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Memantine prodrug as a new agent for Alzheimer's Disease

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Hydrogen sulphide has recently drawn much attention due to its potent anti-inflammatory and neuroprotective roles in brain functions. The purpose of the current study was to exploit these beneficial properties of H₂S to design a new agent for the treatment of Alzheimer's disease (AD). To pursue our aims, we replaced the free amine group of memantine with an isothiocyanate functionality as a putative H₂S-donor moiety. The new chemical entity, named memit, was then tested *in vitro* to determine whether it retains the pharmacological profile of the "native drug", while also providing a source of H₂S in the CNS. Indeed, Memit showed the ability to release H₂S through a cysteine-mediated mechanism, thus generating memantine. Moreover, the new hybrid molecule exerts protective effects against neuronal inflammation and induces a drastic fall in ROS production. In addition, memit was also able to reduce the Aβ(1-42) self-induced aggregation and exerted cytoprotective effect against Aβ oligomers-induced damage in both human neurons and rat microglia cells. Finally, similarly to memantine, the new compound promotes autophagy, a complex process required for cellular homeostasis in cell survival that results to be altered in neurodegenerative diseases. In conclusion, our study revealed that memit is a prodrug of memantine. Further *in vivo* studies will be necessary to fully investigate the synergic or cumulative effects due to the H₂S-releasing moiety and the native drug.

Alzheimer's disease (AD) is a progressive age-dependent neurodegenerative brain disorder that slowly destroys memory and thinking skills. It is the cause of 60% to 70% of cases of dementia and represent one of the main public health issue with a significant impact on the whole society.

Over the past two decades, it has become increasingly apparent that AD neuropathology is characterized by a dramatic reduction of H₂S production in the CNS¹. H₂S is a gaseous transmitter that functions as a smooth muscle relaxant and neuromodulator. It is produced endogenously from the amino acids L-cysteine and homocysteine (HCy) by several enzymes such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT).

CBS, highly expressed in the hippocampus and cerebellum, is the main enzyme responsible for the production of H₂S in the brain². Following a neuronal excitation, CBS produces H₂S which enhances the NMDA receptor-mediated responses thus affecting memory and facilitating the induction of long-term potentiation (LTP)³. Recently, a dramatic decrease of CBS activity and consequently a drastic fall in H₂S levels (about 50%) have been detected in the brain of patients affected by AD, thus suggesting that a reduced production of H₂S may be involved in the cognitive decline associated to AD⁴.

Nowadays, H₂S is also considered a neuromodulator^{2,5} since it interacts with N-methyl-D-aspartate (NMDA), a class of ionotropic glutamate receptors which play critical roles in synaptic plasticity. Even though the mechanism is not yet completely understood, H₂S seems to interact with NMDA receptor both directly, through the sulfhydration of cysteine residues⁶, and indirectly, by regulating the level of intracellular Ca²⁺⁷.

Several *in vitro* and *in vivo* experiments have shown the neuroprotective role of H₂S in AD⁸⁻¹¹. Growing evidences indicate that NaHS, an exogenous H₂S-generating agent, induces neuroprotection against oxidative stress (OS) through at least three main mechanisms: (a) restoration of GSH (glutathione) cellular levels¹²; (b) activation of ATP sensitive potassium channels (K_{ATP}); (c) reduction of mitochondrial ROS production^{13,14}. Recently, it has been also demonstrated that H₂S has protective effects against Aβ-induced cell injury by inhibiting inflammation, promoting cell growth, and preserving mitochondrial function^{2,15}.

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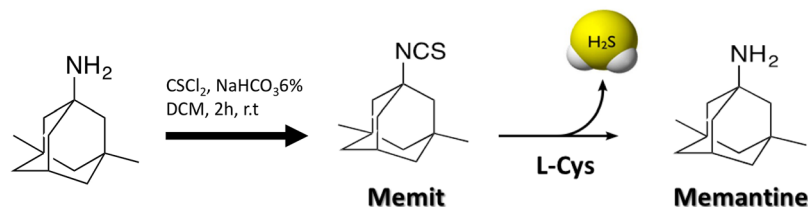


Figure 1. Structures of Memantine and the relative H_2S -donor hybrid memit.

In addition, H_2S exhibits anti-inflammatory and antiapoptotic activities. Indeed, administration of NaHS in a rat model of AD proved to dramatically reduce the release of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin IL-1 β and IL-6 induced by amyloid β -peptides. Moreover, it inhibits the upregulation of COX2 enzyme and the activation of the nuclear factor- κB (NF- κB) in the hippocampus¹⁶, thus reiterating the high potential value of H_2S in AD therapy. Further H_2S neuroprotective mechanisms have been suggested: such as the reduction of lipopolysaccharide (LPS)-induced NO production in microglia via inhibition of p38-MAPK pathway¹⁷.

Taken together all these findings corroborate the functional involvement of H_2S in neurodegenerative diseases¹⁸. Therefore, the restoration of the correct levels of endogenous H_2S is an appealing novel approach for the development of new pharmacological therapies for AD, that are still lacking of an effective treatment. In this paper we decide to investigate the possibility to exploit the H_2S properties to design a new agent for the treatment of AD, following the multitarget directed ligand strategy (MTDL). This approach is currently used for the design of new molecules to be used in several multifactorial pathologies such as cancer¹⁹, cardiovascular²⁰ and neurodegenerative diseases, including AD^{21,22}.

Even though the combination of two or more pharmacophores into a single molecule has been extensively studied, the introduction of an H_2S -donor moiety into the molecular scaffold of a “native” neuroprotective drug has not been explored yet^{9,23–25}. Consistently, to pursue our aim we selected memantine as “native drug” to combine appropriately with a H_2S -generating moiety. Memantine is an excellent example of neuroprotective drug. It is a well-known uncompetitive voltage-dependent NMDA receptor antagonist, targeting principally NMDAR pathological hyperfunction²⁶, and approved in 2003 by FDA as a treatment option for moderate to severe AD.

Starting from memantine, we replaced the free amine group with an isothiocyanate functionality as a putative H_2S -donor moiety (Fig. 1). Indeed, recent studies showed that both synthetic and naturally occurring isothiocyanates generate H_2S with a slow releasing rate^{27,28}. Increasing studies have shown that H_2S -generating agents for clinical use should retain some requirements such as a slow releasing rate, low and long-lasting concentration and favorable pharmacokinetics^{29,30}. All these features are essential to take full advantage of the beneficial effects of H_2S , thus avoiding the onset of toxicity.

Accordingly, a slow-releasing H_2S donor memantine-like molecule, namely Memit, was developed and its neuroprotective properties were investigated in human neuronal-like cells.

Methods

Chemical synthesis. Commercial grade anhydrous solvents were used without further drying. Commercially available chemicals were purchased from Sigma Aldrich and used without further purification. Evaporation was performed in vacuum (rotating evaporator). Anhydrous Na_2SO_4 was used as the drying agent. Flash chromatography was performed on Merck 60 Å high-purity grade silica gel (0.40–63 μm). Reaction was followed by TLC, performed on Merck aluminum silica gel (60 F254) sheets. Spots were viewed under a UV lamp (254 nm). ^1H and ^{13}C NMR spectra were obtained using a Bruker Avance 400 spectrometer and were recorded at 400, and 101 MHz, respectively. Chemical shifts are reported in parts per million (ppm) δ values, coupling constants J are reported in hertz (Hz). Data for ^1H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, integration, coupling constant (Hz). Multiplicities are reported as follows: s = singlet, m = multiplet. Synthesis of memit was reported in Fig. 1. Briefly, to a stirred solution of Memantine (300 mg, mmol 1.67) in DCM (15 ml) and NaHCO_3 6% (15 ml) cooled at 0°C , was added dropwise thiophosgene (1.28 ml, 16.7 mmol). The resulting mixture was stirred at 2 h at r.t., followed by TLC, then the aqueous phase was separated and extracted several times with DCM , dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified through flash chromatography (AcOEt/n -hexane 9:1 as the eluent) to get Memit as a clear oil³¹. ^1H NMR (CDCl_3 -400 MHz): δ 0.84 (s, 6H), 1.12 (s, 2H), 1.24–1.34 (s, 4H), 1.47–1.69 (m, 4H), 1.77–1.78 (m, 2H), 2.11–2.16 (m, 1H) ppm. ^{13}C NMR (CDCl_3 -400 MHz): 129.96 (NCS); 59.71; 49.84; 49.59; 42.24; 42.03; 41.85; 32.78; 32.55; 28.86; 28.75; 29.64.

Cell cultures differentiation into neurons. H9-derived Neural Stem Cells (NSCs) were cultured as reported before^{32,33}. Neurobasal serum-free medium with $5\ \mu\text{M}$ retinoic acid was used to differentiate cells into neurons^{32,33}.

Cellular model of inflammation and viability/proliferation assays in neuronal-like cells. The compounds Memit and Memantine were diluted in DMSO. Neuronal-like cells, differentiated from H9-derived were incubated for 16 h with an inflammatory cocktail of LPS and TNF- α (50 ng/ml), as previously reported³². To verify the protective effects of Memit and Memantine, neuronal-like cells were treated with the two compounds for 24 or 72 h, then the samples were washed and incubated with LPS plus TNF- α for 16 h³² or oligomeric $\text{A}\beta_{1-42}$

for 24 h. In selected experiments, NaHS was used as a positive control. Following incubation, cell viability was determined using the MTS assay³².

Cell proliferation/viability assays on rat microglia cells. Rat microglia cells were isolated from mixed cell culture obtained from Sprague-Dawley rat cortex, by gentle physical shaking, as described^{34,35}. After isolation, cells were seeded into 96 well-plated with fresh culture media and pretreated with Memit or Memantine (10 μ M) for 24 h; before washing and incubation with oligomeric A β _{1–42} for 24 h. Following incubation time, cell viability was determined using the MTS assay³².

ROS Production. ROS activity within the cells was determined using the fluorogenic dye 2',7'-dichlorofluorescein diacetate (H2DCFDA, Molecular Probes, Invitrogen). NSCs were seeded in black 96-multiwell plates (5 \times 10³ cells/well). The cells were treated with Memit or Memantine at the concentration of 10 μ M for 24 h; after the incubation time, cells were washed and incubated with LPS and TNF- α for 16 h. One hour prior to treatment completion, 50 μ M H2DCFDA was added to the same media in the dark at 37 °C. As a positive control, H₂O₂ was added at a concentration of 100 μ M. The fluorescence intensity (excitation 485 nm and emission 520 nm) was normalized based on the number of cells stained with crystal violet³⁶. In selected experiments, NaHS was used as a positive control.

Glioma cell culture and viability experiments. U-87MG cell line was obtained from the National Institute for Cancer Research of Genoa (Italy) and cultured as previously described³⁷. The effects of compound treatment on U87MG cell viability were evaluated using the tetrazolium dye (MTT) colorimetric assay. Briefly, Memantine (10 μ M) or Memit (10 μ M) was added to 96-well plates containing 5,000 U-87MG cells/well. After incubation for 24 h, cell growth was measured by MTT (3-(4,5-dimethylthiazolyl-2)-2-diphenyltetrazoliumbromide) proliferation assay. Absorbance at 570 nm was measured using a Bio-Rad model 550 microplate reader (BioRad Molecular Bioscience Group, Hercules, USA). The data represent the means of three independent experiments.

Western blot analysis. U-87MG cells were seeded in six-well plates at a density of 1 \times 10⁶/well and grown to 70–80% of confluence with standard medium (DMEM-High Glucose). Cells were treated with vehicle (0.1% DMSO) or test compounds (i.e. 10 μ M Memantine or Memit, and 1 μ M Rapamycin) and incubated at 37 °C for 4 and 24 h. After washing with ice-cold PBS cells were lysed and proteins (30–40 μ g) were separated on Criterion TGXTM gel (4–20%) and transferred to Immuno-PVDF membrane (Bio-Rad, Milan, Italy). The membranes were probed overnight at 4 °C with primary antibodies [1:1000, LC3A/B; p62; mTOR, p-mTOR; Akt, p-Akt(Ser 473); β -actin, Cell Signaling]. The primary antibodies were detected using appropriate secondary antibody. The peroxidase was detected using a chemiluminescent substrate (ECL, Perkin Elmer). Immunoreactive bands were quantified performing a densitometric analysis with Image Lab Software (Bio-Rad, Milan, Italy) and normalized to β -actin.

[³H]MK-801 Binding Assay. Crude synaptic membranes were prepared from the cerebral cortex of Sprague-Dawley rats³⁸. Animals used procedures complied with NIH publication n° 80–23 revised in 1996 and approved by the Animals Care Committee of the University of Pisa in compliance with the Directive 2010/63/EU on animal welfare. The pellets were stored at –80 °C for at least 24 h, and washed three more times with Tris-HEPES buffer (Tris 4.5 mM, HEPES 5 mM, pH 7.4) to remove the endogenous amino acids before the binding assay³⁸.

In the binding assay, 50 μ L of membrane preparation (40–50 μ g protein), and 10 μ L of compound were mixed at 25 °C in the presence of 50 μ M L-glutamate (10 μ M) and 50 μ L of glycine (10 μ M). Then, 50 μ L of [³H]MK-801 (final concentration 3 nM) were added to the preparation. Tris-HEPES buffer was added to a final volume of 0.5 mL³⁸. Following incubation time of 2 h at 25 °C, binding was terminated by rapid filtration. Radioactivity was measured using a PerkinElmer liquid scintillation counter. Nonspecific binding was determined in the presence of unlabeled 100 μ M MK-801. The dissociation constant (Kd) of [³H]MK-801 in rat cortex membranes was 4.0 nM. For compound activity determination, aliquots of membrane pellets were incubated with different ligand concentrations of Memit or Memantine (10 nM–10 μ M) in the absence or presence of 4 mM Cysteine for 30 min, and then incubated with 3 nM [³H]MK-801 for 2 h at 25 °C. Samples were then filtered, and the radioactivity was counted.

Thioflavin T fluorescence assay. The ThT stock solution was prepared by adding 8 mg ThT to 10 mL phosphate buffer (10 mM phosphate, 150 mM NaCl, pH 7.0) and filtered through a 0.2 μ m syringe filter. This stock solution should be stored in the dark and is stable for about one week. On the day of the analysis, the stock solution was diluted into PBS to obtain the concentration of 10 μ M. The A β _{1–42} oligomers were prepared by diluting the stock solution in PBS and the dilution was shaken for 48 hours at 37 °C. The cells were seeded in black 96-multiwell plate (3000 c/w) and treated with the compounds Memit or Memantine (10 μ M) for 24 h. Following incubation, the samples were washed and incubated with A β _{1–42} oligomers for 24 h. After this time, 200 μ L of ThT 10 μ M were added to each well in the dark. The ThT fluorescence intensity of each sample was recorded every 5 min using a spectrophotometer by excitation at 355 nm and emission 535 nm.

Determination of H₂S release by amperometric assay. The characterization of the potential H₂S-generating properties of Memit has been carried out by an amperometric approach, through the Apollo-4000 free radical analyzer (WPI) detector and H₂S-selective mini-electrodes. 6 different recordings have been carried out at RT. Following the manufacturer's instructions, a "PBS buffer 10x" was prepared (NaH₂PO₄·H₂O 1.28 g, Na₂HPO₄·12H₂O 5.97 g, NaCl 43.88 g in 500 ml H₂O) and stocked at 4 °C. Immediately before the experiments,

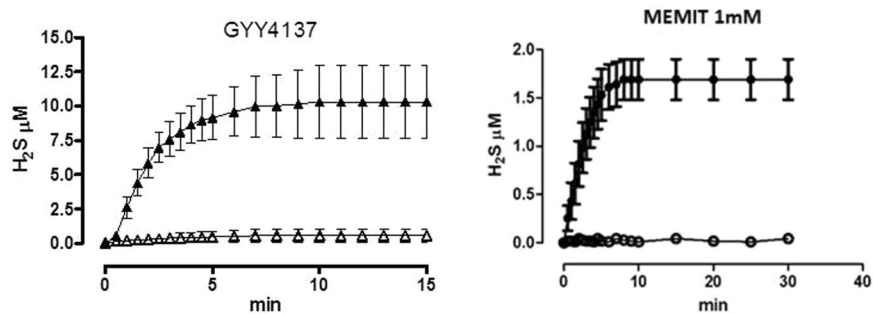


Figure 2. Curves describe the increase of H₂S concentration, with respect to time, following the incubation of GYY4137 (1 mM) and the test compound Memit (1 mM) in the assay buffer, in the absence (white symbol) or in the presence of L-cysteine (black symbol). The vertical bars indicate the SEM.

the “PBS buffer 10x” was diluted using distilled water (1:10) to obtain the assay buffer and the pH adjusted to 7.4. The H₂S-selective mini-electrode was equilibrated in 10 ml of the assay buffer, until the recovery of a stable baseline. Then, 100 µL of a DMSO solution of the tested H₂S-releasing compounds (Memit and positive drug GYY4137) was added, at the final concentration of 1 mM; the final concentration of DMSO in the assay buffer was 1%. The generation of H₂S was observed for 20 min. Preliminary experiments demonstrated that DMSO 1% did not produce any interference on the amperometric recording. When required by the experimental protocol, L-Cysteine (final concentration 4 mM) was added 10 min before the addition of tested compounds. L-Cysteine alone did not produce any amperometric response. The correct relationship between the amperometric currents (recorded in pA) and the corresponding concentrations of H₂S was previously determined by suitable calibration curves, which were obtained by the use of the H₂S donor NaHS (1–3–5–10 µM) at pH 4.0. The curves relative to the progressive increase of H₂S vs time, following the incubation of the tested compounds, were analysed following the same procedure previously described²⁹.

Statistical analysis. The data were analysed and elaborated by Graph-Pad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). All data are presented as the means ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls multiple comparison post hoc test or by Bonferroni’s corrected t-test for post-hoc pair-wise comparisons.

Results

Design and Synthesis. Memit was developed as a novel NMDAR antagonist analogue of memantine provided of H₂S-releasing properties. This new compound was efficiently synthesized by replacing the amine functionality of memantine with an isothiocyanate group through reaction with thiophosgene in heterogeneous phase (NaHCO₃ 6%: DCM 1:1).

Evaluation of Memit H₂S-releasing properties via Amperometric assay. As previously described²⁹, a real-time determination of the H₂S release was achieved by using a H₂S-selective minielectrode incubating reference H₂S donor (GY4137) or Memit in the presence or in absence of L-Cysteine. As shown in Fig. 2, in the absence of L-Cys the H₂S generation from both compounds was almost negligible, whereas after pre-incubation with L-Cys a time-dependent increasing generation of H₂S was observed.

Memit induces neuroprotection in human neuronal cell. Neuronal-like cells, obtained from H9-derived human neural stem cells (NSCs), were incubated with an inflammatory cocktail (LPS plus TNF-α) in order to mimic *in vitro* neuroinflammation³⁹. The effects of Memit (10 µM) and native drug memantine (10 µM) under physiological conditions and under inflammatory stress exposure were investigated by cellular viability experiments (Fig. 3A). The compounds did not alter cellular proliferation “per se” (data not shown). As expected, the inflammatory mixture significantly affected the proliferation of neuronal-like cells. Pre-treatment for 24 or 72 h of neuronal-like cells with the selected compounds significantly counteracted the decrease in cell proliferation elicited by the inflammatory insult. These results suggest that the new compound exerts neuro-protective effects in an experimental model of inflammation.

Memit reduces ROS production. To investigate the scavenger properties of memit, ROS levels were measured in neuronal-like cells under inflammatory conditions. Figure 3B shows that challenging neuronal-like cells with LPS-TNF-α significantly enhanced ROS accumulation. Pre-treatment with compound memit or with the native drug for 24 h almost completely counteracted inflammation-mediated ROS accumulation, thus suggesting that both compounds are able to prevent ROS accumulation.

Memit inhibits Aβ(1-40) self-induced aggregation. Accumulation of extracellular deposits of β-amyloid and abundant neurofibrillary tangles in the brain are correlated with neuron loss and progressive dementia¹⁵. Therefore, in order to evaluate the influence of memit on amyloid fibrils, a Th-T fluorescence assay was performed using NaHS as positive control^{15,16,40}. Memit and Memantine were tested at 10 µM concentration; the results are reported in Fig. 4. As expected, Aβ1-42 significantly enhanced ThT-related fluorescence (165.5 ± 26.5% versus control). All the tested compounds exerted a significant inhibition of ThT-related

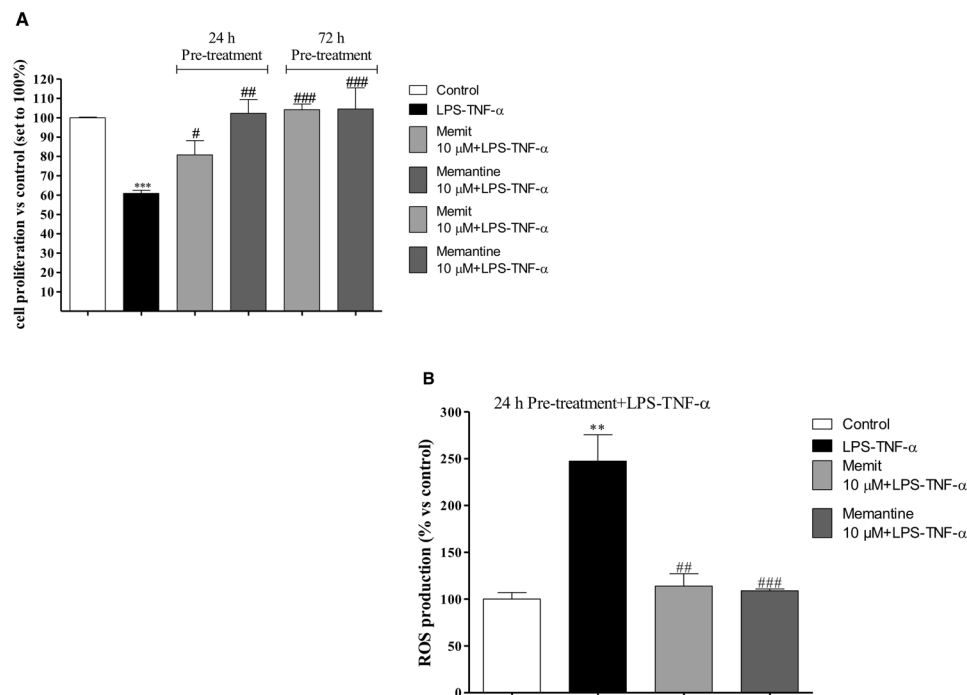


Figure 3. (A) Effect of Memit and memantine treatments on an experimental model of neuroinflammation. The cells were challenged with Memit or memantine (10 μ M) for 24 h or 72 h, and then treated LPS plus TNF- α for an additional 16 h. Cell proliferation was measured by MTS assay. The data are expressed as percentages relative to untreated cells (control), which were set at 100% (mean \pm SEM, N = 3). (B) The cells were treated as in A. After treatment, ROS levels were quantified as described in the experimental section. The data are expressed as percentages relative to untreated cells (control), which were set at 100% (mean \pm SEM, N = 3). * p < 0.01, ** p < 0.001 vs control; # p < 0.05, ## p < 0.01, ### p < 0.001 vs cells treated with LPS/TNF- α .

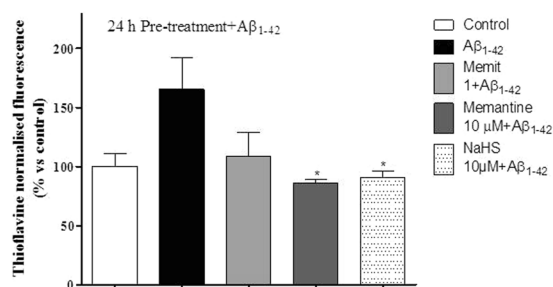


Figure 4. Effect of the anti-amyloidogenic compounds, Memit and memantine, on amyloid oligomers formation. The cells were pre-treated with Memit, Memantine or NaHS (10 μ M) for 24 h. Following incubation time, cells were washed and incubated with A β ₁₋₄₂ oligomers for 24 h. Following incubation, 200 μ l of ThT 10 μ M were added to each well in the dark. The ThT fluorescence intensity of each sample was recorded every 5 min using a spectrophotometer by excitation at 355 nm and emission 535 nm. Values represent mean \pm SEM; * p < 0.05 vs A β ₁₋₄₂.

fluorescence, thus indicating inhibition of A β ₁₋₄₂ self-aggregation. In particular Memit induced an A β ₁₋₄₂ self-aggregation inhibition comparable to its progenitor Memantine.

Memit induces autophagy in U-87MG cell line. Autophagy (ATG) is a complex and finely regulated pathway, essential for the correct cellular physiology as it is involved in the degradation of long-lived cytoplasmic proteins, protein complexes, or damaged organelles. Impairments of the autophagic process are associated with several neurodegenerative disorders⁴¹, it is thus possible that the induction of ATG may be exploited as a strategy to assist neuronal survival. The mammalian target of Rapamycin (m-TOR) is an evolutionarily conserved serine-threonine kinase that is known to play a major role in the modulation of ATG^{42,43}. Therefore, to explore the neuroprotective potential of Memit and Memantine we decided to evaluate their ability to induce ATG in U-87MG cells, a human glioblastoma cell line characterized by a weak ATG machinery due to the upregulation of m-TOR⁴⁴⁻⁴⁶. Expression of protein indicators of autophagy, such as LC3-II⁴⁷, p62⁴⁸ and m-TOR, was detected

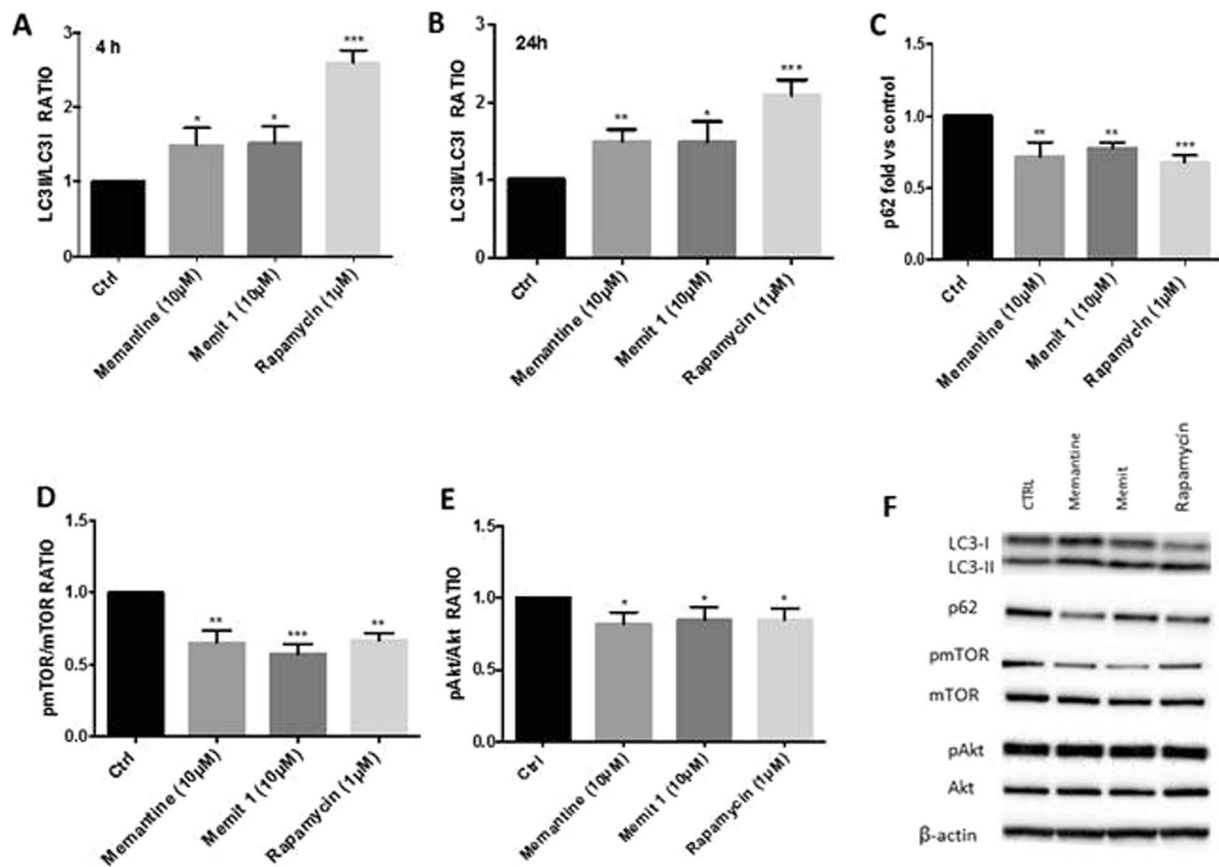


Figure 5. Western blot quantification of LC3II/LC3I ratio, p62, mTOR and Akt as hallmarks of the degree of ATG activation in U87MG cells. Memantine (10 mM), Memit (10 mM) and Rapamycin (1 mM) induced increased LC3II/LC3I ratio at 4h (A) and 24h (B); a significant p62 degradation (C) and decreased p-mTOR/mTOR ratio (D) and pAkt/Akt ratio (E). Results represent mean \pm SEM of three different gels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle treated cells (Control). Representative western blots are shown in Panel F.

by western blotting, using Rapamycin as a positive control. As shown in Fig. 5 a significant up-regulation of LC3II expression was observed after 4h treatment with 10 μ M Memit or Memantine (Fig. 5A), and also remained almost unchanged until 24h treatment (Fig. 5B), suggesting that both Memit and Memantine are able to stimulate the ATG flux. Consistently, after 4h treatment parallel decreased expressions of p62, which is degraded during autophagy, and p-mTOR were observed (Fig. 5C,D). Next, we decided to address whether the observed induction of autophagy in U-87MG cells caused by treatment with 10 μ M Memit or Memantine was accomplished via inhibition of the survival PI3K/Akt/mTOR pathway⁴⁹. To this end, we analyzed the phosphorylation status of Akt in U-87MG cells treated with test compounds. As shown in Fig. 5E, 4h treatment with Memit and Memantine (10 μ M) resulted also in a significant reduction of the phosphorylation levels of Akt-Ser 473 (* $P < 0.05$, ** $P < 0.01$, versus vehicle treated cells), suggesting that, similarly to Rapamycin, both compounds may induce autophagy through the inhibition of the mTOR phosphorylation by the PI3K/AKT/mTOR pathway (full length Western blots for Fig. 5F are shown in Supplementary Fig. S1). In addition, cellular viability was determined using the MTT colorimetric assay. No significant alterations of cell viability were observed in U-87MG cells treated for 24h with (10 μ M) test compounds, as compared to vehicle (0.1% DMSO) (Fig. 6).

Evaluation of Memit binding activity on NMDAR. Memit was tested to assess its ability to inhibit native-target activity (i.e. NMDAR). Binding to NMDAR was assessed using a radioligand assay on cortex membranes based on the displacement of [³H]MK-801³⁸, in the presence or absence of Cys (Table 1). Interestingly, while Memantine showed a significant displacement of [³H]MK-801 both in presence or in absence of Cys ($K_i = 954.8 \pm 74.2$ nM and 328.8 ± 10.9 nM respectively), our H₂S donor compound revealed a significant activity only in the presence of an organic source of H₂S (Fig. 7 and Table 1). Importantly, the K_i value for memit (458.4 ± 77.7 nM) was comparable to that obtained for Memantine. These data demonstrate that memit could be considered a pro-drug of Memantine, released in the presence of Cys, and that the chemical modification did not alter the compound ability to bind NMDA.

Of note, Memit itself increased significantly its affinity for NMDA receptor in the presence of Cys (Fig. 7, Table 1), suggesting that Cys is involved in the regulation of the receptor affinity/functionality. Consistent with our hypothesis, crucial cysteine residues have been suggested to be involved in the modulation of NMDA protein

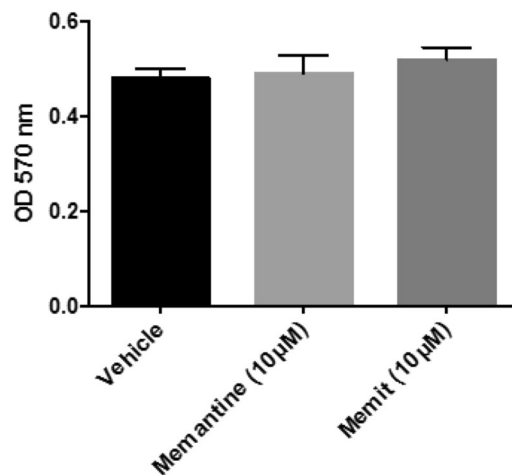


Figure 6. Cell viability assay. MTT assay showing U-87MG cells viability after 24 h treatment with tested compounds. Results are expressed as the mean \pm SEM of twelve biological replicates.

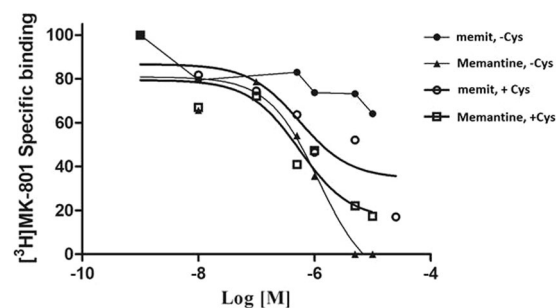


Figure 7. Displacement of specific $[^3\text{H}]$ MK-801 binding in rat cortex membranes in the absence or presence of cysteine (Cys). Rat cortex membranes were incubated with the indicated compounds and $[^3\text{H}]$ MK-801, in the absence or presence of Cys, Data are reported as percentage of radioligand specific binding and are the means \pm S.E.M. of three different experiments (performed in duplicate).

Compound	K _i , nM (-Cys) ^a	K _i , nM (+Cys 4 mM, 30 min) ^a
Memit	28.0% ^b	458.4 \pm 77.7
Memantine	954.8 \pm 74.2	328.8 \pm 10.9

Table 1. Displacement of specific $[^3\text{H}]$ MK-801 binding in rat cortex membranes in the absence or presence of cysteine (Cys). Data are reported as the means \pm S.E.M. of three different experiments (performed in duplicate). ^aThe K_i values are means \pm SEM derived from an iterative curve-fitting procedure (Prism program, GraphPad, San Diego, CA). ^bPercentage of inhibition is reported for Memit in the absence of Cys.

activity and signaling events via other reactions of thiol groups⁵⁰. In particular, the presence of cysteine in the binding buffer could involve reactions that affect ligand binding allosterically, as can be also observed by the K_i values of reference drug in the presence and in absence of Cys⁵⁰.

Memit protects neuronal-like cells and microglia from A β (1-42) induced injury. Several studies have proved that H₂S and NMDA ligands have protective effects on A β -induced cellular injury^{15,16}. On this basis, the effect of Memit on injured neurons was assessed. As expected, oligomeric A β caused a significant reduction of neuronal viability (Fig. 8, panel A). When cells were pre-challenged with Memit, a marked cyto-protective effect was observed (Fig. 8, panel A). Similar results were obtained probing cells with Memantine (Fig. 8, panel A).

Microglia, together with astrocytes, forms the main active immune defense of the CNS. During the inflammation process, induction of NF- κ B occurs, accompanied by a release of inflammatory mediators such as TNF- α , IL-6 and nitrite ions. Notably, also levels of CBS and H₂S result down-regulated^{151,52}. Consistently, to further determine the neuroprotection elicited by our hybrid, we decided to evaluate effects of Memit and Memantine on rat microglia cells. Cells were pretreated with the two compounds (10 μ M concentration) followed by incubating A β ₁₋₄₂ oligomers. As depicted in Fig. 8 (panel B), pre-treatment with Memit and Memantine completely restored

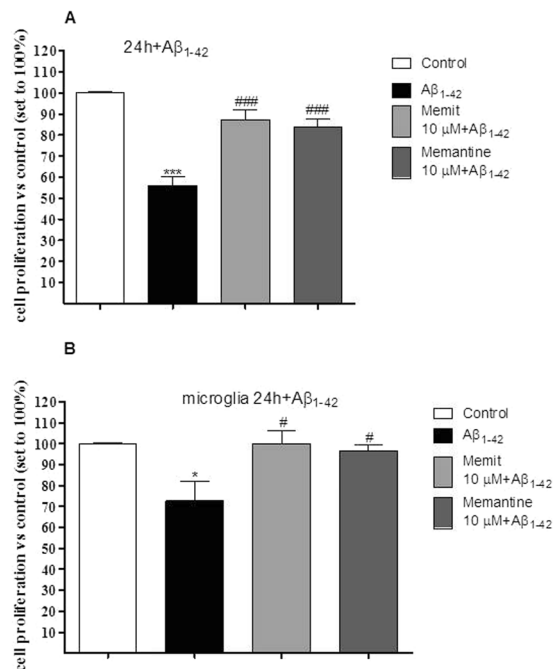


Figure 8. Neuronal-like cells (panel A) or rat microglia cells (panel B) were pre-treated with Memit or Memantine (10 μ M) for 24 h. After washing, the cells were challenged with A β ₁₋₄₂ for 24 h. At the end of treatments, cell proliferation was measured by MTS assay. The data are expressed as percentages relative to untreated cells (control), which were set at 100% (mean \pm SEM, N=3) * p <0.05 vs control; # p <0.05 vs cells treated with A β ₁₋₄₂.

cell proliferation. These data demonstrate that the compounds can protect both neuronal cells and microglia from A β -induced injury.

Discussion

The goal of the present work was to develop a new and improved molecule for the treatment of neurodegeneration by modifying the structure of a well-established drug that is already approved for therapeutic use. Thus, starting from memantine, a compound currently prescribed for the treatment of moderate-to-severe Alzheimer's disease, we created a new chemical entity, named memit, by introducing an isothiocyanate moiety capable of releasing H₂S into its scaffold.

Although the pharmacological profile of memantine has been extensively studied (moderate affinity, uncompetitive, voltage-dependent, NMDA receptor antagonist that exhibits fast on/off kinetics⁵³), the conjunction with an H₂S-donor moiety creates a new hybrid molecule whose properties need to be fully investigated. Putatively such a molecule should exert neuroprotective and anti-inflammatory effects through both hydrogen sulphide- and NMDA-mediated mechanisms. This could result in a delaying of the neurodegeneration process.

Therefore, our initial studies were directed to assess whether memit still retains the pharmacological profile of the "native drug", while also being a source for H₂S in the CNS. To evaluate its H₂S donor properties we used an amperometric approach²⁹, which provides a real-time determination of H₂S-release, either in the absence or in the presence of an organic thiol, such as L-cysteine. The results of the amperometric analysis on memit showed a prolonged and persisting L-cysteine mediated release, similar to that exhibited by the reference slow H₂S-releasing agent GYY4137, a phosphinodithioate derivative.

Next, we investigated the neuroprotective activity of memit in human neuronal-like cells and in rat microglia cells, with the former representing the main active immune defense of the CNS. In neuronal cells, the neuroprotection was evaluated following an LPS-TNF α insult, known to induce oxidative stress and consequently apoptosis and cell death. The new drug demonstrated to protect cells from both oxidative stress and cytotoxicity induced by LPS-TNF α . The observed effects can be attributed to both NMDA binding and H₂S release. Indeed, hydrogen sulfide has been proven to attenuate neural injury and cytokine release in rats⁵⁴ and memantine has been shown to mitigate colchicine-induced inflammation in the hippocampus⁵⁵.

In addition, Memit was able to reduce the A β (1-42) self-induced aggregation, with effects comparable to those of its progenitor memantine. Accordingly, memit and the native drug were also able to reduce A β oligomers-induced damage in rat microglia cells. Moreover, the observed reduction in A β accumulation elicited by Memit was shown to protect neuronal cells from the peptide-induced toxicity.

Consistent with our data, an over activation of NMDA receptor has been linked to abnormal A β deposition in AD and memantine has been demonstrated to decrease A β secretion in human cells or transgenic mice^{56,57}.

Interestingly, H₂S has been also proven to inhibit protein fibrillation of A β peptides by forming trisulfide bridges⁵⁸ and therefore it may contribute to Memit-elicited properties.

The observed cytoprotection from A β -induced toxicity was then tested in microglia cells. Recent evidences have highlighted the pivotal role of these cells in different neuropathologies, including AD⁵⁹. Notably, pharmacological studies suggest that promoting microglia polarization plays a key role in maintaining tissue homeostasis through different mechanisms including the suppression of inflammation⁵⁹. In our hand, both memit and the native drug were also able to reduce A β oligomers-induced damage in rat microglia cells, suggesting the two drugs as promising protective agents for non-neuronal cells too.

Moreover, since a large body of evidence underlies the existence of a correlation between neurodegeneration and altered autophagic flux⁶⁰, we also decided to investigate the ability of memit and memantine to induce autophagy in U87-MG cells. Our western blot analysis revealed that both compounds were able to significantly affect the expression of common autophagy markers such as LC3II, p62, mTOR and Akt, suggesting that autophagy could be an additional mechanism to strengthen their efficacy as neuroprotective drugs. These data are of particular relevance considering the role of AKT/mTOR pathway in the modulation of autophagy and the clearance of protein oligomers in neurodegenerative diseases⁴⁹.

Finally, we also tested the ability of memit to bind to the NMDA receptor, the main target of memantine. Interestingly, we found that memit displayed an affinity comparable to that of the native drug only when cysteine was present in the assay medium, while no binding was observed in the absence of cysteine. These observations suggest that memit behaves as a prodrug, and that the binding is due to the production of memantine. In this sense, it has to be mentioned the reported facilitating effects of hydrogen-sulfide on NMDA receptors^{61,62}. Nevertheless, the functional consequences of H₂S-mediated NMDA receptor activation in neurons and stem cells is not strictly linked to neurotoxicity. For example, these facilitating actions are age- and receptor subunit-specific⁶¹ and can finally promote synaptic plasticity. Furthermore, H₂S has been demonstrated to enhance NMDA receptor-mediated currents and facilitate the induction of hippocampal long-term potentiation (LTP)⁶³. Finally, activation of NMDA receptors increases proliferation and differentiation of hippocampal neural progenitor cells⁶⁴ and thus does not show toxic effects on differentiated cells.

Further studies will be needed to elucidate changes in NMDA receptor functionality that can be mediated by Memit-released hydrogen sulfide.

Conclusion

Unfortunately, to date the etiopathology of neurodegeneration is still not well understood and the therapies that are currently available lack in efficacy, as they are unable to completely block neuronal damage. Consequently, there is an enormous need for new therapeutic tools that are at least capable of slowing down the progression of the pathology. The work we presented here describes the synthesis and the preliminary *in vitro* evaluation of an innovative multi-functional prodrug. To the best of our knowledge, memit is the first compound capable of producing H₂S in the CNS that was designed starting from the well-known drug memantine. The new compound we have developed has the potential of restoring H₂S levels in the CNS and, like memantine, has the ability of inducing autophagy, thus eliciting neuroprotective effects. However, further *in vivo* investigations will be necessary to fully appreciate the synergistic or cumulative effects due to the H₂S-releasing moiety and the native drug.

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Author Contributions

S.S. carried out the synthesis. S.D., D.P., L.B., V. Citi carried out the experiments and analyzed the data. S.R. design and coordinate the project. V.C. and C.M. revised the manuscript. All authors discussed the results and contributed to the final manuscript. S.S., S.R. and G.C. wrote the manuscript.

Additional Information

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