

## Review Article

# Recent Advances in the Treatment of Neurodegenerative Diseases Based on GSH Delivery Systems

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Neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), are a group of pathologies characterized by a progressive and specific loss of certain brain cell populations. Oxidative stress, mitochondrial dysfunction, and apoptosis play interrelated roles in these disorders. It is well documented that free radical oxidative damage, particularly on neuronal lipids, proteins, DNA, and RNA, is extensive in PD and AD brains. Moreover, alterations of glutathione (GSH) metabolism in brain have been implicated in oxidative stress and neurodegenerative diseases. As a consequence, the reduced GSH levels observed in these pathologies have stimulated a number of researchers to find new potential approaches for maintaining or restoring GSH levels. Unfortunately, GSH delivery to the central nervous system (CNS) is limited due to a poor stability and low bioavailability. Medicinal-chemistry- and technology-based approaches are commonly used to improve physicochemical, biopharmaceutical, and drug delivery properties of therapeutic agents. This paper will focus primarily on these approaches used in order to replenish intracellular GSH levels, which are reduced in neurodegenerative diseases. Here, we discuss the beneficial properties of these approaches and their potential implications for the future treatment of patients suffering from neurodegenerative diseases, and more specifically from PD and AD.

## 1. Introduction

Neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis make up a group of pathologies characterized by a separated etiology with distinct morphological and pathophysiological features. These disorders are defined by a multifactorial nature and have common neuropathological hallmarks such as (a) abnormal protein dynamics with defective protein degradation and aggregation; (b) oxidative stress and free radical formation; (c) impaired bioenergetics and mitochondrial dysfunctions; (d) neuroinflammatory processes [1, 2]. It is difficult to establish the correct sequence of these events, but it has been shown that the oxidative damage to the brains of affected individuals is one of the earliest pathological markers. Oxidative and nitrosative stresses arise from the imbalance between the increased production of both the

reactive oxygen species (ROS) and the reactive nitrogen species (RNS) and the cellular antioxidant defense systems [3]. At low levels, ROS function as signaling intermediates for the modulation of cellular activities but, at higher concentrations, they contribute to neuronal membrane damage. The ROS mainly involved in neurodegeneration are the superoxide anion ( $O_2^-$ ), the hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $HO^\bullet$ ). RNS, such as nitric oxide (NO), can react with  $O_2^-$  to produce peroxynitrite ( $ONOO^-$ ), a powerful oxidant that may decompose itself to form  $HO^\bullet$  [4]. Cells normally employ a number of defense mechanisms against free radical such as enzymes (Cu/Zn- and Mn-superoxide dismutase, GSH peroxidase, GSH reductase, catalase, and methionine sulfoxide reductase) and low-molecular-weight antioxidants (vitamin E, ascorbate, and GSH) [5]. Macromolecules such as lipids, proteins, and DNA undergo damage and subsequently cell death mainly

by apoptosis when the antioxidant defense network is not sufficient [6].

## 2. GSH Depletion in PD and AD

The brain is especially vulnerable to free radical damage because of its high oxygen consumption rate, high content of lipids, and relative paucity of antioxidant enzymes compared with other organs [7]. Significant biological changes, related to a condition of oxidative stress, have been found in brain tissue of individuals affected by PD, AD, and other diseases [5, 8–11]. In particular, data from *postmortem* studies of brains from patients with PD suggest that oxidative stress plays a role in neural degeneration of the pigmented dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) [12]. The normal metabolism of dopamine can generate free radicals and other ROS. Furthermore, in the human SNpc the autooxidation of dopamine leads to neuromelanin and can generate quinone and semiquinone species and ROS [13]. Finally, enzymatic oxidation of dopamine catalyzed by monoamine oxidase leads to formation of  $H_2O_2$ , which can react with  $Fe^{2+}$  and form the highly reactive radical  $HO^\bullet$  via the Fenton reaction [14]. All these unfavorable events contribute to alter the antioxidant defenses suggesting that the oxidative stress plays an important role in PD. The strongest alteration in the antioxidant defense is a decrease in GSH concentration [15–17]. According to *postmortem* studies, GSH levels in the SNpc of PD patients are remarkably lower than those of healthy subjects (60% compared to control subjects) while oxidized glutathione (GSSG) levels are slightly increased [18, 19]. Although GSH is not the only antioxidant molecule reported to be altered in PD, it is hypothesized that the magnitude of its depletion is the earliest indicator of nigrostriatal degeneration [20]. Moreover, striatal DA content and GSH levels are not altered in areas of the brain other than SNpc, or in other diseases affecting dopaminergic neurons [21–24]. GSH loss in PD is also accompanied by a reduction in mitochondrial complex I activity, which is regionally selective for the SNpc in PD and does not occur in related basal ganglia degenerative disorders [25]. These findings suggest that decreased nigrostriatal GSH levels can initiate or facilitate a cascade of further oxidative stress with consequent degeneration of dopaminergic neurons in idiopathic PD [26, 27].

AD is characterized by the loss of pyramidal neurons in the hippocampus and cortex, as well as cholinergic neurons in the basal forebrain. The etiology of AD is not completely known yet, although there are different hallmarks that seem to play significant roles in the disease, such as  $\beta$ -amyloid ( $A\beta$ ) deposits,  $\tau$ -protein aggregation, oxidative damage in cellular structures, and low levels of acetylcholine (ACh) [28, 29]. In AD patients there is a strong evidence that  $A\beta$ -associated free radicals and the resultant oxidative stress are a part of the mechanism that is involved in the pathogenic cascade that leads to neurodegeneration in AD brain [30]. Furthermore, alterations to GSH metabolism have been found in these pathological conditions [16]. In this context,

Gu et al. [31] reported that GSH levels are depressed in AD cingulate cortex and AD *substantia innominata*, while Liu et al. [32] found these reduced levels only in red blood cells of male AD patients. Nevertheless, increased GSH levels have been observed by Adams et al. [33] in the midbrain and in the caudate nucleus, while normal GSH contents have been determined by Perry et al. [34]. Presumably, dissenting results are due to differences in techniques or difficulty in sample collection after death of AD patients. In any case, it has been observed that GSH protects cultured neurons against oxidative damage resulting from  $\beta$ -peptide and 4-hydroxynonenal (HNE), a lipid peroxidation product that is increased in AD [35]. A significant decrease in Cu and significant increases in Zn and Fe were found in AD hippocampus and amygdala, while Cu, Fe, and Zn are elevated in senile plaques of AD. These metal ions can catalyze free radical reactions and contribute to oxidative damage observed in AD brain [36]. GSH protects these areas through formation of metal complexes via nonenzymatic reactions and may also be beneficial for normalizing the adverse effects of iron accumulation in the aging brain [37].

## 3. Antioxidant Neuroprotection in PD and AD

Neuroprotective antioxidants are considered a promising approach to slow down the progression and limit the extent of neuronal cell loss in neurodegenerative disorders [38–41]. These agents were classified by Behl and Moosmann according to their mode of action in (a) compounds that prevent the formation of free radicals; (b) compounds that chemically interfere with formed free radicals; (c) compounds which limit the damage extent to the cell by alleviating the secondary metabolic burden of increased levels of free radicals [42]. N-acetylcysteine, lipoic acid, GSH, and its thiol derivatives belong to the last class of neuroprotective antioxidants. In this context, the GSH system is especially important for cellular defense against ROS in brain cells, acting directly in detoxification of radicals in non-enzymatic reactions and working as a substrate for various peroxidases [43]. Astrocytes appear to play a key role in the GSH metabolism in the brain since astroglial GSH export is essential for providing GSH precursor to neurons. Normally astrocytes release GSH and protect it against oxidation by releasing a protecting factor into the medium. Astroglial release of GSH is the first step in the supply of the GSH precursor cysteine to neurons. The extracellular GSH is processed by  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) and aminopeptidase N (ApN) to generate the cysteine, which limits the synthesis of GSH in neurons. Alterations of the release rate of GSH from astrocytes and reduced activities of the ectoenzymes may contribute to a lowered antioxidant defense in neurons and to an increased susceptibility to oxidative stress, both involved in the progression of neurodegenerative diseases [44]. Alterations of GSH metabolism in brain have been found in neurodegenerative disorders as PD and AD [5, 45–47]. The causes of GSH depletion are not well understood yet, but their consequences are quite serious. GSH depletion can inhibit complex I, E1 ubiquitin ligase, and proteasome activity; it can also exacerbate oxidative stress and activate

the JNK pathway, leading to an inflammatory response. All these effects cause dopaminergic neuronal death and accumulation of proteins into Lewy bodies in patients affected by PD [48]. Furthermore, an emerging evidence indicates that the total antioxidant capacity (including GSH, ascorbic acid, uric acid, and bilirubin) has shown to be reduced by 24% in plasma samples from AD patients [49]. An increased number of mutations in mitochondrial DNA have been found in AD, such as increased concentrations of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to DNA. These deletions or point mutations, which may result from oxidative stress, can cause mitochondrial dysfunction and trigger apoptotic cell death. In addition to DNA damage, several mitochondrial key enzymes involved in ROS detoxification are also affected. *In vivo* studies on animal models of AD have also shown the implication of mitochondria in the disease pathogenesis [50]. In this regard, several groups have focused their efforts on developing neuroprotective strategies targeting mitochondria. Some of the major mitochondrial targets used as therapeutics against ROS-mediated damage are members of the quinone family. An ubiquinone derivative, mitoquinone mesylate or MitoQ, has been used to prevent oxidative damage in AD [51]. MitoQ consists of CoQ10 linked to a triphenylphosphonium ion, which has a positive charge; therefore, it accumulates in mitochondria, which have a strongly negative membrane potential (about  $-120$  mV). More precisely, MitoQ is adsorbed in the inner mitochondrial membrane facing the matrix. This ROS-enriched region provides a real potency to MitoQ. In addition, MitoQ prevents AD-like pathology in mouse cortical neurons in cell culture, attenuates  $\beta$ -amyloid-induced neurotoxicity, and prevents increased production of ROS.

Mitochondrial dysfunction, oxidative stress, glutamate excitotoxicity, and formation of high-molecular-weight aggregates also define the most common adult-onset motoneuron disease: amyotrophic lateral sclerosis (ALS) caused by the progressive degeneration of motoneurons in the spinal cord, brain stem, and motor cortex [52]. Dominant mutation in Cu/Zn-superoxide dismutase (SOD1) causes familial forms of ALS. In order to investigate the role of GSH in this pathology, knockout mice for the glutamate-cysteine ligase modifier (GCLM) subunit were used. Results suggested that the lack of GCLM significantly accelerates disease and mitochondrial pathology in hSOD1 mice [53].

A promising therapeutic intervention in the above reported diseases could be the antioxidant neuroprotection [54]. In this context, the increase of GSH availability in neurons is a logical therapeutical target in neural impairment related to oxidative stress. Due to the difficulty in elevating GSH directly as described by Zeevalk et al. [55], other strategies to raise brain levels of this antioxidant have been investigated [56]. In this paper we will focus on the medicinal chemistry and technological approaches aimed at maintaining or restoring GSH levels in PD and AD patients. Particular attention will be paid to different strategies for increasing GSH levels by supplying GSH codrugs and GSH nanocarrier systems able to cross the cellular membrane more easily than GSH.

#### 4. Medicinal-Chemistry-Based Strategies to Increase GSH Levels

Medicinal-chemistry-based strategies include analogues [57, 58], as well as prodrugs and codrugs approaches [59]. While each of these strategies may be equally promising to increase GSH levels, this paper will mainly focus on codrugs approach since the other medicinal-chemistry-based strategies have been previously discussed [56, 60–62]. The codrug approach consists in linking, via a covalent chemical linkage, two different pharmacophores with similar or different pharmacological activities in order to improve physicochemical, biopharmaceutical, and drug delivery properties of therapeutic agents. The resulting codrug has to be stable at gastrointestinal level and transported to the target site of action where it provides the two parent drugs following hydrolysis [63].

The codrug approach has been used for the treatment of PD and AD joining antioxidant or chelating molecules with a therapeutic compound (antiparkinson or anti-alzheimer's drugs) [64–66]. In particular, codrugs containing antioxidant molecules such as GSH, N-acetyl-cysteine, methionine, and cysteinyl derivatives have been synthesized in order to permit a targeted delivery of antioxidant directly to specific groups of neurons where cellular stress is associated with PD and AD. The dual advantage of these antioxidant molecules lies in the fact that the antioxidant portion, in addition to acting as a scavenger directly or indirectly of free radicals, can be used as a carrier. In fact, GSH and cysteinyl derivatives can be used as BBB shuttles for delivery of antiparkinson or anti-alzheimer's drugs since the presence of GSH transporters at the BBB is well documented [67, 68]. In this context, the research of new codrugs for the treatment of PD and AD has gained our attention. L-Dopa-GSH codrugs (LD-GSH, 1-2), obtained via an amide bond between LD and the C- and N-terminal GSH, respectively, have been synthesized and evaluated as potential anti-Parkinson agents with antioxidant properties (Figure 1) [69]. These codrugs permit a targeted delivery of GSH directly to SNpc neurons of PD patients and contribute in attenuating the damage caused by the prooxidant effects of traditional LD therapies. Codrugs 1-2 showed good stability toward gastrointestinal simulated fluids and released LD in rat and human plasma after enzymatic hydrolysis. Furthermore, they prolonged the plasma LD levels and were able to induce sustained delivery of dopamine (DA) in rat striatum with respect to equimolar dose of LD. Taken together, these results demonstrated the possible therapeutic application of codrugs 1-2 in PD, being able to protect against the oxidative stress deriving from autoxidation and the MAO-mediated metabolism of DA [69].

Later, More and Vince [70] reported two GSH bioconjugates (3-4) containing a metabolically stable urea analogue of GSH resistant to the enzyme  $\gamma$ -GT (Figure 2). The antioxidant portion has been covalently joined to the therapeutic drugs, as DA and adamantine, via a heterodisulfide linkage. This suitable junction is stable in plasma and able to release DA or adamantine, and the antioxidant portion due to the abundance of the enzyme disulfide reductases in the

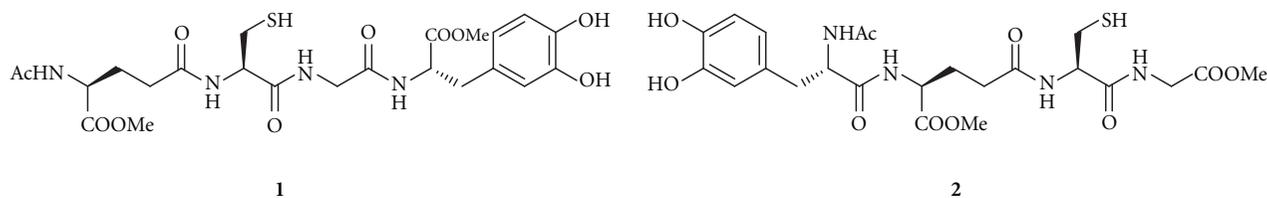


FIGURE 1: Chemical structures of GSH codrugs 1-2.

brain [71]. More importantly, these bioconjugates cross the BBB through recognition by GSH transporters on the luminal side of BBB [68]. Studies successfully confirmed the carrier-mediated transport of conjugates 3-4 in an *in vitro* BBB model and their ability to release the active drug at the target site, thus representing an innovative approach for the targeted delivery of anti-Parkinson drugs into the CNS using the GSH transport system [70]. In particular, the MDCK cell monolayer has been used to study the bioconjugates 3-4 transport. At concentration of 100  $\mu$ M, the transport of bioconjugates 3-4 from the apical to the basal side was greater than the transport in the reverse direction. Moreover, to ensure that the codrugs 3-4 were not being metabolized as they crossed the MDCK cell monolayer, the integrity of codrugs 3-4 was confirmed by HPLC studies [70]. These experiments successfully demonstrated the GSH-carrier-mediated transport of the bioconjugates 3-4 in an *in vitro* BBB model.

Another bioconjugate that could use the GSH transporters on the luminal side of BBB might be the hybrid 5 (Figure 3). This molecule is characterized by the replacement of cysteine with methionine in order to obtain stable GSH analogue at  $\gamma$ -GT [72]. Furthermore, the GSH analogue has been linked to LD to obtain CNS drug delivery. This compound was demonstrated to cross unaltered the acidic environment of the stomach, to be stable enough to be absorbed from the intestine, to have radical scavenging activity, and to release LD in human plasma after enzymatic hydrolysis. Taken together, these data suggest a therapeutic potential of 5 in pathological events associated with free radical damage and decreasing DA concentration in the brain [72].

Recently, Ehrlich et al. [73] designed and synthesized a library of new GSH codrugs (called UPF peptides) with powerful hydroxyl radical scavenging activities. They have been obtained via an amide bond between GSH and tyrosine derivatives as shown in Figure 4. In particular, the enzyme free hydroxyl radical scavenging assay showed that substitution of  $\gamma$ -glutamyl moiety (UPF1, 4-methoxy-L-tyrosinyl- $\gamma$ -L-glutamyl-L-cysteinyl-glycine, 6) with  $\alpha$ -glutamyl moiety (UPF17, 4-methoxy-L-tyrosinyl- $\alpha$ -L-glutamyl-L-cysteinyl-glycine, 7) improved hydroxyl radical scavenging activity of about 500-fold [74]. UPF1 (6) is an effective and potential agent that diminishes neuronal injury in global cerebral ischemia [75]; it acts as a free radical scavenger or a modulator of G-protein in frontocortical membrane preparations. Although the exact mechanisms of the protective action of UPF1 still remain unclear, it can

possibly act as a scavenger or a signal molecule increasing GSH levels or the GSH redox ratio; UPF1 could be a promising lead for the design of powerful antioxidants for the treatment of conditions associated with reduced GSH levels [76]. Unfortunately, the role of UPF peptides for the treatment of PD has not been studied yet. It could be interesting to investigate the activity of UPF peptides in patients affected by PD since these peptides contain the tyrosine moiety, the metabolic precursor of DA.

Few data are available in the literature about GSH codrugs for the treatment of AD. We recently synthesized Ibuprofen-GSH (IBU-GSH, 8) obtained via amide bond between GSH and IBU, a nonsteroidal anti-inflammatory drug (NSAID) (Figure 5) [77]. NSAIDs treatment reduces AD risk, delays disease progression, and reduces microglia activation [78]. In particular, Lim et al. [79] reported that six months of treatment of a transgenic animal model of AD with IBU resulted in a significant reduction of amyloid plaque burden and total A $\beta$  peptide levels. Furthermore, IBU treatment led to a reduction of plaque-associated microglia and a corresponding attenuation in proinflammatory cytokine levels in brain [80]. Codrug 8 possessed good stability toward human plasma enzymatic activity and displayed *in vitro* free radical scavenging activity in time- and concentration-dependent manner. More importantly, it antagonizes the deleterious and cognitive effects of  $\beta$ -amyloid(1-40) in a rat model for AD, as also confirmed by behavioral tests of long-term spatial memory. In conclusion, IBU-GSH might permit targeted delivery of IBU and GSH directly to neurons, where oxidative stress and inflammatory processes are associated with AD [77].

Almost all the codrugs (1-2, 5, 9-14) have been tested for their chemical and enzymatic stabilities in order to check both their stability in aqueous medium and their sensitivity towards enzymatic cleavage in rat and human plasma (Tables 1 and 2) [69, 72, 81]. Stability studies were performed at 37°C in isotonic sodium phosphate buffer (pH 7.4), in simulated gastric fluid (SGF, pH 1.3), and in rat and human plasma diluted to 80% with isotonic sodium phosphate buffer (pH 7.4). All codrugs showed good stability toward gastrointestinal hydrolysis ( $t_{1/2} > 20$  h) (Table 1). On the contrary, in rat and human plasma the codrugs (1-2, 5, 9-14) underwent rapid bioconversion of the codrugs into their constituents (Table 2).

Sulfur-containing amino acids have gained great attention as source of thiols for GSH synthesis [82]. A series of multifunctional thiol codrugs (9-14) were synthesized to overcome the prooxidant effect associated with LD

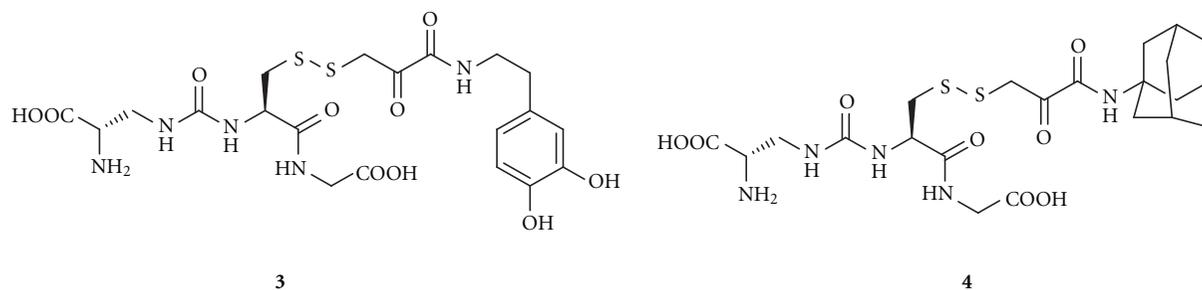


FIGURE 2: Chemical structures of GSH codrugs 3-4.

TABLE 1: Kinetic data for chemical hydrolysis of codrugs 1-2, 5, and 9-14 at 37°C.

Compd	pH 1.3 <sup>a</sup>		pH 7.4 <sup>a</sup>	
	$t_{1/2}$ (h)	$K_{obs}$ (h <sup>-1</sup> )	$t_{1/2}$ (h)	$K_{obs}$ (h <sup>-1</sup> )
1	20.14 ( $\pm 0.73$ )	0.034 ( $\pm 1.2 \times 10^{-3}$ )	7.22 ( $\pm 0.31$ )	0.096 ( $\pm 4.1 \times 10^{-3}$ )
2	28.12 ( $\pm 1.21$ )	0.025 ( $\pm 1.1 \times 10^{-3}$ )	12.23 ( $\pm 0.49$ )	0.057 ( $\pm 2.3 \times 10^{-3}$ )
5	20.67 ( $\pm 0.83$ )	0.094 ( $\pm 0.3 \times 10^{-3}$ )	10.80 ( $\pm 0.40$ )	0.018 ( $\pm 0.8 \times 10^{-3}$ )
9	301.0 ( $\pm 10.5$ )	0.002 ( $\pm 0.07 \times 10^{-3}$ )	46.2 ( $\pm 0.90$ )	0.015 ( $\pm 0.3 \times 10^{-3}$ )
10	290.6 ( $\pm 5.8$ )	0.002 ( $\pm 0.04 \times 10^{-3}$ )	48.0 ( $\pm 1.70$ )	0.015 ( $\pm 0.45 \times 10^{-3}$ )
11	296.3 ( $\pm 11.8$ )	0.002 ( $\pm 0.08 \times 10^{-3}$ )	30.2 ( $\pm 1.40$ )	0.023 ( $\pm 1.04 \times 10^{-3}$ )
12	292.1 ( $\pm 4.4$ )	0.002 ( $\pm 0.03 \times 10^{-3}$ )	26.9 ( $\pm 0.70$ )	0.026 ( $\pm 0.65 \times 10^{-3}$ )
13	292.8 ( $\pm 8.8$ )	0.002 ( $\pm 0.06 \times 10^{-3}$ )	48.50 ( $\pm 0.70$ )	0.005 ( $\pm 0.25 \times 10^{-3}$ )
14	293.4 ( $\pm 14.7$ )	0.002 ( $\pm 0.1 \times 10^{-3}$ )	21.3 ( $\pm 0.60$ )	0.033 ( $\pm 0.99 \times 10^{-3}$ )

<sup>a</sup>Values are means of three experiments, and standard deviation is given in parentheses.

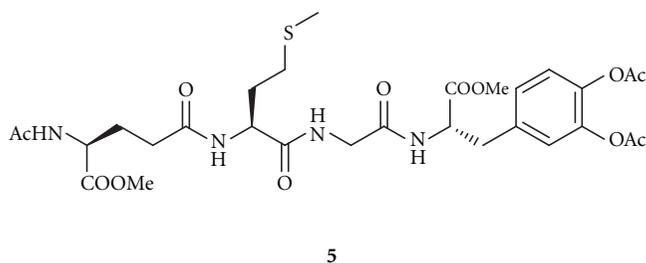


FIGURE 3: Chemical structure of GSH hybrid 5.

therapy in parkinsonian models (Figure 6) [81]. In this regard, thiol antioxidants (N-acetyl-L-cysteine, methionine, dithiothreitol) prevent DA autoxidation, production of dopamine-melanin, and inhibition of dopamine-induced apoptosis [83]. Moreover, they increase levels of intracellular cysteine, the limiting amino acid in GSH biosynthesis, thus potentiating the natural cellular defense mechanisms against oxidative damage. The multifunctional codrugs 9-14 proved to be good radical scavengers. The LD and DA striatal level profiles indicate that codrugs 11 and 12 were able to induce sustained delivery of both LD and DA in rat striatum with respect to equimolar doses of LD [82].

In addition, Minelli et al. [84] showed that administration of codrug 11 to mice treated with Z-Ileu-Glu(OtBu)-Ala-Leu-CHO (PSI), used as a PD model, resulted in a reduction in dopaminergic neuronal death and a significant

raise in GSH levels. In particular, codrug 11 could control the LD-induced oxidative stress in primary mesencephalic cultures and in newborn mice pups since in both cases GSH content results increased. Using newborn mice pups, characterized by incomplete formation of BBB, Minelli et al. [84] found that buthionine-[S,R]-sulfoximine-(BSO-) mediated GSH depletion prevented the increase of GSH levels promoted by codrug 11, supporting the role of GSH for codrug-11-induced protection. To investigate whether heme oxygenase (HO) activity was related to GSH levels, ZnPPIX was used as HO inhibitor. Compared to untreated control, and LD-treated newborn mice, brain GSH levels were increased by ZnPPIX indicating that HO activity was not essential to GSH synthesis. An injection of codrug 11 induced a significant increase in GSH levels that was markedly reduced by BSO indicating the essential role of  $\gamma$ -glutamylcysteine synthetase in increasing GSH brain levels. Codrug 11 exhibited *in vivo* protective effect against LD-induced stress through a mechanism via Nrf2 activation leading to a decrease in ROS generation and an increase in GSH. Therefore, this codrug might offer benefits in the treatment of PD and provide a potential alternative to LD therapy by avoiding nigrostriatal oxidative degeneration [84].

Maher et al. [85, 86] demonstrated that the conjugation of catechins with cysteine generates antioxidant compounds (15-20) with enhanced neuroprotective activity (Figure 7). The thiol conjugates 15-20 were active in protecting HT-22 nerve cells ( $EC_{50}$  between 36 and 65  $\mu$ M) from oxidative

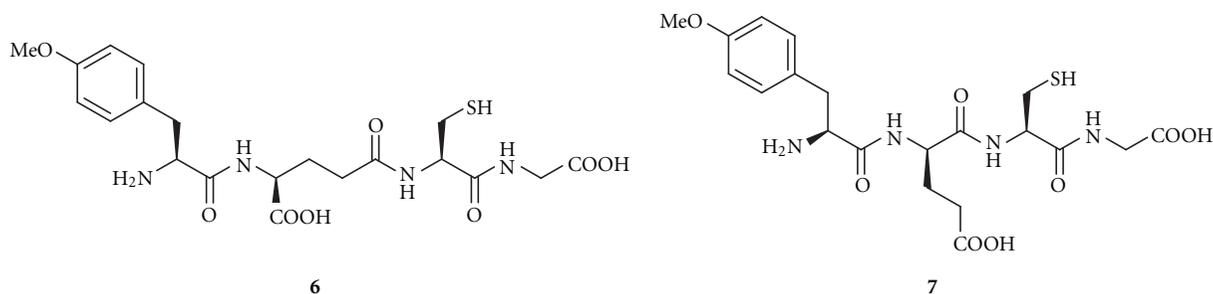


FIGURE 4: Chemical structures of GSH codrugs 6-7.

TABLE 2: Rate constants for the hydrolysis of codrugs 1-2, 5, and 9-14 in 80% rat plasma and 80% human plasma at 37°C.

Compd	Rat plasma <sup>a</sup>		Human plasma <sup>a</sup>	
	$t_{1/2}$ (min)	$K_{obs}$ ( $\text{min}^{-1}$ )	$t_{1/2}$ (min)	$K_{obs}$ ( $\text{min}^{-1}$ )
1	immediate hydrolysis	—	3.2 ( $\pm 0.1$ )	0.217 ( $\pm 6 \times 10^{-3}$ )
2	2.7 ( $\pm 0.1$ )	0.257 ( $\pm 8 \times 10^{-3}$ )	15.1 ( $\pm 0.4$ )	0.046 ( $\pm 1 \times 10^{-3}$ )
5	4.7 ( $\pm 0.1$ )	0.150 ( $\pm 0.01$ )	7.3 ( $\pm 0.3$ )	0.100 ( $\pm 0.01$ )
9	46.8 ( $\pm 1.4$ )	0.010 ( $\pm 0.20 \times 10^{-3}$ )	69.6 ( $\pm 1.4$ )	0.015 ( $\pm 0.44 \times 10^{-3}$ )
10	36.6 ( $\pm 1.6$ )	0.019 ( $\pm 0.85 \times 10^{-3}$ )	65.4 ( $\pm 1.6$ )	0.011 ( $\pm 0.26 \times 10^{-3}$ )
11	115.2 ( $\pm 11.0$ )	0.002 ( $\pm 0.07 \times 10^{-3}$ )	315.0 ( $\pm 4.6$ )	0.006 ( $\pm 0.24 \times 10^{-3}$ )
12	93.0 ( $\pm 10.2$ )	0.003 ( $\pm 0.17 \times 10^{-3}$ )	263.4 ( $\pm 1.9$ )	0.007 ( $\pm 0.14 \times 10^{-3}$ )
13	55.8 ( $\pm 10.5$ )	0.003 ( $\pm 0.11 \times 10^{-3}$ )	203.4 ( $\pm 0.6$ )	0.030 ( $\pm 0.75 \times 10^{-3}$ )
14	69.6 ( $\pm 3.5$ )	0.010 ( $\pm 0.50 \times 10^{-3}$ )	90.0 ( $\pm 2.7$ )	0.008 ( $\pm 0.24 \times 10^{-3}$ )

<sup>a</sup> Values are means of three experiments, standard deviation is given in parentheses.

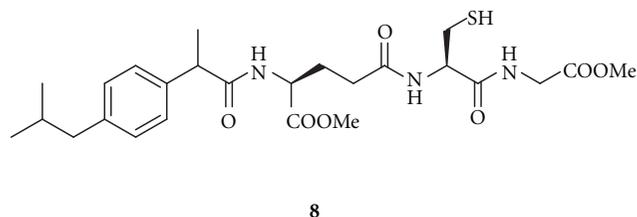


FIGURE 5: Chemical structure of GSH codrug 8.

stress-induced death. Although all the conjugates were able to scavenge mitochondrial generated ROS inside the cells, the majority of their neuroprotective activity seems to be dependent on their ability to maintain GSH levels. These compounds were able to maintain cellular GSH levels by enhancing the uptake of cystine/cysteine into cells by a mechanism that uncouples the uptake from system  $x_c^-$ , a  $\text{Na}^+$ -independent cystine/glutamate antiporter [87]. System  $x_c^-$  transports cysteine into cells in a 1 : 1 exchange with glutamate. The importance of this system for the maintenance of the GSH levels in cells is demonstrated by the loss of GSH and subsequent cell death in nerve and other types of cells following exposure to millimolar concentrations of extracellular glutamate. Treatments able to maintain GSH levels, in presence of an induced stress by GSH loss, have a significant potential for the treatment of neurodegenerative diseases.

## 5. Technology-Based Strategies to Increase GSH Levels

The effectiveness of exogenous antioxidants to protect tissues from oxidative stress *in vivo* depends on the antioxidant used, its physicochemical and biopharmaceutical properties, and its bioavailability at the site of action [88, 89]. With the aim of improving the physicochemical, biopharmaceutical and drug delivery properties of neuroprotective antioxidants, the technology-based strategy could be useful for the treatment of several diseases in which oxidative stress plays an important role [59, 90]. Particularly, this approach could be adopted in order to selectively deliver antioxidants to tissues in sufficient concentrations to reduce the oxidative damage. In order to afford neuroprotection and to facilitate the delivery of GSH across the BBB, several GSH delivery systems, such as liposomes, nanoparticles, and dendrimers, were developed.

Liposomes are considered as carrier systems for therapeutically active compounds due to their unique characteristics such as capability of incorporating hydrophilic and hydrophobic drugs, good compatibility, low toxicity, lack of immune system activation, and targeted delivery of bioactive compounds to the site of action [91]. Liposome technology has been recently used in the treatment of neurodegenerative diseases. In this context, GSH has been encapsulated in liposomes in order to replenish intracellular GSH and provide neuroprotection in an *in vitro* model of PD [92]. The formulation of GSH has been encapsulated in lipid vesicles

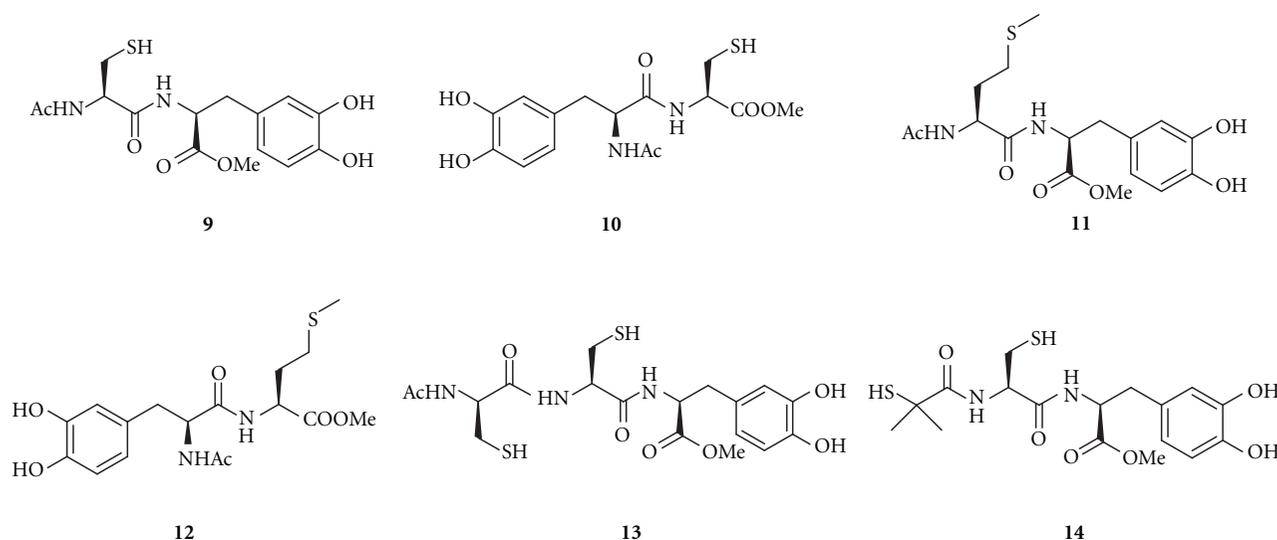


FIGURE 6: Chemical structures of cysteinyl codrugs 9–14.

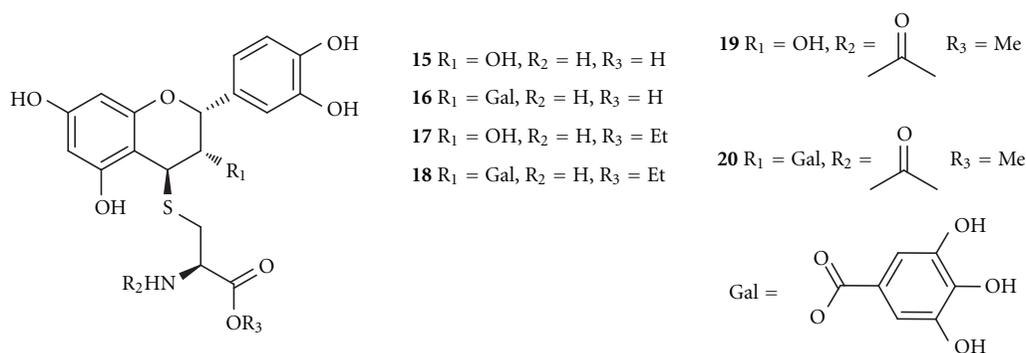


FIGURE 7: Chemical structures of thiol conjugates 15–20.

made of lecithin and glycerol and then tested on mixed mesencephalic cultures treated with paraquat plus maneb. Zeevalk et al. [92] observed that liposomal GSH was taken up into neurons and astrocytes via an endosomal process, and subsequently the endosomes containing liposomal-GSH were fused with lysosomes (Figure 8).

In these conditions, GSH was hydrolyzed and its constituent amino acids (glutamate, cysteine, and glycine) released from lysosomes could be used for the GSH biosynthesis. The results obtained by Zeevalk et al. [92] suggested that this formulation was 100-fold more potent than nonliposomal-GSH in providing substrates for the maintenance of intracellular GSH in neuronal cells. Moreover, liposomal-GSH dose-dependently provided complete neuroprotection of dopaminergic neurons treated with paraquat plus maneb with an  $EC_{50}$  of  $10.5 \mu\text{M} \pm 1.08$ . These findings suggest that liposomal-GSH represents a promising therapeutic strategy for neuronal maintenance in pathologies characterized by GSH depletion.

Nanoparticles (NPs) are solid colloidal particles made of polymeric materials ranging in size from 1–1000 nm. NPs are used as carrier systems in which the drug is dissolved,

entrapped, encapsulated, adsorbed, or chemically linked to the surface [93]. The advantages of NPs are high drug-loading capacity and resistance against chemical and enzymatic degradation. Coating NPs with hydrophilic polymer is a promising strategy in order to prolong their presence in plasma and the therapeutic effect. The surface modification of NPs can be achieved using polyethylene glycol (PEG) or polysaccharides such as chitosan, dextran, pectin, and hyaluronic acid [94, 95]. NPs have been employed for delivering GSH to CNS. In this regard, a series of NPs containing GSH (GS-PEG-GS, **21**) were prepared with PEGs of various molecular weights (Figure 9) [96]. PEG was used because of its well-established biocompatibility, low immunogenicity, low antigenicity, and low toxicity [97].

Unfortunately, GS-PEG-GS nanoparticles were not able to exert their antioxidant activity because the thiol groups were consumed during Michael addition. Thus, a disulfide bridge was proposed for antioxidant delivery in order to release GSH when the pH was low enough to enable thiol formation. Disulfide-linked GSH NPs (GS-SPEGS-SG, **22**) were synthesized and tested on SH-SY5Y cells challenged with  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$ , a compound that induces oxidative

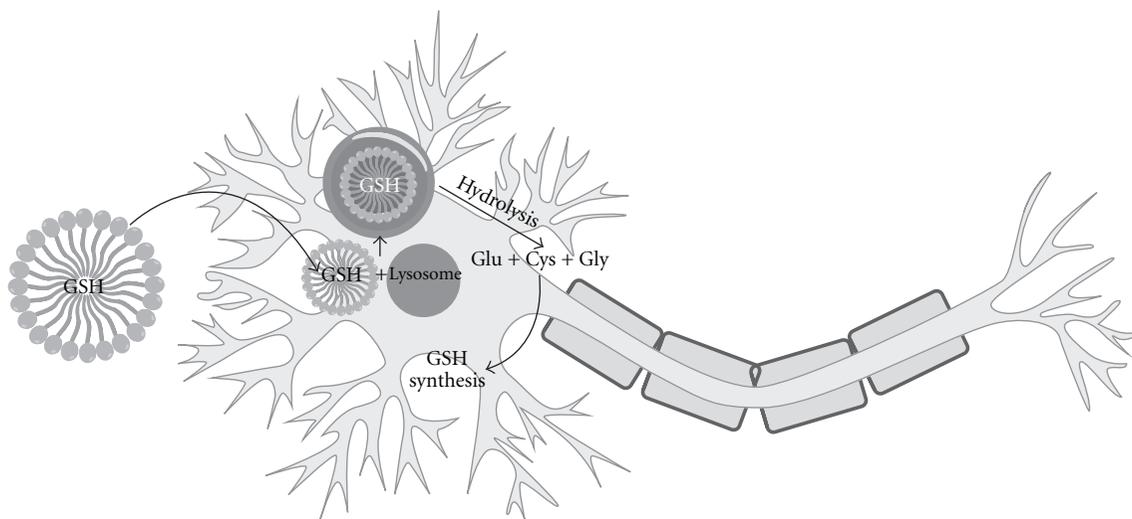


FIGURE 8: Liposomal GSH delivery to neurons and relative hydrolysis following fusion with lysosome.

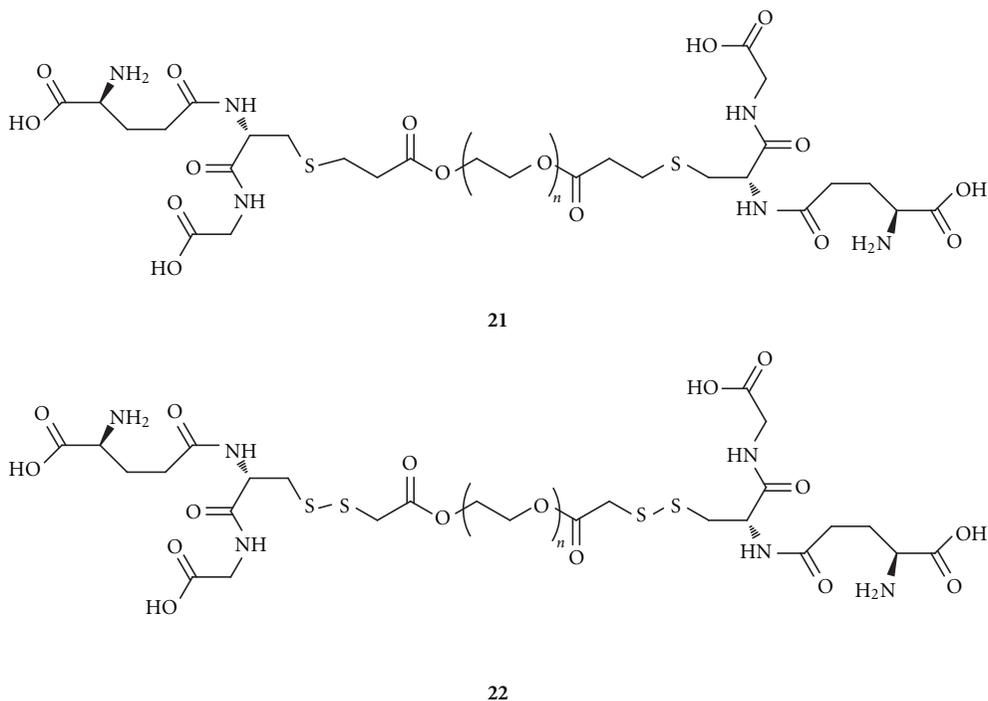


FIGURE 9: Chemical structures of GS-PEG-SG (21) and GS-SPEGS-SG (22).

stress. The GS-SPEGS-SG NPs were 100% at protecting SH-SY5Y cells at 250  $\mu$ M from oxidative stress, while the GS-PEG-GS did not offer protection [96]. According to these data, this approach could be employed in treating diseases typically associated with increased ROS levels.

Chitosan-GSH nanoparticles (CS-GSH NPs) have been developed by Koo et al. [98] as delivery system for enhancing stability and bioavailability of GSH. Chitosan (CS) nanodelivery system offers many advantages: (a) it is not toxic; (b) it is biodegradable; (c) it is biocompatible; (d) it has good bioadhesibility and water dispersibility [99, 100]. Thus,

CS-GSH was synthesized using a radical polymerization method, and CS-GSH NPs were prepared by ionic gelation of CS-GSH with sodium triphosphate (TPP) (Figure 10). The resulting NPs showed a good entrapment and loading efficiency. Furthermore, to investigate the CS-GSH NPs stability under oxidative stress, the effect of the presence of  $H_2O_2$  on their thiol groups was evaluated. The reduction of thiol groups of the CS-GSH NPs under oxidative stress resulted in being 1.5-fold lower than that of free GSH [98]. These results suggest that CS-GSH NPs could be used as effective delivery carriers of GSH under oxidative insults, but

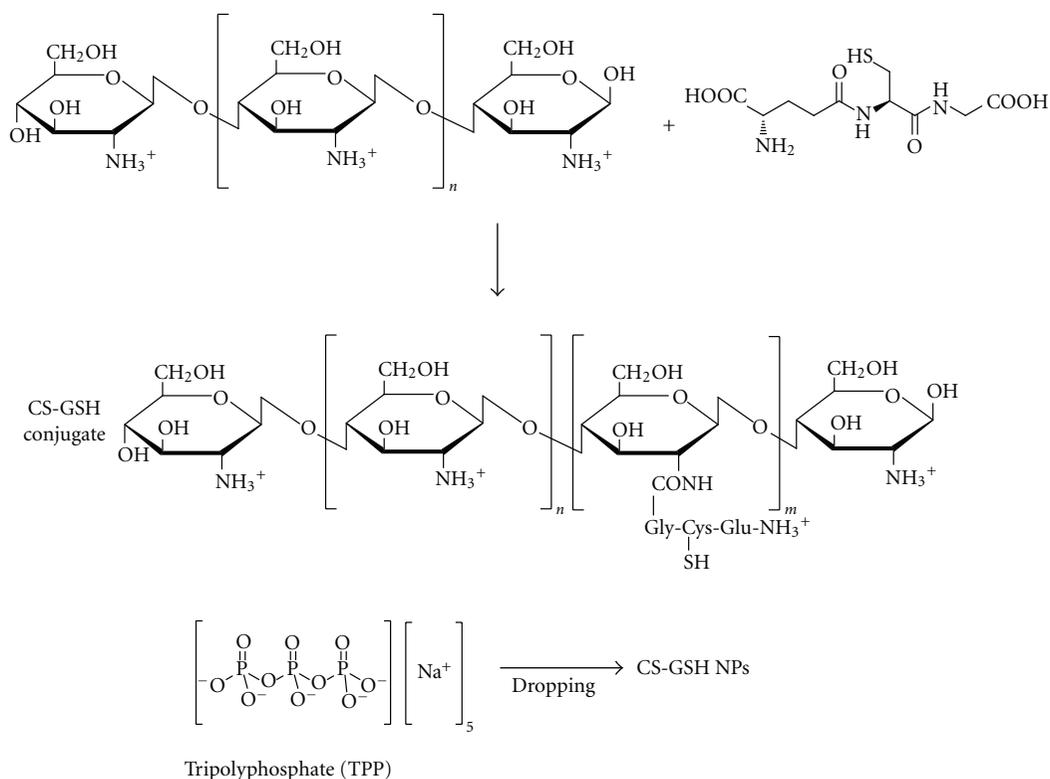


FIGURE 10: Scheme for GS-GSH NPs preparation as reported by Koo et al. [98].

further studies on animal models of PD and AD are necessary in order to evaluate their true efficacy.

The use of appropriate nanocarrier systems for GSH may be useful because they are noninvasive systems and protect the molecule to be delivered against inactivation mechanism and clearance. However, they are characterized by limiting factors as safety and toxicity. At present, few data are available about the utilization and human application of nanocarrier systems for transport across the BBB and CNS delivery. Thus, the future clinical study of GSH delivery systems for neurodegenerative diseases is strongly recommended.

## 6. Conclusions

The drug delivery to CNS is a complex and challenging task requiring close collaboration of several scientific areas including pharmaceutical and technological sciences, biological chemistry, and pharmacology. In this context, this paper has investigated multidisciplinary approaches such as the codrug and nanocarriers strategies that could be used to treat neurodegenerative disorders associated with GSH deficiency. GSH and cysteinyl codrugs have been designed on the basis of combining suitable groups of the antioxidant portion with available drugs without altering their inherent pharmacodynamic properties with improved physicochemical properties of drugs. This promising approach has also been used to resolve the issues like permeation, solubility of drug, stability, drug resistance, oral absorption, and brain delivery but still

there are very few well-established candidates that have been approved for clinical applications.

Novel experimental neuroprotective strategies include formulations containing GSH, such as nanocarrier systems. This approach could be adopted in order to selectively deliver GSH to tissues in sufficient concentrations to reduce the oxidative damage, but few data are available about clinical studies.

Although the potential use of these strategies needs further exhaustive studies, they may offer a promising therapeutic alternative for reducing the GSH functional loss related to human diseases such as PD and AD.

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