblast.

Our results show a massive induction of HO-1 mRNA in lungs 2 days after blast exposure in accordance with our previous study (16). Heme oxygenase 1 is a stress protein induced by a variety of stimuli such as oxidative stress, endotoxins, cytokines, and its main substrate, hemin (32). It catalyzes the initial and rate-limiting step in the catabolism of heme to yield biliverdin, carbon monoxide, and iron. Whereas CO is supposed to be responsible for the antiinflammatory effect of HO-1 (inhibition of expression of cytokines and iNOS), biliverdin may contribute protection by its potent antioxidant effect. It was shown that upregulation of HO-1 expression by injection of hemin significantly increased survival rate in
animals exposed to a high level of blast 170 kPa (33). We suggested that the HO-1 induction is a part of adaptive and protective mechanisms leading to the resolution of oxidative and inflammatory lung damage after exposure to moderate (nonlethal) levels of blast. Protection related to HO-1 may include multiple anti-inflammatory and antioxidant mechanisms such as downregulation of proinflammatory cytokines and adhesion factors, resulting in suppression of neutrophil infiltration in inflamed tissue or downregulation of iNOS expression by degradation of heme.

Our primary objective in this study was to evaluate the effect of a novel antioxidant NACA on inflammation of the lungs exposed to BOP. Reactive oxygen species accumulate in lungs after blunt trauma as a result of reactions catalyzed by iron released from hemoglobin and by inflammatory cells infiltrating the airways. Overproduction of ROS or depletion of the protective antioxidant mechanisms results in damage to endothelial and epithelial cells by multiple mechanisms such as an increase in endothelial and epithelial permeability or modification of pulmonary vascular function (34). Activation of oxidative stress in blast lung injury was demonstrated by increased protein oxidation/nitration in lung tissue (16) and by increased EPR signal and depletion of antioxidants levels in lungs (15). Pharmacological intervention to lung inflammation can be focused at several levels such as blocking of neutrophil infiltration, downregulation of proinflammatory cytokine activation, or elimination of ROS by antioxidants. Previous studies showed that administration of some exogenous antioxidants reduced airway inflammation in animal models of acute lung injury (13). The most extensively studied is a low molecular weight thiol antioxidant N-acetylcysteine (NAC) providing a substrate cysteine for glutathione biosynthesis and reducing its oxidized form GSSG to GSH (35). Additionally, NAC is a potent free radical scavenger as a result of its nucleophilic reaction with ROS (36). In endotoxic animals, NAC decreased neutrophil-aggregating activity, reduced pulmonary hypertension, and attenuated vascular permeability (17). Bioavailability of the NAC is very low, and a very high dose of the drug (500 mg/kg) is required for its protective effect on lung inflammation (37). A newly designed amide form of NAC is NACA, in which the carboxyl group is neutralized is expected to be more hydrophobic and membrane permeable (20). N-Acetylcysteine amide was shown to have more efficient membrane permeation (21, 22), high metal chelating activity, and better scavenging antioxidant properties (23) compared with NAC. Recently, it was shown that NACA administered before challenge significantly ameliorated inflammation, decreased vascular permeability, and decreased pulmonary resistance in the
mouse model of allergic airway disease (24). Although numerous pharmacological interventions have conferred protection in animal models of acute lung injury, they were administered before the insult. In our study, NACA was administered immediately after BOP to evaluate possible therapeutic use of the drug in a relevant real-world scenario. The dose of 100 mg/kg was comparable to that used by others (24). N-Acetylcysteine amide was administered two times in 30-min intervals immediately after blast to quench ROS produced by release of hemoglobin and then 24 h later for the culmination of infiltration of neutrophils and activation of inflammatory response in lungs (16). Our finding that NACA was effective when administered after exposure to blast is especially important because it is obviously impossible to anticipate the exact time and location of blast.

The data show that the NACA significantly ameliorated inflammation in the lungs at 2 days after blast. Myeloperoxidase activity in the lung tissue as an index of neutrophil infiltration was significantly reduced, but not eliminated, by NACA. Similarly, surface CD1 lb mRNA was significantly decreased but did not return to baseline with NACA. On the other hand, the increase in MIP-1, MCP-1, and CINC-1 mRNA 2 days after blast was completely eliminated by NACA. The differential inhibition of neutrophil infiltration versus chemokine mRNA inhibition indicates that the mechanism by which NACA exerts its beneficial effect in this model is related to free radical scavenging and inhibition of neutrophil-mediated oxidant injury, rather than by an effect on inflammatory cell migration. A similar mechanism of protection was observed in endotoxin lung damage by treatment with NAC (31).

In addition to the direct oxidative effect, ROS have been implicated as second messengers in the activation of redox-sensitive signaling pathways and transcription of numbers of genes (38). Among the transcription factors involved in modulating proinflammatory responses, nuclear factorY.B is the most important redox-sensitive transcription factor (38). Nuclear factorY.B binding motif is present in the promoter regions of many genes that encode proinflammatory cytokines, especially chemokines that are important in the recruitments of neutrophils, macrophages, and lymphocytes (38). The administration of both NAC and NACA resulted in significant reduction of nuclear factorY.B activation and attenuated lung inflammation caused by increased production of ROS (24, 31).

N-Acetylcysteine amide protection against inflammation in our model was not related to the activation of antioxidant defense enzymes HO-1, MnSOD, or GR. Instead, NACA administration prevented the activation of HO-1 mRNA observed 2 days after blast and had
no effect on MnSOD and GR mRNA transcription. In lung fibroblasts, HO-1 was induced during oxidative stress, and this induction paralleled a decrease in intracellular GSH, and treatment with NAC reduced expression of HO-1. This supports previous observations of the mechanism of NACA’s antioxidant effect by restoration of GSH level in lung inflammation and our observation of inhibition of HO-1 mRNA activation. We also examined whether the protection by NACA related to the augmentation of endogenous antioxidant glutathione could be related to the activation of GR. Glutathione reductase is a crucially important cellular enzyme in the GSH redox cycle. Glutathione reductase in the presence of NADPH, a supplier of reducing equivalents, recycles GSSG back to GSH to increase the antioxidant pool required for detoxification. Our data indicate that NACA antioxidant effect was not related to the activation of GR expression.

The results of this study support previous observations that a moderate level of blast results in severe isolated lung contusions and transient injury with a relatively rapid recovery. Administration of antioxidant NACA was shown to facilitate lung recovery from inflammatory damage, and this protection could be vital in situations of more severe lung blunt trauma with progression to ALI/ARDS. This may include situations of repetitive exposure to blast or to additional insults that can exacerbate pulmonary injury as in the Btwo-hit hypothesis(40).


**Example 15: Assessment of NACA in the rodent blast overpressure (BOP) model**

In this study, we undertook an assessment of NACA in the rodent blast overpressure (BOP) model. Interim results which we previously sent were interesting. In the first series of experiments we looked at preventing mortality with NACA administered just prior to BOP injury. We have previously submitted for your review the data regarding rats treated (IP) with 500 mg/kg NACA 30 minutes prior to exposure to near lethal levels of BOP (~160 kPa).
Under the conditions of this experiment, NACA significantly increased the fraction of animals surviving (see Figure 14).

We subsequently examined if NACA could limit the morbidity that developed after BOP injury. In this case, rats were exposed to BOP injury and treatment was given after the injury. As previously mentioned, endpoints in this study included gross lung tissue pathology and histopathology including a lung injury score (0=no damage to 5=extensive damage), genes indicative of inflammation were assessed by measuring mRNA levels in lung tissue, and total body weight was assessed daily. Brains were also harvested and we are in the process of evaluating the potential beneficial effects of NACA on blast induced brain injury as this is a key issue facing our military today. In this model, we expected the BOP injury to cause an increase in reactive oxygen species (ROS) and expression of inflammatory genes in lung tissue. The preliminary results we had previously submitted showed that at 8 days post-blast, IL-1, MMP-9, TNFa, and HO-1 were all significantly up-regulated in placebo treated animals as compared to controls (placebo-treated animals not exposed to blast). Treatment with NACA prevented the increase in mRNA levels and was comparable to controls (see Figure 15).

We have almost completed the additional analyses regarding this experiment in which rats were exposed to BOP then treated with 100 mg/kg NACA (IP) 30 minutes, 60 minutes, and 24 hours afterwards. Other animals were exposed to BOP then treated with vehicle or were not exposed to BOP. The results shown below are the most recent findings which include data from the cohort assessed two days after BOP injury (this is part of the same experiment which assessed eight days after BOP injury.

The above new data demonstrate that NACA-treated animals exhibited less upregulation of MIP (Macrophage Inflammatory Protein) a so-called monokine that is involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes and MCP (monocyte chemoattractant protein) which induces monocytes to leave the bloodstream and enter the surrounding tissue. This suggests a dampening on the acute response which may be protective. In addition, the decreased expression of hemeoxygenase (HO-1), an enzyme important in the catabolism of heme that is induced under conditions of oxidative stress, may indicate a relative decrease in the amount of hemorrhaging observed in NACA-treated lungs of animals, especially at two (2) days after exposure to blast (BOP). We currently have a limited number of histopathology slides completed. The preliminary indication is that the overt inflammatory response is more
restricted in the NACA-treated animals as evidenced by less infiltration of bronchiolar lesions. See Figures 16-19.

In brief summary, the new additional data together with the previously submitted data further indicate that NACA may effectively prevent morbidity and mortality resulting from blast overpressure when given before or after injury.

**Example 16: Post-Injury Administration, of a Cell-Permeant Glutathione Precursor, N-Acetylcysteine Amide, Increases Tissue Sparing and Reduces Oxidative Stress Following Traumatic Brain Injury**

Traumatic brain injury (TBI) is a silent epidemic, resulting in over one million new cases annually in the United States; unfortunately, treatment options have been limited and no approved pharmacological treatment has been effective thus far. TBI has been characterized as a biphasic injury, which includes a primary blunt force injury and a prolonged secondary injury cascade occurring in the penumbra surrounding the injury. Associated with this secondary injury cascade is glutamate induced excitotoxicity resulting from increased intracellular Ca\(^{2+}\) levels. Mitochondria act as calcium sinks during normal cellular functioning, however excessive calcium uptake during excitotoxic insult results in reduced mitochondrial membrane potential, increased reactive oxygen species (ROS) production, and decreased ATP production. Increased ROS production can overwhelm endogenous antioxidant systems ultimately leading to mitochondrial dysfunction and neuronal cell death. To date no antioxidant therapy has been successful in treating TBI mainly due to the inability of the compound to enter into the mitochondrial matrix, which is the major site of ROS formation and oxidative damage following TBI.

Glutathione, a thiol which acts as the primary intracellular antioxidant, plays a critical role in the attenuation of excessive ROS production. It has been shown that following injury both cellular and mitochondrial levels of glutathione are decreased, and that the loss of mitochondrial glutathione has been associated with increased tissue damage. Recently, several studies have evaluated the efficacy of using the novel antioxidant N-acetylcysteine amide (NACA), due to its permeability to both cellular and mitochondrial membranes. NACA, a glutathione precursor, can increase levels of glutathione by reducing oxidized glutathione and supplying the rate limiting substrate for glutathione biosynthesis. NACA, the amide form of N-acetylcysteine, has a neutralized carboxylic group making it more lipophilic, which enables it to be cellular and mitochondrial membrane permeable. It has
also been shown to chelate copper, attenuate MAPK activity, and decrease oxidative stress. Previous studies have also shown that NACA crosses erythrocyte membranes and upon entering replenish intracellular glutathione levels. Also, neuronal cell line studies have shown that NACA reduces the levels of ROS induced by glutamate and decreases lipid peroxidation.

Due to the abundance of evidence that NACA provides beneficial support to endogenous antioxidant systems after oxidative insult, it may be able to attenuate the rampant cascade of secondary injury after TBI. Therefore, in our current studies we have investigated the ability of NACA to spare tissue and decrease oxidative stress when administered after a controlled cortical impact model of TBI.

Methods

Animals and Experimental Design

All animal procedures were approved by the University of Kentucky institutional animal care and use committee. Adult Sprague-Dawley rats were given a moderate (1.5 mm) unilateral controlled cortical impact (CCI) injury as previously described. In order to assess tissue sparing following TBI rats were divided into three groups (N=6/group): (I) N-acetylcysteine amide loaded pump (18.5 mg/kg/hr) and a single 150 mg/kg bolus injection of N-acetylcysteine amide (30 min post injury) (II) N-acetylcysteine amide (18.5 mg/kg/hr) and CsA (2.5 mg/kg/hr) loaded pump and a single bolus injection of N-acetylcysteine amide (150 mg/kg) and CsA (20 mg/kg) (30 min post injury) (III) Vehicle loaded pump and single vehicle bolus injection (30 min post injury). The miniosmotic pumps were assembled and implanted immediately after injury as previously described with few modifications and remained in the animals for 7 days. In order to assess oxidative damage following TBI, another set of rats received a moderate CCI injury. The second experiment divided rats into two groups (N=3/group): (I) N-acetylcysteine amide loaded pump (18.5 mg/kg/hr) and a single 150 mg/kg bolus injection of N-acetylcysteine amide (30 min post injury) (II) Vehicle loaded pump and single vehicle bolus injection (30 min post injury).

Tissue Sparing Assessment

At 7 days post injury animals from the first experiment were anesthetized with pentobarbital (95 mg/kg body weight) and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde at pH 7.4. After removal, the brains were placed in paraformaldehyde-sucrose (15%) for an additional 24 hrs. Coronal sections (50 µm) were then cut using a freezing microtome throughout the rostral caudal extent of the damaged
hemisphere and stained with cresyl violet. Cortical damage was assessed using the unbiased stereological Cavalieri method as previously described\textsuperscript{12}. Tissue sparing was expressed as a percentage of the contralateral cortex within each animal, by dividing the ipsilateral cortical volume by the contralateral cortical volume.

**Oxidative Damage Assessment**

To assess oxidative damage, 50 μm\textsubscript{1} coronal tissue sections from the rats in the second experiment were stained for 4-hydroxynonenenal (HNE), a lipid peroxidation marker, and for 3-nitrotyrosine (3-NT), a protein nitrosylation maker. Adducts were reduced by exposing tissue to 0.1 M NaBH\textsubscript{4} in MOPS (Sigma) at pH 8.0 for 10 minutes followed by rinsing sections 3 times with PBS for 5 minutes. Sections were incubated in 0.3 % H\textsubscript{2}O\textsubscript{2} for 30 minutes at room temperature; sections were then washed 3 times for 5 minutes in PBS. Sections were then blocked using 5 % goat serum, 0.25 % Triton X-100, and 1 % milk in a PBS solution for 2 hours at room temperature. Sections were then immunoreacted with primary antibody (rabbit anti-HNE polyclonal antibody, Calbiochem) (mouse anti-3-NT monoclonal antibody, Upstate). Sections were then rinsed with PBS and incubated with secondary antibody for 2 hours (800 secondary goat anti-rabbit antibody, Rockland) (IR Dye 700 D conjugated goat anti-mouse IgG antibody). Sections were rinsed with water and mounted. Imaging was preformed on Li-core Odyssey machine.

**Results**

**N-acetylcysteine amide is Neuroprotective following TBI**

Following TBI there is an increase in ROS production that overwhelms endogenous antioxidant systems leading to tissue damage and oxidative stress\textsuperscript{2}. In order to attenuate this cascade of damage we supplemented this antioxidant system with NACA by exogenously administering antioxidant (NACA) after moderate TBI. We found that all animals had cortical damage at the site of injury (FIG 1), however animals treated with NACA showed increased tissue sparing compared to animals receiving vehicle (FIG 2). Indeed, there proved to be a significant increase in tissue sparing in both the animals that received NACA and the co-administered NACA + CsA when compared to animals administered vehicle. However, when comparing the NACA treated rats with the NACA and CsA treated rats there was no significant difference in tissue sparring, therefore indicating a lack of synergistic effect with co-administration of the two compounds.
N-acetylcysteine amide Decreases Oxidative Stress Following TBI

When we investigated the effect of NACA on oxidative stress in a second set of animals following TBI, we found that there was a significant decrease in 4-HNE levels in the NACA treated animals compared to vehicle (FIG 4). However, there was no significant difference in 3-NT levels between NACA treated animals and vehicle treated animals.

Discussion

The results of this study clearly demonstrate the important role of oxidative stress in TBI neuropathology, and that NACA could be used as a potentially effective treatment for TBI. Our results show increased tissue sparing with post-injury administration of the antioxidant NACA following TBI. Co-administration of CsA (the gold standard neuroprotective compound) and NACA did not significantly increase tissue sparing more when compared to NACA administered alone, indicating that NACA is as effective as CsA for treatment of TBI.

TBI is classically associated with increased ROS production and oxidative damage; therefore we measured oxidative markers to assess the effect of NACA on tissue. FINE levels were significantly reduced in injured animals treated with NACA when compared with vehicle treated animals. 3-NT levels were not significantly reduced among vehicle treated and NACA treated rats. NACA was not expected to reduce 3-NT levels, as observed, due to the absence of support for the reduction of peroxynitrite by glutathione. From these data it is shown that NACA significantly reduces oxidative stress following TBI.

As our results clearly demonstrate, post-injury administration of NACA following TBI is neuroprotective. This neuroprotective effect was produced due to the ability of NACA to reduce oxidative stress and increase tissue sparing. These data also highlight the critical role that ROS plays in the neuropathology of TBI. Our results indicate that NACA has enormous potential to be translated into an antioxidant therapy for the clinical treatment of TBI.


**Example 17: NAC inhibits Dengue replication in human monocytic cells**

Human monocytic cells or baby hamster cells were exposed to Dengue virus in a presence or absence of NAC for 3 days. Secreted virus was collected and seeded to naive baby hamster cells and overlaid by methylcellulose. Three days later plates were stained for plaques. Titration of the virus demonstrated that 5 mM NAC inhibited Dengue replication by 1000 folds. Separate study has shown NAC has no effect on cell viability of either human monocytic cells or baby hamster cells. See Fig. 25.

NACA is metabolized to NAC in the body. See Fig. 36.

**Example 18: NACA inhibits HIV replication**

We tested the effects of NAC on cytokine-induced HIV expression, namely chronically infected U1 cells where the virus is silent in unstimulated conditions, but can be promptly reactivated by different stimuli. No cytotoxicity was detected by TOVA.

No induction of HIV expression by TOVA was observed (the low levels RT counts are barely above background). A concentration-dependent inhibition of HIV expression induced by TNF-a was seen. This was strongest at the lowest concentrations (10 μM) suggesting that the effect could be even greater by further dilutions (e.g., 0.5 μM, 1 μM, 5 μM). Further, this effect is occurring with about 1,000-fold less concentrations than NAC. (not shown). Some inhibition was noted also with IL-6 as stimulant.

FIGs. 26-27 show that NACA (a.k.a. TOVA) blocked the cytokines and HIV replication and that strong blockage was seen at a dosage of NACA that was 1/1000 the dose previously reported for NAC (Poli 1992).
Example 19: N-acetylcysteineamide (NACA) prevents inflammation animals exposed to poison gas, smoke inhalation or diesel engine exhaust

Diesel exhaust particles (DEPs), a by-product of diesel engine exhaust (DEE), are one of the major components of air borne particulate matter (PM) in the urban environment. DEPs are composed of soot, polycyclic aromatic hydrocarbons (PAHs), redox active semi-quinones, and transition metals, which are known to produce pro-oxidative and pro-inflammatory effects, thereby leading to oxidative stress-induced damage in the lungs. Similar particulate matter may be present in smoke. The objective of this study was to determine if N-acetylcysteineamide (NACA), a novel thiol antioxidant, confers protection to animals exposed to DEPs from oxidative stress-induced damage to the lung. To study this, male C57BL/6 mice, pretreated with either NACA (250 mg/kg body weight) or saline, were exposed to DEPs (15mg/m3) or filtered air (1.5-3 h/day) for nine consecutive days. The animals were sacrificed 24 h after the last exposure. NACA-treated animals exposed to DEP had significant decreases in the number of macrophages and the amount of mucus plug formation in the lungs, as compared to the DEP-only exposed animals. In addition, DEP-exposed animals, pretreated with NACA, also experienced significantly lower oxidative stress than the untreated group, as indicated by the glutathione (GSH), and malondialdehyde (MDA) levels and catalase (CAT) activity. Further, DEP-induced toxicity in the lungs was reversed in NACA-treated animals, as indicated by the lactate dehydrogenase levels. Taken together, these data suggest that the thiol-antioxidant, NACA, can protect the lungs from DEP-induced inflammation and oxidative stress related damage.

Diesel engine exhaust (DEE) is a complex mixture of organic and inorganic gases (NOx, SOx, CO), and particulate matters (PMs). Diesel exhaust particles (DEPs), a by-product of DEE, are one of the major components of airborne particulate matter in the urban environment. Epidemiological studies have demonstrated a strong association between particulate matter and lung diseases like asthma and chronic obstructive pulmonary disease (Nel et al., 1998; Peterson and Saxon, 1996; Diaz-Sanchez, 1997; Li et al., 1996). DEPs have mean diameters of 0.2 μm or less, which render them easily respirable and capable of being deposited in the airways and the alveoli. Further, DEPs contain carbon, with large surface areas which readily adsorb chemicals like polyaromatic hydrocarbons (PAH), quinones, aldehydes, and heavymetals like iron, copper, chromium, and nickel (Schuetzle, 1983; Schuetzle and Frazier, 1986; Draper, 1986). Organic compounds, like PAH and quinone,
which constitute about 30% of the weight of DEPs, have been reported to be potential carcinogenic risks for humans (Vostal, 1983; Rosenkranz, 1993). In addition, these organic compounds have also been reported to generate reactive oxygen species (ROS) in macrophages (Nel et al., 1998; Kumagai et al., 1997; Hiura et al., 1999a; Squadrio et al., 2001). Macrophages, which are the first line of defense in the lungs and contain enzymes (like cytochrome P4501A1) which aid in the conversion of xenobiotics, have also been reported to generate ROS (Park et al., 1996; Kumagai et al., 1997; Ng et al., 1998). Further, ROS have also been linked to the activation of stress-activated protein kinases (SAPKs), which regulate the expression of proinflammatory genes in macrophages (Ng et al., 1998; Hiura et al., 1999a), indicating that oxidative stress is one of the mechanisms by which DEPs exert their toxic effects in the lungs.

Oxidative stress occurs when pro-oxidants outweigh antioxidant levels in the cells. Glutathione (GSH) is one of the major intracellular thiol antioxidants that provide protection against oxidative stress in the body. Alteration in the GSH metabolism has been recognized as a key feature of many inflammatory diseases of the lung (Lee et al., 2007). Thiol antioxidants, like N-acetylcysteine (NAC), which increase GSH by affecting the cysteine levels in the body, have been shown to be effective in treating the oxidative and inflammatory effects of allergens in the respiratory tract (Astiet et al., 1995; Whitekus et al., 2002; Lee et al., 2004). However, the antioxidant NAC has a negatively charged carboxyl group at physiological pH, which limits its ability to cross the cell membrane.

2. Materials and methods

2.1. Materials

C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Nacetylcysteineamide was provided by Dr. Glenn Goldstein (David Pharmaceuticals, New York, NY, USA). N-(1-pyrenyl)-maleimide (NPM) was purchased from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were purchased from Sigma (St. Louis, MO), unless stated otherwise.

2.2. Generation of diesel exhaust particles

DEPs were generated from a diesel generator (Model No. 4039T, John Deere, IL). The diesel exhausts from the generator were diluted with clean dry air, one part exhaust to six parts air, and fed to the exposure chamber. The particle size distribution and total concentration of the DEPs were monitored using a Cambustion DMS500 (Cambridge, UK), a
state-of-the-art fast particulate spectrometer (Reavell et al., 2002). The aerosol characteristics of the DEP, as calculated from the size distribution were: mean diameter 38 nm, geometric standard deviation 1.36, total number concentration $D_2.1 \times 10^6$ cm$^{-3}$, and mass concentration 0.23 mg/m$^3$ using a soot density of 2 g/cm$^3$ (Virtanen et al., 2002).

2.3. Animal experiments

Male C57BL/6 mice (30-35 g, 7 weeks old) were housed in a controlled temperature (20-23 °C) and controlled-humidity (D55%) animal facility, with a 12 h light and dark cycle. The animals had unlimited access to rodent chow and water, and were utilized after 1 week of acclimatization. All animal procedures were conducted under an animal protocol approved by the Institutional Animal Care and Use Committee of the Missouri University of Science and Technology. The mice were divided into two major groups: an experimental and a control group. The model was developed based on previous reports of diesel exposure in in vivo models (Dong et al., 2005). The animals in the experimental group were further divided into four groups ($n = 4$ each): DEPs exposed for 1.5 h, DEPs exposed for 3 h, NACA+ DEPs exposed for 1.5 h and NACA+ DEPs exposed for 3 h, respectively. The animals in the control group ($n = 4$ each) were divided into a control and a NACA-treated group. All of the animals were pretreated (oral gavage) with either NACA (250 mg/kg body weight) or saline 30 min before exposure to DEPs or clean filtered air, on alternate days. The animals in the experimental group were exposed to DEPs and the control animals were exposed to clean filtered air (3 h) for nine consecutive days (Fig. 28). The mice were sacrificed 24 h after the last exposure by urethane injection. All mice were weighed at the beginning and at the end of the study. Following sacrifice, the lungs were harvested and divided into two parts, with two slices fixed in 10% buffered formalin and the remaining tissue being stored in an antioxidant buffer [8.6 mM sodium phosphate dibasic (Na$_2$HP0$_4$), 26.6 mM sodium phosphate monobasic (NaH$_2$P0$_4$), 50 M butylhydroxytoluene (BHT), 10 mM aminotriazole, 0.1 mM diethyltriamine pentaacetic acid (DTPA)] at -80 °C for further analysis.

2.4. Evaluation of histology of the lung

For histological examination, 10% formalin-fixed lung tissues were embedded in paraffin, cut into sections, and stained with hematoxylin and eosin (H&E). To quantitate the degree of inflammation in the lung, the number of macrophages per five high power field (40×) were counted.

2.5. Determination of GSH and cysteine (CYS) levels
The levels of GSH and CYS in the tissue were determined by RP-HPLC, according to the method developed in our laboratory (Winters et al., 1995). The HPLC system (Thermo Electron Corporation) consisted of a Finnigan Spectra System vacuum membrane degasser (model SCM1000), a gradient pump (model P2000), autosampler (model AS3000), and a fluorescence detector (model FL3000) with _ex = 330nm and _em = 376 nm. The HPLC column used was a Reliasil ODS-1 C18 column (5-μm packing material) with 250mmx4.6mm i.d. (Column Engineering, Ontario, CA).

The mobile phase (70% acetonitrile and 30% water) was adjusted to a pH of 2 with acetic acid and o-phosphoric acid. The NPM derivatives of CYS and GSH were eluted from the column isocratically at a flow rate of 1 ml/min. The tissue samples were homogenized in a serine borate buffer, centrifuged, and 250_1 of the supernatant was added to 750_1 of ImM NPM. The resulting solution was incubated at room temperature for 5min, and the reaction was stopped by adding 10_1 of 2N HCl. The samples were then filtered through a 0.45-μm filter and injected into the HPLC system.

2.6. Determination of lactate dehydrogenase (LDH)

Lactate dehydrogenase levels were determined according to the method described by Uitto et al. (1972). Briefly, a cytosolic fraction of the tissue homogenate was added to a solution containing 50mM potassium phosphate buffer and 10mM sodium pyruvate. The reaction was initiated by adding the NADH (Nicotinamide adenine dinucleotide-reduced), and the absorbance was recorded at 340 nm. The activity of LDH was determined spectrophotometrically by measuring the rate of NADH disappearance.

2.7. Determination of malondialdehyde (MDA)

The MDA levels were determined according to the method described by Draper et al. (1993). Briefly, 550_1 of 5% trichloroacetic acid (TCA) and 100_1 of 500ppm butylated hydroxytoluene (BHT) in methanol were added to 350_1 of the tissue homogenates, and boiled for 30 min in a water bath. After cooling on ice, the mixtures were centrifuged, and the supernatant collected and mixed 1:1 with saturated thiobarbituric acid (TBA). The mixture was again heated in a water bath for 30 min, followed by cooling on ice. 500_1 of the mixture was extracted with 1ml of α-butyrol acetate and centrifuged to facilitate the separation of phases. The resulting organic layers were first filtered through 0.45-μm filters and then injected into the HPLC system (Shimadzu, US), which consisted of a pump (model LC-6A), a Rheodyne injection valve and a fluorescence detector (model RF 535). The column was a 100mmx4.6mm i.d. C18 column (3-μm packing material, Astec, Bellefonte, PA). The mobile
phase used contained 69.4% sodium phosphate buffer, 30% acetonitrile, and 0.6% tetrahydrofuran. The fluorescent product was monitored at _ex = 515nm and _em = 550 nm. Malondialdehyde bis(dimethyl acetal), which gives malondialdehyde on acid treatment, was used as a standard.

2.8. Catalase (CAT) activity assay

Catalase activity was measured according to the method described by Aebi (1984). Briefly, the activity of catalase was measured spectrophotometrically (240 nm) in the supernatant of the total lung homogenate, following the exponential disappearance of hydrogen peroxide (H2O2, 10mM). The catalase activity was calculated from $A_{60} = A_{\text{initial}} - kt$, where $k$, is the rate constant, $A_{\text{initial}}$ is the initial absorbance and $A_{60}$ is the absorbance at 60 s.

2.9. Determination of protein

Protein levels of the tissue samples were measured by the Bradford method (Bradford, 1976). Concentrated Coomassie Blue (Bio-Rad, Hercules, CA) was diluted 1:5 (v/v) with distilled water. 20 μl of the diluted tissue homogenate was then added to 1.5ml of this diluted dye, and absorbance was measured at 595nm using a UV spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Bovine serum albumin (BSA) was used as the protein standard.

2.10. Statistical analysis

Group comparisons were performed using the one-way analysis of variance (ANOVA) test and the TUKEYS post hoc test. Statistical analyses were made using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of DEP and NACA on inflammation in the lung

The effect of DEPs on inflammation in the lungs was studied. Macrophages, which are the first line of defense in the lungs, were found to be significantly increased in DEP-exposed animals, as compared to the control or NACA-alone treated groups (Figs. 2 and 3). In the DEP-exposed group, a significant increase in the number of macrophages was observed in the animals exposed to DEPs for 3 h, as compared to those exposed for only 1.5 h (Fig. 30). In addition to the increase in number, the animals in the 3 h DEP-exposed group also had significantly higher DEP-laden macrophages and mucus formation in the bronchiole
(Fig. 31). However, DEP-exposed animals that were pretreated with NACA had significantly fewer macrophages and less mucus formation, as compared to the untreated group.

3.2. Effect of DEP and NACA on GSH and cysteine levels in the lungs

The effect of DEP on GSH levels in the lungs was studied. Compared to the controls and the NACA-alone treated group, the DEP-treated animals had significantly (D40%) lower GSH levels in their lungs. The animals exposed to DEPs for 3 h had lower GSH levels than those exposed for 1.5 h. However, pretreatment of the animals with NACA increased the GSH levels significantly (by about 20%) in DEP-exposed animals, indicating that NACA was conferring protection to these animals (Fig. 32A). In addition, a significant increase in the level of cysteine was also observed in DEP-exposed animals that had been pretreated with NACA, as compared to the DEP-alone exposed group (Fig. 32B). This indicated that, in addition to replenishing GSH levels in the lungs, NACA was also able to provide surplus cysteine and GSH to the DEP-exposed animals.

3.3. Effect of DEP and NACA on lipid peroxidation in the lungs

Lipid peroxidation is an important consequence of oxidative stress, and can be estimated by measuring the levels of malondialdehyde (MDA)—a stable by-product of lipid peroxidation. DEP-exposed animals had about a 1.5-fold increase in MDA levels, as compared to the levels in the controls (Fig. 33). An increase in the dose-dependent MDA level was also observed in animals exposed to DEPs, although the difference was not significant. The animals in the NACA-treated group experienced significantly less lipid peroxidation than the animals did in the DEP-only treated group. The MDA levels in the NACA-treated group were similar to the levels in the controls.

3.4. Effect of DEP and NACA on antioxidant enzyme activity

Antioxidant enzymes like Catalase (CAT) are involved in detoxification of peroxides in the body. Exposure of animals to DEPs decreased the levels of CAT activity in the lungs, as compared to the levels in the lungs of the control or NACA-alone treated group (Fig. 34). However, partial reversal in the loss of CAT activity was observed in DEP-exposed animals that had been pretreated with NACA.

3.5. Effect of DEP and NACA on cytotoxicity in the lungs

LDH levels were measured to assess the toxicity of DEP in the lungs. Compared to those in the control group, the DEP-exposed animals showed a significant increase in the LDH levels in the lungs, indicating that DEPs were toxic to the lungs (Fig. 35). However,
DEP exposed animals that were pretreated with NACA had lower LDH levels than the untreated animals did, though the difference was not significant.

4. Discussion

Diesel exhaust particles (DEPs), a by-product of diesel engine exhaust (DEE), are one of the major air pollutants worldwide. As a consequence of the small size of these particles, they can easily penetrate and settle deep inside the lungs. In addition, DEPs are composed of soot, PAH, redox active semi-quinones, and traces of transition metals, which are also known to produce pro-oxidative and pro-inflammatory effects, thereby inducing oxidative stress and damage in the lungs. Thiol antioxidants have been shown to be effective in treating oxidative effects in the respiratory tracts (Asti et al., 1995; Whitekus et al., 2002). In this study, we investigated the role of the novel thiol antioxidant, NACA, in protecting the lungs from DEP-induced oxidative damage. We determined the oxidative stress parameters by measuring the levels of GSH, cysteine, and lipid peroxidation by-product (MDA), and the activity of the antioxidant enzyme catalase, in animals exposed to DEP/filtered air. The cytotoxic effects of DEP on lungs were assessed by measuring the lactate dehydrogenase levels and the number of macrophages in these animals. The lungs are constantly exposed to xenobiotics and PM that can damage them. Macrophages, an important target for DEPs (Alsberg et al., 1985; Goldsmith et al., 1997; Hiura et al., 1999b, 2000), are the first lines of cellular defense in the lungs, and clear the airspace by phagocytosis of the toxic or allergic particles that evade the respiratory tract. After phagocytosis, macrophages respond in a hierarchical fashion to increasing particle load and incremental levels of oxidative stress (Li et al., 2001). The results from this study show a significant increase in the number of macrophages in animals exposed to DEPs. A dose-dependent increase in particle load in the macrophages was also observed.

These effects suggest that the macrophages were ingesting the DEPs in an effort to clean the xenobiotics from the lungs. Several enzymes, like cytochrome P4501A1, that are present in the macrophages, have been reported to aid in the detoxification of the xenobiotics in the lungs (Harris et al., 1978; Whitlock et al., 1996; Kumagai et al., 1997; Nel et al., 1998). However, activation of these enzymes contributes to the generation of reactive oxygen species (ROS, Nel et al., 1998) that cause damage to the lungs. DEP-exposed animals that were pretreated with NACA had a significant decrease in the number of macrophages, indicating the potent role of this antioxidant in reducing oxidative-stress related damage.
In addition to DEPs, DEE is also composed of a variety of gases especially NOx (90% NO and 10% N02), CO, and S02 (Reed et al., 2004). Among all of the gases, NOx and CO have been reported to potentiate inflammation and oxidative damage in lung (Weinberger et al., 2000), and also reduce the capacity of hemoglobin to transfer oxygen (Kampa and Castanas, 2008). Previous studies characterizing DEE have shown that in similar systems, PM emission around 100-300 g/m3 yields NO in the range of 5.8-17.9 ppm (Reed et al., 2004). Since in our experiment, we recorded a PM level of 230 g/m3, it is expected that the NO levels should be <13 ppm, and hence the effect of these gases were not considered in the current study.

Although the lungs have a well-developed antioxidant system (Rahman and MacNee, 1996), any increase in ROS or depression in the antioxidant system (a condition termed as oxidative stress) has been reported to result in epithelial cell damage, cell shedding, and bronchial hyperactivity (Hulsmann et al., 1994; Cortijo et al., 1999). Studies with animal models have indicated that oxidative stress contributes to airway hyper-responsiveness by increasing damage to the oxidant-sensitive -adrenergic receptors, as well as decreasing mucociliary clearance (Adam et al., 1999). Consistent with these observations, DEP-exposed animals had a significant decrease in mucous clearance. However, pretreatment of the DEP-exposed animals with NACA significantly improved this condition, indicating that NACA attenuates the hyper-responsiveness in the lungs of DEP exposed animals by reducing oxidative stress.

Cellular redox status in the body is regulated by a predominant non-protein thiol known as glutathione (GSH, -glutamylcysteinyl-glycine). GSH, a direct scavenger of ROS (Yamamoto and Zhu, 1998), has been reported to be a vital component in defending the airspace epithelium from damage in response to oxidants and inflammation. In addition, GSH redox status is also critical for the transcriptional regulation of many pro-inflammatory genes (Biswas and Rahman, 2008). Decreases in GSH levels in the lungs have been reported in various pulmonary diseases like idiopathic pulmonary fibrosis and acute respiratory distress syndrome (Rahman et al., 1999; Rahman and MacNee, 2000). Recently, GSH has also been reported to attenuate IL-13-induced asthma in mice (Lowry et al., 2008). This indicates that low levels of GSH may render individuals susceptible to the deleterious effects of exposure to inhaled toxicants and may also perpetuate inflammatory responses in their lungs. The results from our study show that animals exposed to DEPs have significant decreases in their GSH levels, indicating that exposure to DEPs induces oxidative stress in
these animals. In the DEP-exposed group, however pretreatment of the animals with NACA, increased their GSH levels. Further, NACA-treated animals also had significant increases in their cysteine levels, as compared to the untreated group. This indicates that NACA is capable of facilitating intracellular GSH biosynthesis by either reducing extracellular cystine to cysteine, or by supplying sulfhydryl (-SH) groups that can stimulate GSH synthesis, as evidenced in the case of its sister molecule, NAC (Issels et al., 1988).

In addition to GSH, other antioxidant enzymes exist in the mammalian system that catalyzes reactions designed to remove free radicals and other oxidant species from the body. Catalase (CAT) is one such enzyme found in peroxisomes that aids in removal of hydrogen peroxide. The results from our study indicate that exposure of animals to DEPs significantly decreases the CAT levels. Pretreatment with NACA increases the CAT levels, thereby protecting the lungs by removing hydrogen peroxide and superoxide radicals. Free radicals, produced by oxidative stress, also attack lipids, especially polyunsaturated fatty acids in the cell membranes and lead to the formation of by-products like MDA (Karbownik and Reiter, 2000). Our studies indicate that exposure to DEPs induced a significant increase in MDA levels in these animals, as compared to the NACA-treated group, thereby pointing to the role of this antioxidant in protecting animals from DEPs-induced damage. Lipid peroxidation is known to disturb the integrity of cellular membranes and lead to the leakage of cytoplasmic enzymes, such as lactate dehydrogenase (Drent et al., 1996). Increases in the release of LDH have been reported to occur as a result of ischemia, starvation, dehydration, injury and chemical poisoning (Moss and Henderson, 1986; Lott and Nemensanszky, 1987). According to our studies, increases in LDH levels were observed in animals exposed to DEPs, and pretreatment of the animals with NACA resulted in a decrease in the LDH levels in the lungs. These results demonstrate a positive correlation between LDH activity and MDA levels in the NACA-treated animals and the untreated DEP-exposed animals, suggesting that the release of LDH was a result of damage to the cell membranes due to lipid peroxidation. In addition, cytotoxicity in the lungs may also be attributed to increased apoptosis of the macrophages (Hiura et al., 1999b) due to DEPs. Apoptosis of the macrophages involves shedding of the apoptotic bodies after death, which spreads the toxic chemicals to the neighboring cells. This further induces cytotoxicity, as these apoptotic bodies contain active chemicals (PAHs), and their uptake by the surrounding inflammatory cells may further induce cytotoxicity and contribute to pathogenesis of respiratory diseases (Hiura et al., 1999b).
In summary, data from the present study indicates that, after exposure to DEE, DEPs enter into the lungs, where they are engulfed by the macrophages. These particle-laden macrophages induce oxidative stress in the lungs, as indicated by decreases in GSH and catalase levels, and increases in MDA levels (Fig. 36). Administration of NACA however, resulted in significant reductions in macrophages and oxidative stress-induced damage, thereby suggesting a therapeutic potential for this novel antioxidant.

References


Example 20: N-acetylcysteine amide (NACA) protects renal proximal tubular epithelial cells against iohexol induced apoptosis by inactivation of p38 MAPK and iNOS signaling pathway.

Radio-contrast media (RCM) used in radiological procedures can cause contrast-induced nephropathy (CIN), eventually leading to acute renal failure (ARF) [1]. CIN occurs in 5% of patients with normal renal function and in 50% of patients with preexisting renal dysfunction [2]. No specific treatment is available other than adequate intravenous volume expansion with isotonic crystalloid, use of a low-or iso-osmolality contrast agent, and restraint on the amount of contrast agent [1].

The exact pathogenesis of CIN is poorly understood, while vasoconstriction, direct tubular toxicity, and increased oxidative stress have been postulated as the major causes [2-4]. Several studies have reported that RCM induce caspase-dependent apoptosis in glomerular and renal tubular epithelial cells in vitro [3, 5, 6]. Although these findings suggest that apoptosis is involved in the pathophysiology of CIN, the mechanism that initiates apoptosis after exposure to RCM is still poorly understood[7]. Caspase-dependent apoptosis may be mediated by the free radical nitric oxide (NO) [8-10]. Interrelationship between NO and reactive oxygen species (ROS) is an emerging theme in the understanding of the biochemistry and physiology of NO, particularly within the kidney [11]. Superoxide reacts with NO to form peroxynitrite, which may mediate the cytotoxic effects of NO and cause cell apoptosis via oxidant injury [11, 12]. Therefore various antioxidant compounds have been used to scavenge ROS after exposure to RCM [13]. N-acetylcysteine (NAC, a thiol-containing free-radical scavenger) has been reported to have a weak protective effect [14]. The aim of the present study was therefore (1) to examine whether NO-dependent renal tubular epithelial apoptosis plays a role in the RCM-induced nephropathy, and (2) to investigate whether N-acetylcysteine amide (NACA), with better membrane penetration, is more effective than NAC in preventing RCM-induced renal cell injury.

Materials and Methods

All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Cell culture medium and plastic ware were from Invitrogen Life Technologies (Carlsbad, CA, USA). N-acetylcysteine amide (NACA) was provided David Pharmaceuticals, NY, USA. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay kit was from Promega (Madison, WI, USA). The iodinated
radiographic contrast agents used in this study was iohexol (Omnipaque™ 300 mg iodine/ml, abbreviated as mg/l/ml) purchased from Amersham Health (Princeton, NJ, USA).

Cell culture and treatments: LLC-PK1 cell line from porcine proximal tubule (European Collection of Animal Cell Cultures) was cultured in medium 199 containing penicillin (50 U/ml) and streptomycin (50 µg/ml), supplemented with 10% fetal calf serum (FCS) in an atmosphere of 5% CO₂ in air at 37°C. Experiments were performed on cells of passages 5 to 12. The cells were plated at a density of 1x10⁵/well on 6-well tissue culture plates and were grown to over 90% confluence before exposure to various agents.

The selective iNOS inhibitor Aminoguanidine (AG, 10 mM, freshly dissolved in PBS), p38 MAPK inhibitor SB203580 (10 µM, freshly dissolved in DMSO), and NAC (10 mM, freshly dissolved in culturing media) were added 30 min before cells exposure to iohexol. NACA (12 mM, freshly dissolved in culturing media) and the nonselective iNOS inhibitor N (q)-nitro-l-arginine methyl ester (L-NAME, 20 mM, freshly dissolved in PBS) were added to the cell culture at the same time of adding iohexol.

Morphology: Cell morphological changes were routinely checked under phase contrast microscope before and after cells exposure to various agents.

MTT assay: The cells were seeded on 96-well plates at a density of 1x10⁴ cells per well, cultured at 37°C for 20 h. Cells were exposed to iohexol with different concentrations ranging from 12.5 to 150 mg I/ml at 37°C for 2, 8, or 24 h. After treatments with or without the contrast medium, 15 µL Dye Solution was added to each well followed by incubation for 4 h at 37°C. Subsequently, 100 µL Solubilization Solution/Stop Mix was added to each well. The plate was left overnight in a sealed container with a humidified atmosphere to completely solubilize the formazan crystals. The absorbance was measured at 570 nm against 650 nm as the reference.

MTT conversion rate (MTT %) was calculated as follows:

\[
\text{MTT} \% = \left( \frac{\text{A}_{570\text{nm}} \text{sample}}{\text{A}_{570\text{nm}} \text{blank}} \right) \times 100\%
\]

Analyses of cell apoptosis (PI staining) by fluorescence activated cell sorter (FACS): Apoptotic cells were identified by diminished DNA content below the Go/Gi population of normal diploid cells[16]. In order to count the cells in sub-G₁/Gj peak, FACS was used by staining cell with propidium iodide (PI). This analysis has also been used to quantify apoptosis in various cell lines as previously described [16-19]. Briefly, cell pellets were fixed in 70% ethanol at -20°C for at least 24 h. After washing twice with ice-cold PBS, cells were incubated in RNase A/PBS (1 mg/ml) at 37°C for 30 min. DNA was stained with PI for 15
min at room temperature and analyzed by flow cytometry on a linear scale using a Becton-Dickinson FACS Calibur apparatus (San Jose, CA, USA).

Cells within a distinct sub- G₀/G₁ peak were considered apoptotic, because they showed DNA condensation and fragmentation, resulting in reduced PI staining [20]. Approximately 10,000 counts were made for each sample. Apoptotic rate was calculated by evaluating the percentage of events accumulated in the sub- G₀/G₁ position with ModFit LT for Mac 3.0 software (Becton Dickinson and Company, CA, USA). Experiments were carried out in triplicates and repeated three times.

Results: Western blotting: After the various treatments, cells were lysed for 15 min in lysis buffer (containing 62.5 mmol/L Tris-HCl [pH 6.8], 6 mol/L urea, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol) and sonicated 3×3 s. The protein concentration of the extracts was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as the standard. Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) using a 6-12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in TBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. Membranes were incubated with primary antibody at 4 °C overnight and then with secondary antibody for 1 h. The primary antibodies were diluted in TBST with 5% w/v nonfat dry milk or 5% w/v BSA. Membranes were washed three times in TBST for 10 min between each step. Antibody labeling was detected using an enhanced chemi-luminescence reagent (NEN™, MA, USA) according to the manufacture’s instructions. Protein expression was quantified by ImageJ software after scanning the film.

The specific primary antibodies included the following: anti-iNOS (Sigma, 1:5000); anti-eNOS (Sigma, 1:5000); anti-PARP (Santa Cruz Biotechnology, 1:1000); anti-phospho-p38 MAPK (Santa Cruz Biotechnology, 1:1000); anti-Bcl-2(Santa Cruz Biotechnology, 1:1000); anti-Bax(Santa Cruz Biotechnology, 1:1000); anti-Mcl-1 (Santa Cruz Biotechnology, 1:1000); anti-P-actin(Santa Cruz Biotechnology, 1:1000). All the experiments were performed at least three times under the same conditions.

Statistical analysis: All data are presented as mean±SD. Statistical significance was assessed with one-way ANOVA (SPSS statistical analysis software, version 15.0, Inc., Chicago, IL, USA). In some cases, a t test was performed between two groups. Differences were considered significant if p<0.005 and highly significant if p<0.001.

Results
Iohexol-induced cell death in a time- and dose-dependent manner: To investigate the cytotoxicity of RCM in LLC-PK1 cells, we incubated the cells with iohexol in concentrations ranging from 12.5 mg l/ml to 150 mg l/ml for 2-24 h. As shown in Fig. 38, iohexol induced a time- and dose-dependent cell death as assayed by inhibition of MTT conversion rate and by morphology. With a concentration of 25 mg l/ml, no significant cell death was observed before 8 h incubation, while 75 mg l/ml concentration induced significant cell death after 2 h incubation (p<0.001 versus control) (Fig. 38 A). Major morphological changes were cell shrinkage, membrane blebbing, and the appearance of brightly rounded bodies under phase contrast microscopy, implying the occurrence of apoptosis (Fig. 38 C-D). In the following experiments we therefore used a concentration of 75 mg l/ml and 2 hours incubation.

Iohexol-induced cell death displays apoptotic features: To further confirm that the Iohexol-induced cell death is primarily caused by apoptosis, a biochemical hallmark of apoptosis i.e. cleavage of caspase substrate PARP, was investigated by Western blotting [21]. As shown in Fig. 39 A, cleavage of PARP was observed after exposure to 75 mg l/ml iohexol. We also observed sub-G0/G1 peak by FACS in iohexol-exposed cells (Fig. 39 B). The apoptotic rate was increased as compared to control (30.01%±1.45% versus 4.70%±0.46%, p<0.001).

Next we investigated whether increased up-regulation of iNOS is crucial for iohexol-induced apoptosis. As shown by Western blotting (Fig. 40 A), iNOS protein expression was up-regulated after 2 h incubation with iohexol, while eNOS protein expression was not effected (Fig. 40 B).

To further confirm the involvement of iNOS activation in iohexol-induced cell apoptosis, we exposed cells to iohexol in the presence of a selective iNOS inhibitor (AG) or a nonselective iNOS inhibitor (L-NAME) to examine the effects on cell morphology, PARP cleavage, and apoptosis rate by FACS. We found that both AG and L-NAME reduced cell death in a dose-dependent manner, with maximal inhibition at 10 mM and 20 mM respectively, as assayed by morphological changes (Fig. 41 A) and PARP cleavage (Fig. 41 B). Furthermore, as shown by FACS in Fig. 41 C, AG and L-NAME significantly decreased the percentage of apoptotic cells to 14.92%±1.00% and 17.56%±1.57% respectively, as compared with 30.01%±1.45% induced by iohexol (p<0.001). Furthermore, as shown in Fig. 42 A, iNOS activation induced by iohexol was inhibited by AG and L-NAME (p<0.001 versus iohexol treatment). Neither AG nor L-NAME had an effect on eNOS protein expression as assessed by Western blotting (p>0.005 versus iohexol treatment) (Fig. 42 B).
We also performed Western blotting to detect the expression of anti-apoptotic proteins (Bcl-2, Mcl-1) and a pro-apoptotic protein (Bax). As shown in Fig.43 A-B, iohexol induced a significant decrease in the expression of Bcl-2 and Mcl-1 protein and a concomitant significant increase in Bax protein expression (Fig.43 C). Treatment with 10 mM AG and 20 mM L-NAME inhibited Bax activation and increased anti-apoptotic factors Bcl-2 and Mcl-1 expression (Fig.43 A-C).

Iohexol-induced apoptosis involves activation p38 MAPK: We postulated that the up-regulation of iNOS protein expression in iohexol-induced apoptosis follows p38 MAPK activation. As assayed by Western blotting, we found that the phospho-p38 MAPK was increased in LLC-PK1 cells after exposure to iohexol (Fig.40C). To further evaluate the involvement of p38 MAPK activation in iohexol-induced cell apoptosis, a specific p38 MAPK inhibitor (SB203580) was used. Addition of SB203580 (10 µM) 30 min before the exposure to 75 mg l/ml iohexol protected LLC-PK1 cells from apoptosis, as assayed by morphological changes (Fig.41 A) and PARP cleavage (Fig.41 B). Furthermore, as shown in Fig.41 C, treatment with SB203580 significantly decreased the percentage of apoptotic cells to 24.73% ±1.29% as compared to 30.01%±1.45% induced by iohexol (p<0.001) and attenuated the iohexol-induced increase in iNOS protein expression (Fig.42 C). In contrast, SB203580 had no effect on eNOS expression (Fig.42 D). Meanwhile, treatment with 10 µM SB203580 inhibited the iohexol-induced Bax activation and increased anti-apoptotic factors Bcl-2 and Mcl-1 expression (Fig.44 A-C).

NACA protects LLC-PK1 cells against iohexol-induced apoptosis: Damage by oxygen radicals is a possible mechanism for CIN. We investigated the effect of the new antioxidant NACA on iohexol-induced apoptosis. We found that NACA reduces cell death in a dose-dependent manner, with maximal inhibition at 12 mM as assayed by morphological changes (Fig.42 B) and PARP cleavage (Fig.42 C). Thereafter, the concentration of 12 mM NACA was used in all the following experiments. In addition, the protective effect of NACA was also confirmed by the decrease in apoptotic DNA fragmentation (Fig.41 C).

We also compared the protective effects of NAC and NACA on iohexol-induced apoptosis. Cells were treated with NAC in concentrations ranging from 5 to 20 mM. We found a slight protective effect of 10 mM NAC on iohexol-induced cell damage assayed by morphological changes (Fig.41 A), while 20 mM NAC was toxic (data not shown). Therefore, we compared the effects of 10 mM NAC and 12 mM NACA on apoptotic rate as assessed by FACS. As shown in Fig.41 C, 10 mM NAC decreased the percentage of
apoptotic cells from 30.01%±1.45% to 27.04%±0.31% (p<0.005). Interestingly, the protection of NACA (24.25%±0.56%) was more pronounced than the NAC protection (p<0.005 versus NAC treatment, p<0.001 versus iohexol treatment).

Given that p38 MAPK constitutes a critical component of apoptotic signaling in LLC-PK1 cells, we examined the effect of NACA on p38 MAPK activation induced by iohexol. Pretreatment of the cells with 12 mM NACA inhibited p38 MAPK activation (Fig.45 A). Moreover, the increase in iNOS protein induced by iohexol was also partially blocked by 12 mM NACA (Fig.42 A), but there was no significant effect on eNOS protein expression (Fig.42 B).

To investigate the possibility that alterations in pro-apoptotic and anti-apoptotic protein expression was associated with the NACA-induced protection, we examined the protein levels of Bax, Bcl-2, and Mcl-1. As seen in Fig.45 B-D, the exposure to iohexol resulted in a significant increase in Bax expression and a decrease in Bcl-2 and Mcl-2 expression, and NACA could reverse the iohexol-induced alteration in the expression of Bax, Bcl-2, and Mcl-1.

Discussion

CIN may be a serious problem, particularly when RCM are used in patients with renal dysfunction. In the present study, we used renal proximal tubular epithelial cells in culture to test the in vitro tubular toxicity of iohexol, the most widely used RCM [22, 23]. We report that the low-osmolar nonionic contrast agent iohexol induces apoptosis in renal proximal tubular epithelial cells and we demonstrate that RCM-induced apoptosis may be partially blocked by a new antioxidant, NACA.

In clinical practices, iohexol is commonly administered at a dose of 1.5 ml/kg, resulting in plasma concentrations of 15-20 mg I/ml [6]. Since 70-80% of the glomerular ultra-filtrate is reabsorbed in the proximal convoluted tubule, the RCM concentration in this nephron segment will range between 25 and 100 mg I/ml[24]. Thus, the concentration used in the present study (75 mg I/ml) may be of clinical relevant.

Apoptosis is an important mechanism in mammalian development, homeostasis and immune responses [25]. As in other forms of ARF[1], apoptosis of renal tubular cells has been observed in CIN. It has recently been reported that RCM induce caspase-dependent apoptotic injury both in glomerular cells and renal tubular epithelial cells, and proximal tubular cells seem more sensitive to RCM cytotoxicity comparing with distal tubular cells [3,
5, 6]. Although these findings suggest that apoptosis is associated with the pathophysiology of CIN, the selective regulation of apoptosis is not fully understood [7].

NO is a reactive nitrogen species that interacts with ROS and can mediate apoptosis [11, 12]. Since iNOS-derived NO is a key molecule in tubular epithelial apoptosis induced by different toxic agents [8-10, 26, 27], we investigated whether iNOS-derived NO contributes to the toxic effects of iohexol. Interestingly, our results demonstrate that iNOS is upregulated in iohexol induced apoptosis, and that inhibition of iNOS prevents apoptosis.

NO produced by eNOS is generally considered as a protective factor during inflammation and oxidative stress [28]. Several studies reported that cell apoptosis mediated by iNOS activation could be accompanied by eNOS depression [26] or activation [29]. On the contrary, we found that eNOS protein expression was unchanged up to 2 h after iohexol exposure. Similar to our data, iNOS activation without concomitant effect on eNOS has also been observed in rats with volume-overload heart failure [30] and LPS-induced endotoxemia [31]. These results indicate that iohexol induced apoptosis is mainly due to NO-production due to iNOS activation.

As a member of MAP kinase superfamily, p38 MAPK is activated in response to a variety of environmental stresses and inflammatory signals and promote apoptosis and growth inhibition [32, 33]. In the present study, we found that p38 MAPK was activated in LLC-PK1 cells after exposure to iohexol, and that p38 MAPK inhibitor SB203580 significantly suppressed cell apoptosis. These results provide further evidence that p38 MAPK is a stress signal in renal proximal tubular epithelial cells. Furthermore, p38 MAPK inhibitor SB203580 could also suppress iNOS protein expression indicating that p38 MAPK is an activator of iNOS. To the best of our knowledge, this is the first study showing that RCM-induced cell death could be triggered by p38 MAPK-mediated activation of iNOS.

One mechanism by which NO can induce apoptosis is through increased expression or translocation of the pro-apoptotic protein Bax from cytosol to mitochondria [34, 35]. Both ionic contrast media (sodium iotalamate) [36] and nonionic iodinated contrast material (ioversol) [3] can cause apoptosis in renal tubular cells by enhancing Bax expression and reducing expression of the anti-apoptotic proteins Bcl-2. In our iohexol-exposed cells, we found that the expression of Bcl-2 and of another anti-apoptotic protein, Mcl-1, are down-regulated, while the pro-apoptotic protein Bax is up-regulated. Recently, Mcl-1 has been found being a crucial molecule for cisplatin-induced apoptosis in renal proximal tubular epithelial cells [37].
Our results demonstrate that NACA may partially protect LLC-PK1 cells from iohexol-induced apoptosis by suppression of p38 MAPK activation and iNOS protein expression. In addition, we observed that NAC could partially inhibit iohexol-induced apoptosis consistent with previous studies that NAC has a slight action against CIN [14]. Our results may indicate that NACA is more effective than NAC. One possible explanation is that NACA has better membrane permeation and NACA could thus be expected to be even more effective than NAC in vivo.

Summary: The pathogenesis of contrast-induced nephropathy (CIN) is still poorly understood and apoptosis via oxidative stress has been proposed as one possible mechanism.

We therefore studied apoptotic signaling mechanism in CIN and also tested whether the new antioxidant N-acetylcysteine amide (NACA) could prevent CIN. LLC-PK1 cells were exposed to most widely used contrast agent iohexol for different times and doses. Cytotoxicity was assessed with morphology and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cell death was also analyzed by the DNA content analysis and PARP cleavage. Protein expression was assessed with western blotting. We observed cell death with apoptotic features in dose- and time-dependent manner. Initiation of iohexol-induced apoptosis was mediated by up-regulation of Bax and inhibition of Bcl-2 and Mcl-1, which was preceded by p38 MAPK activation and induction of iNOS. Inhibitors of p38 MAPK and iNOS partially abolished iohexol-induced apoptosis. Furthermore, we found that cells pretreated with 12 mM NACA were partially rescued from iohexol-induced cell death by inhibiting p38 MAPK and iNOS signaling pathway, and by reversing the effects of iohexol on Bcl-2, Mcl-1, and Bax expression. This study demonstrates that apoptosis occurs during CIN. Apoptosis is associated with activations of p38 MAPK and iNOS. Pretreatment with the antioxidant NACA could prevent iohexol-induced cell apoptosis by blocking the p38 MAPK/iNOS signaling pathway.

In summary, we have demonstrated an apoptotic cascade triggered by p38 MAPK-mediated iNOS in renal tubular proximal epithelial cell exposed to iohexol. Moreover, the new antioxidant NACA prevents iohexol-induced cell apoptosis by blocking the p38 MAPK/iNOS signaling pathway.
Example 21. Directing dopaminergic fiber growth along a preformed molecular pathway from embryonic ventral mesencephalon transplants in the rat brain.

The purpose of this study was to determine what molecules might promote the long-distance growth of dopaminergic axons from transplanted embryonic ventral mesencephalon (VM) tissue. Two molecules were selected for this study: glial cell-line derived neurotrophic factor (GDNF) and GPI-linked GDNF receptor α1 (GFRα1). To create an expression pathway along the corpus callosum, adenovirus encoding GDNF, a combination of GDNF and GFRα1, or green fluorescent protein (GFP) was injected at 0.5 and 1.5mm from the midline in both sides of the corpus callosum with the volume increasing from the right to the left to create a gradient effect. In all groups, GDNF adenovirus was also injected on the right side 2.5mm from the midline at the desired transplant site. Three to four days later, a piece of VM tissue from embryonic day 14 rats was injected at the transplant site. All rats also received daily subcutaneous injections of N-acetyl-L-cysteinamide (NAC Amide, 100ug per rat), a cell-permeate antioxidant and glutathione precursor, to reduce viral toxic effects and oxidative stress, beginning immediately after virus injection and ending one day after transplantation. Chondroitinase ABC (lOU/ml, 2ul) was injected at the transplant site right before VM transplantation to decrease the subsequent expression of chondroitin sulfate proteoglycans. Two weeks after transplantation, the rats were perfused and brains dissected out. Coronal sections were cut and immunostained with antibody to tyrosine hydroxylase (TH) to identify and count dopaminergic fibers in the corpus callosum. In GFP-expressing pathways, TH+ fibers grew out of the transplants for a short distance in the corpus callosum. Very few TH+ fibers grew across the midline. However, pathways expressing GDNF supported more TH+ fiber growth across the midline into the contralateral hemisphere. Significantly greater numbers of TH+ fibers grew across the midline in animals expressing a combination of GDNF and GFRα1 in the corpus callosum. These data suggest that expression of GDNF or a combination of GDNF and GFRα1 can support the long-distance dopaminergic fiber growth from a VM transplant, with the combination having a superior effect. The next step will be to determine whether creating a path expressing GDNF and GFRα1 between the substantia nigra and striatum can aid reconstruction of the nigrostriatal pathway and lead to functional recovery in models of Parkinson disease.
Example 22. With NAC amide, multi-organ tissue damage was blocked and histological review showed minimal tissue damage in all organ system. Further there was no anemia (no decrease in blood cell count).

Experiments were performed using a model system as described in Seixas et al., 106 (37); 15837-15842, (2009)). DBA2 mice infected with red blood cells containing Plasmodium chabaudi chabaudi (day 0) develops severe liver failure and dies. Both NAC and NAC amide (NACA) were administered starting on day 4 post infection, twice a day (15 mg/kg body weight, i.p.) and continued with the administration until day 15. Although NAC and NACA afford similar level of protection as assessed by mortality, when mice were sacrificed 20 days after infection, the NAC treated group were more anemic, compared to the NACA treated mice, which appeared normal. The data illustrated that NACA is very efficient in suppressing this lethal outcome, in a similar manner to NAC. NACA, thus, acts therapeutically in terms of suppressing the lethal outcome of this disease when administered after infection. However, the data also suggests that the protective effect of NACA is higher as compared to that of NAC. That is because with NAC amide, multi-organ tissue damage was blocked and histological review showed minimal tissue damage in all organ systems. Further there was no anemia (no decrease in blood cell count). These effects were not seen with NAC.

Reference


WHAT IS CLAIMED IS:

1. A method for preventing tissue damage in a human subject exposed to a high-energy impulse blasts, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to high-energy impulse blasts.

2. The method according to claim 1, wherein the method is for prophylactic treatment of lung injury due to exposure to a high-energy impulse blasts.

3. A method for preventing pulmonary inflammation after exposure to blast overpressure or related conditions in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing pulmonary inflammation or related conditions after exposure to blast overpressure.

4. The method of claim 3, wherein the related conditions are contusions or barotrauma-like injury to air-filled organs, wherein the air-filled organs are ears, lungs, and the gastrointestinal tract.

5. A method for preventing blunt chest trauma in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing blunt chest trauma.

6. A method for preventing lung contusion in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing lung contusion.

7. A method for preventing traumatic brain injury in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing traumatic brain injury.

8. A method for preventing malaria in a human subject infected with a malarial parasite, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for prevention of malaria.

9. The method according to claim 8, wherein the method is for prophylactic treatment of malaria.
10. A method for inhibiting replication of HIV virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication.

11. A method of preventing HIV/AIDS in a human subject infected with HIV virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for prevention of HIV/AIDS.

12. The method according to claim 11, wherein the method is for prophylactic treatment of HIV/AIDS.

13. A method for inhibiting replication of dengue virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication.

14. A method for preventing dengue fever in a human subject infected with dengue virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for prevention of dengue fever.

15. The method according to claim 14, wherein the method is for prophylactic treatment of dengue fever.

16. A method for preventing tissue damage in a human subject exposed to radiological materials, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to radiological materials.

17. The method according to claim 16, wherein the method is for prophylactic treatment of radiological exposure.

18. A method for preventing tissue damage in a human subject exposed to airborne particulate matter, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to airborne particulate matter.

19. A method for preventing tissue damage in a human subject exposed to toxic gas or fumes, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for preventing tissue damage due to exposure to toxic gas or fumes.
20. A method for inhibiting replication of an influenza virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication.

21. A method for preventing influenza in a human subject infected with an influenza virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for prevention of influenza.

22. The method according to claim 21, wherein the method is for prophylactic treatment of influenza.

23. A method for inhibiting replication of a virus in a human subject infected with the virus, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, in a dose effective for inhibiting the viral replication.

24. A method for preventing infection by a virus in a human subject, comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective.

25. The method according to claim 24, wherein the method is for prophylactic treatment of viral infection.

26. A method for improving survivability after exposure to a high energy impulse blast or blast overpressure comprising administering to a human subject in need thereof N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing pulmonary inflammation after exposure to a high energy impulse blast or blast overpressure.

27. A method for preventing pulmonary damage after exposure to blast overpressure in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing pulmonary inflammation after exposure to blast overpressure.

28. A method for preventing multiple organ damage or related conditions after exposure to blast overpressure in a human subject in need thereof comprising administering N-acetylcysteine amide (NAC amide), or a pharmaceutically acceptable salt, ester, or derivative thereof, to the subject in a dose effective for preventing organ damage or related conditions after exposure to blast overpressure.
29. The method of claim 28, wherein the related conditions are contusions or barotrauma-like injury to air-filled organs, wherein the air-filled organs are ears, lungs, and the gastrointestinal tract.

30. The method of claim 28, wherein the organs are one or more organs selected from the group consisting of: lung, heart, brain, liver, kidneys, and gastrointestinal tract.

31. The method according to any one of the preceding claims, wherein the dose for administration is 50-10,000 mg per dose, or in an equivalent amount.

32. The method according to any one of the preceding claims, wherein the dose for administration is 25-500 mg per dose, or in an equivalent amount.

33. The method according to any one of the preceding claims, wherein NAC amide is delivered orally via a capsule.
N-Acetylcysteine (NAC)

N-Acetylcysteine amide (AD4)

FIG. 1A

FIG. 1B
FIG. 2A

Control

NACA only (750 μM)

FIG. 2B

Glutamate only (10 mM)

Glutamate (10 mM) + NACA (750 μM)

FIG. 2C

FIG. 2D
FIG. 3

% LH release

Control    Glu(10mM)    AD4    Glu+AD4 (750 μM)

*  **  ***
FIG. 6

Comparision of survival rates of SD-rats after X-ray radiation with NAC or TOVA treatments

[Bar chart showing survival rates for different groups: XRT only, XRT+Dox (pre-treated), XRT+Dox (post-treated), Control (no XRT and no drug treatment), NAC only, TOVA only, XRT+NAC (post-treated), XRT+TOVA (post-treated).]
* p<0.05, control vs NACA
FIG. 15

Control group was not exposed to BOP injury.
Control group was exposed to BOP injury.
Treated groups (NACA & Placebo) were exposed to BOP injury.

Fold change in mRNA levels (mean)

* p<0.05, t-test, NACA group vs Placebo Group

- Control
- NACA
- Placebo

MMP-9
MnSOD
TNF-a

IL-1β
HO-1
Bcl-2
Bcl-x
FIG. 16

MIP in Lungs

MIP mRNA (relative units)

Control

2 days

8 days
FIG. 19

HO-1 in Lungs

Control

* 8 days
2 days

HO-1 mRNA (relative units)

0 2 4 6 8 10 12
### FIG. 25

**NAC inhibits Dengue replication in human monocytic cells**

<table>
<thead>
<tr>
<th></th>
<th>No infection</th>
<th>DENV 10 moi</th>
<th>DENV 0.1 moi</th>
<th>DENV 0.001 moi</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Human monocytic cells**

<table>
<thead>
<tr>
<th></th>
<th>No NAC</th>
<th>+ NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baby hamster kidney cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Human monocytic cells or baby hamster cells were exposed to Dengue virus in a presence or absence of NAC for 3 days. Secreted virus was collected and seeded to naïve baby hamster cells and overlaid by methylcellulose. Three days later plates were stained for plaques. Titration of the virus demonstrated that 5 mM NAC inhibited Dengue replication by 1000 folds. Separate study has shown NAC has no effect on cell viability of either human monocytic cells or baby hamster cells.
FIG. 27A

U1 + TNF-alpha (1 ng/ml)

RT Activity (cpm/ul)

Concentration NAC (uM)
FIG. 27B

U1 + IL-6 (10ng/ml)

RT Activity (cpm/ul)

0 uM
10 uM
35 uM
110 uM
333 uM
1000 uM

Concentration of NAC (uM)
FIG. 28

Days

↑ NACA pretreatment

↑ Exposure to DEP or clean filtered air

★ Animals sacrificed
FIG. 32

(A) GSH (% control)

(B) Cysteine (% control)
FIG. 33

Graph showing MDA (nm/100mg protein) for different treatments:
- Control
- NACA
- DEP 1.5h
- DEP 3h
- NACA+DEP 1.5h
- NACA+DEP 3h

Significance markers:
- *: Significant difference
- #: Significant difference
- ###: Highly significant difference
FIG. 34

Catalase activity

Control, NACA, DEP 1.5h, DEP 3h, NACA + DEP 1.5h, NACA + DEP 3h
FIG. 35

LDH (nmol/mg of protein)

Control | NACA | DEP 1.5h | DEP 3h | NACA+DEP 1.5h | NACA+DEP 3h

* indicates significant difference.
FIG. 36

DEP
↓
Lung
↓
Macrophages: engulfs the DEP

NACA
←→

Generates oxidative stress

GSH ↓ MDA ↑ Catalase
↓
Disturbance in protective pathway:
inflammation, allergic reactions

↑ Cytotoxicity (LDH)
↓
Lung damage
**FIG. 38**

![Bar chart showing absorbance levels with acetaminophen concentrations.](chart.png)

*** p < 0.05 compared to control
FIG. 39

![Bar graph showing absorbance levels for different acetaminophen concentrations.](image)

* p < 0.05 compared to control
FIG. 41

- 20mM ACP
- ACP+5mM NAC
- ACP+5mM MAC
- # p<0.05 compared to control.
- * p<0.05 compared to control.
FIG. 42

* p < 0.05 compared to control, *** p < 0.05 compared to treated group

Absorbance
FIG. 43

* p < 0.05 compared to control, # p < 0.05 compared to treated group
* p < 0.05 compared to control, # p < 0.05 compared to treated group