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Original Contribution

Novel *S*-acyl glutathione derivatives prevent amyloid oxidative stress and cholinergic dysfunction in Alzheimer disease models

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ABSTRACT

Oxidative stress-mediated neuronal death may be initiated by a decrease in glutathione (GSH), whose levels are reduced in mitochondrial and synaptosomal fractions of specific CNS regions in Alzheimer disease (AD) patients. Currently, the use of GSH as a therapeutic agent is limited by its unfavorable pharmacokinetic properties. In this study, we designed the synthesis of new *S*-acyl glutathione (acyl-SG) thioesters of fatty acids via *N*-acyl benzotriazole-intermediate production and investigated their potential for targeted delivery of the parent GSH and free fatty acid to amyloid-exposed fibroblasts from familial AD patients and human SH-SY5Y neuroblastoma cells. Cell culture supplementation with acyl-SG derivatives triggers a significant decrease in lipid peroxidation and mitochondrial dysfunction in a fatty acid unsaturation degree-dependent fashion. Acyl-SG thioesters also protect cholinergic neurons against Aβ-induced damage and reduce glial reaction in rat brains. Collectively, these findings suggest that acyl-SG thioesters could prove useful as a tool for controlling AD-induced cerebral deterioration.

Alzheimer disease (AD) is an age-related neurodegenerative disorder that is characterized clinically by progressive loss of memory and cognitive functions and neuropathologically by localized neuronal death and loss of synaptic connections within selective brain regions, all of which results in severe dementia [1]. The main pathogenic factor associated with AD seems to be amyloid-β peptide (Aβ) oligomers that tend to accumulate extracellularly as amyloid deposits. Recent studies have shown that soluble, prefibrillar Aβ oligomers, rather than plaque load, correlate better with cognitive impairment and neuronal dysfunction, suggesting that the oligomeric form may be the main toxic Aβ species involved in AD [2,3]. Several hypotheses have been proposed to explain AD pathogenesis and disease progression, including the amyloid cascade, excitotoxicity, oxidative stress, and inflammatory processes [4–7]. Nevertheless, a growing body of evidence suggests a critical role for Aβ peptide metabolism and oxidative stress in the pathophysiology of AD [8,9]. The relationship between Aβ and free radical production is a positive feedback loop: not only can oxidative processes convert nonaggregated Aβ into aggregated Aβ in vitro, Aβ itself is also a source of free radicals [10]. In fact, several studies have shown that Aβ stimulates reactive oxygen species (ROS) production in microglia and triggers microglial release of NO_2 radicals in rodents [11].

It has been suggested that several individual antioxidants and free radical scavengers, such as ascorbate, vitamin E, and protein sulfhydryls, or a combination of these, can be neuroprotective and decrease the risk of AD or slow its progression [12,13]. One of the endogenous free radical scavenger systems in the brain is associated with glutathione (GSH), which is the most abundant intracellular nonprotein thiol and is able to detoxify various oxidants by direct scavenging of free

Abbreviations: acyl-SG, *S*-acyl glutathione; AD, Alzheimer disease; APP, amyloid precursor protein; Aβ, amyloid-β peptide; BODIPY, 4,4-difluoro-3*a*,4*a*-diaza-*s*-indacene; ChAT, choline acetyltransferase; CM-H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; DHA-SG, docosahexaenoic-SG; DMSO, dimethyl sulfoxide; FAD, familial Alzheimer disease; GFAP, glial fibrillary acidic protein; GSH, glutathione; IBA-1, ionized calcium-binding adaptor molecule 1, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MUFA, monounsaturated fatty acid; NBM, nucleus basalis magnocellularis; NGS, normal goat serum; PS-1, presenilin-1; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RT, room temperature; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

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radicals or by acting as a cosubstrate in GSH–peroxidase-catalyzed reactions [14]. Because GSH metabolism is altered in affected brain regions of AD patients and its levels decreased in experimental models of AD [15–17], it is generally accepted that restoring GSH levels may help to manage AD [18]. The impact of GSH deficiency in numerous pathologies has prompted several researchers to investigate new alternative strategies for maintaining or restoring GSH levels in these patients [19]. Currently, the use of GSH as a therapeutic agent is limited by its unfavorable biochemical and pharmacokinetic properties. Indeed, it has a short half-life in human plasma and does not easily cross cell membranes or the blood–brain barrier, so administration of high doses is necessary for it to be of therapeutic value [19,20]. In an effort to improve GSH bioavailability, by dietary or pharmacological intake, the prodrug approach seems to be the most promising, and some GSH precursors, prodrugs, carriers, analogs, and mimetics have already been developed [21]. Anyway, drugs currently used in the treatment of cognitive impairment and dementia have very limited therapeutic value.

Recent studies demonstrated that elevated intake of unsaturated fatty acid (UFA)—monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids— and high levels of antioxidant compounds and very low saturated fatty acid (SFA) intake could act synergistically in improving cognitive performance and possibly in preventing or delaying the onset of dementia [22]. Moreover, the fatty acid composition of neuronal membranes showed an increase in MUFA content and a decrease in PUFA content with advancing age, suggesting an increased demand for UFAs during aging [23]. The protective effects of dietary UFAs could be related to the role of fatty acids in maintaining the fluidity of synaptosomal membranes and the activity of certain membrane-bound enzymes and receptors, thereby regulating neuronal transmission [22].

Considering the importance of developing new antioxidant compounds and their relevance in the treatment of oxidative stress-related disorders, in this paper we report an efficient and straightforward method for the synthesis of a series of novel *S*-acyl glutathione (acyl-SG) thioesters. In particular, these acyl-SG thioesters can cross plasma membranes and result in the intracellular release of the reduced form of GSH and free fatty acid, thus synergistically triggering a significant decrease in intracellular lipid peroxidation and mitochondrial dysfunction in primary fibroblasts from familial AD (FAD) patients and human SH-SY5Y neuroblastoma cells experiencing oxidative injury. These novel acyl-SG derivatives also protect cholinergic neurons against Aβ-induced damage and reduce glial reaction in rat brains. Based on our results, acyl-SG thioesters appear to be novel prodrugs that could be useful in the treatment of AD and other oxidative stress-related disorders.

Materials and methods

General procedure for the synthesis of S-acyl glutathiones **3a–3e**

A solution of 1-acyl-1*H*-benzotriazoles **2a–2e** (1 eq) dissolved in acetone or MeOH (see the supplementary materials and methods for further details) was added to a solution of GSH (1 eq) in H_2O and the reaction mixture was stirred at room temperature (RT) (Scheme 1). After 10 min, an aqueous saturated $NaHCO_3$ solution (2 eq) was added dropwise to the mixture at a rate of 0.2 ml/min. The reaction progress was monitored by thin-layer chromatography (disappearance of **2a-e,** $R_f = 0.89–0.73$ with petroleum ether:ethyl acetate 1:1 as eluent; appearance of benzotriazole, $R_f = 0.38$), which indicated the completion of the reaction within 1 h. The solvent was evaporated under reduced pressure and 85% H_3PO_4 or CH_3COOH was added until the mixture reached pH <4. The precipitate formed was collected on a Buchner funnel and the filter cake washed several times with H_2O and ethyl acetate to afford the desired *S*-acyl glutathiones **3a–3e** in 27–86% yield as white or light yellow solids.

As a control, the *N*-linoleoyl derivative of ophthalmic acid (H-γ-Glu-Abu-Gly-OH), a tripeptide without redox capacity, was also synthesized using a literature procedure on 1-linoleoyl-1*H*-benzotriazole, **2c**.

Cell cultures and rat model

Primary FAD fibroblasts were obtained from punch biopsies of the upper arm, taken from Italian Alzheimer patients bearing amyloid precursor protein (APP) Val717Ile or PS-1 Leu392Val mutations, as previously reported [24]. Patient clinical assessment was carried out according to published guidelines, and the AD diagnosis fulfilled the *Diagnostic and Statistical Manual of Mental Disorders* criteria [25,26]. The local ethics committee approved the protocol of study, and written consent was obtained from all patients. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and a solution of 1% penicillin and streptomycin and subjected to a number of passages ranging from 15 to 20. Human SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM F-12 Ham containing 25 mM Hepes and $NaHCO_3$ (1:1), and supplemented with 10% FBS, 1.0 mM L-glutamine, and antibiotics in a 5.0% CO_2-humidified atmosphere at 37 °C. Cells were grown until they reached 80% confluence and used for a maximum of 20 passages.

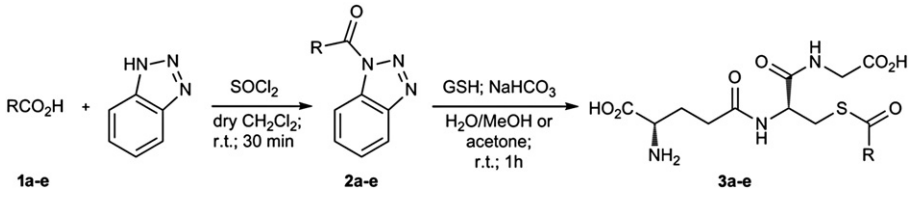
Three-month-old (220–250 g) male Wistar rats (Harlan Nossan, Correzzana, Italy) were housed in Macrolon cages until surgery and maintained on a 12-h light/dark cycle at 23 °C. All animal manipulations were performed in vivo, according to the European Community guidelines for animal care (DL 116/92).

Aβ oligomer preparation and treatment

Prefibrillar aggregates of the Aβ42 peptide were obtained by dissolving aliquots of Aβ42 in DMSO to a concentration of 5 mM, incubated in ice-cold F-12 medium to a concentration of 100 μM for 24 h at 4 °C, and then centrifuged at 14,000 *g* for 10 min, according to Lambert's protocol [27]. The supernatant, defined as the amyloid β-derived diffusible ligand (ADDL) preparation, comprises a fibril-free solution of oligomers (ADDLs), as routinely assessed by tapping mode atomic force microscopy. When oligomers were added to cell culture medium at their final concentration, no disassembly or microscopic differences in aggregate structure occurred [28].

Determination of intracellular GSH uptake

Primary FAD fibroblasts and human SH-SY5Y neuroblastoma cells were incubated with 5.0 μM acyl-SG thioesters or increasing



Scheme 1. Synthesis of acyl-SG thioesters **3a–e.** The two-step sequence utilized for chemical synthesis of *S*-acyl glutathiones is shown.