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A Chemical Chaperone-Based Drug Candidate is Effective in a Mouse Model of Amyotrophic Lateral Sclerosis (ALS)

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective death of motor neurons and skeletal muscle atrophy. The majority of ALS cases are acquired spontaneously, with inherited disease accounting for only 10% of all cases. Recent studies provide compelling evidence that aggregates of misfolded proteins underlie both types of ALS. Small molecules such as artificial chaperones can prevent or even reverse the aggregation of proteins associated with various human diseases. However, their very high active concentration (micromolar range) severely limits their utility as drugs. We synthesized several ester and amide derivatives of chemical chaperones. The lead compound **14**, 3-((5-((4,6-dimethylpyridin-2-yl)methoxy)-5-oxopentanoyl)oxy)-*N*,*N*-dimethylpropan-1-amine oxide shows, in the micromolar concentration range, both neuronal and astrocyte protective effects in vitro; at daily doses of 10 mg kg^{-1} **14** improved the neurological functions and delayed body weight loss in ALS mice. Members of this new chemical chaperone derivative class are strong candidates for the development of new drugs for ALS patients.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in humans, affecting people at any time during adulthood, although the average age of onset begins in the mid-fifties.[1] Patients usually die within 3–5 years of symptoms onset, usually due to respiratory failure.[1] The only available FDA-approved drug, riluzole, offers only a marginal delay in disease progression.[1] About 90% of ALS patients do not have any genetic abnormalities (sporadic form of the disease, sALS), supporting the idea that perhaps ALS is a multifactorial disease with both environmental and genetic components. Fewer than 10% of ALS cases have a familial history (familial ALS, fALS) and are linked to certain mutations in various genes.[2] One of the most common mutations found in fALS is a mutation in the gene for superoxide dismutase 1 (SOD1).[2] More than 120 mutations in *SOD1* are linked to the disease.[2] Interestingly, *SOD1* encodes an enzyme that plays an essential role in human physiology as a scavenger of superoxide radicals. However, not all of the ALS-linked SOD1 mutants are physiologically inactive.[3] Moreover, *SOD1* knockout mice do not develop ALS, while the expression of mutant SOD1 causes the disease.[3] The large number of ALS-causing *SOD1* mutations suggests that almost any alteration in the structure of SOD1 will be cytotoxic, leading specifically to ALS and not to other diseases. All other fALS-related mutations are not ALS specific. Mutations in those genes are associated with other neurological disorders as well, in particular with frontotemporal dementia.[4] *SOD1* is the only gene, that when mutated in almost all possible positions, leads to ALS. The disease pathology and symptoms in SOD1-linked fALS patients closely resemble those of sALS patients. In light of the linkage of the *SOD1* gene to fALS, it was hypothesized that there may be a common molecular basis for both forms of the disease.[5] There are many reports that oxidized/misfolded wild-type SOD1 (^{WT}SOD1) induces the selective death of motor neurons.[6] Accordingly, several studies have investigated the possible role of structural changes in mutated SOD1 that cause toxic effects in motor neurons.[7] Indeed, several common features of nearly all SOD1 mutants associated with ALS have been found, in particular,

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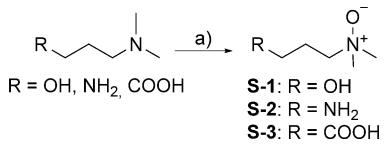
the increased formation of aggregates.[8] It has also been shown that different factors, among them oxidative stress, lead to alterations in appropriate folding of SOD1 in motor neurons following the loss of Cu^{2+} and Zn^{2+} ions, accelerating aggregation of the protein.[9] Indirect evidence supports the idea that toxic forms of oxidized/misfolded ^{WT}SOD1 are secreted from glia cells into the extracellular space, leading to motor neuron death.[10]

Artificial chaperones including polyols, trimethylamino *N*-oxide (TMAO), tauroursodeoxycholic acid, taurine, 4-phenylbutyric acid, and various amino acid derivatives have been shown to reverse the mislocalization and aggregation of proteins associated with different human diseases.[11] The mechanisms by which chemical chaperones function are not fully understood. The major limitation in using chemical chaperones as drugs is their very high active concentration, in the millimolar range. For example, the active concentrations of TMAO needed for chaperone-like effects in cell cultures is in the range of 1.0 mM.[12] Tauroursodeoxycholic acid shows an effect on aggregation of misfolded proteins at a concentration range of 0.2–1 mM in vitro and at a more acceptable dose in vivo, ~500 mg per day p.o. (the compound is used therapeutically for the treatment of cholestatic liver diseases).[13] 4-Phenylbutyric acid is active in vitro at concentrations of 0.5–5 mM and in vivo at daily doses of 0.5–20 g.[14] In the search for small molecules that can prevent the formation of toxic aggregates at concentrations lower than those required by available compounds, we decided to prepare more lipophilic, yet still water-soluble derivatives of known chemical chaperones. In this way we planned to decrease their active concentrations and allow them to serve as ALS drug candidates.

Results and Discussion

TMAO and 4-methoxyphenylbutyric acid have poor membrane permeability.[15,16] Because good blood–brain barrier (BBB) permeation is mandatory for any compound in the treatment of ALS, the conjugation of these chemical chaperones to membrane-permeating moieties such as aromatic amines (methylpyridines and imidazole) was attempted.[15–17] We used a methylated derivative of 4-phenylbutyric acid (a known chemical chaperone) to avoid the effect of the negatively charged phenol moiety on membrane penetration, hypothesizing that within the cell, the methyl ether will be transformed back to the phenolic form by the well-known metabolic oxidative O-dealkylation reaction.[18–20] Amide and ester bonds were chosen for the formation of linkers between chemical chaperones and aromatic amine moieties.

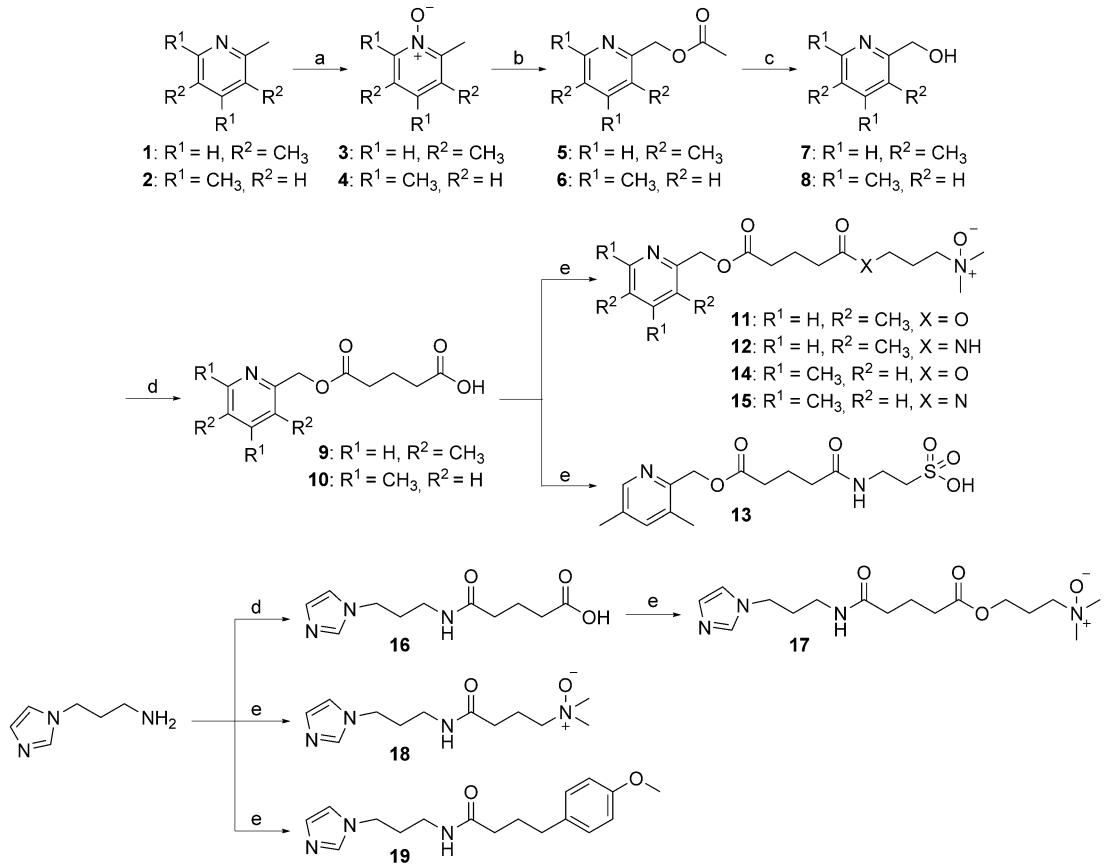
Precursor compounds **S-1**–**S-3** were synthesized as outlined in Scheme 1. The tertiary amines in three starting molecules were oxidized with a 3% hydrogen peroxide solution. Altogether, eight different derivatives of TMAO, taurine, and 4-phenylbutyric acid were synthesized as shown in Scheme 2. Commercially available 2,3,5-trimethylpyridine (**1**) and 2,4,6-trimethylpyridine (**2**) were oxidized by hydrogen peroxide into the corresponding *N*-oxides **3** and **4** followed by conversion of the methyl group at the second position into acetyl esters **5** and **6**,



Scheme 1. *Reagents and conditions*: a) 3% aq. H_2O_2, 72 h, RT.

using the Boekelheide rearrangement. The ester was hydrolyzed in the presence of sodium methoxide in methanol to obtain the corresponding primary alcohols **7** and **8**. Glutaric anhydride was used to form the cleavable linker between the lipophilic pyridine-based moiety and the chemical chaperones. After esterification of **7** and **8**, the free carboxylic acid was introduced to obtain **9** and **10**. Compound **9** was conjugated in the presence of the coupling reagent PyBOP to the chemical chaperone, 3-hydroxy-*N*,*N*-dimethylpropan-1-amine oxide (Scheme 2) to obtain the ester derivative **11**. The identical reaction between **9** and 3-amino-*N*,*N*-dimethylpropan-1-amine oxide resulted in the amide derivative **12**. Finally, **9** was conjugated in a similar way with taurine to obtain the corresponding amide **13**. Subsequently, **10** was converted using the same synthetic approach into the ester derivative of 3-hydroxy-*N*,*N*-dimethylproban-1-amine oxide (**14**) or into the amide derivative of 3-amino-*N*,*N*-dimethylproban-1-amine oxide (**15**). In addition, three imidazole-based compounds of TMAO and 4-phenylbutyric acid (methyl ether) were synthetized. Briefly, commercially available 1-(3-aminopropyl)imidazole was conjugated with glutaric anhydride, leading to the formation of an ester derivative of 3-hydroxy-*N*,*N*-dimethylpropan-1-amine oxide to obtain **17**. The 1-(3-aminopropyl)imidazole was also directly conjugated with carboxylic acid derivative of TMAO: 3-carboxy-*N*,*N*-dimethylproban-1-amine oxide to obtain the corresponding amide **18**. The latter compound was also synthesized via a direct conjugation between the 1-(3-aminopropyl)imidazole and 4-(4-methoxyphenyl)butyric acid. The synthesis of the corresponding amide **19** was successfully accomplished in a similar manner.

The resulting compounds were screened in the ALS in vitro model, ^{hSOD1G93A}NSC-34 motor neuron-like cells.[21] This model was established based on experimental data obtained from human SOD1/G93A transgenic mice that become paralyzed due to motor neuron loss and die at the age of 5–6 months.[22] ALS-related pathophysiological properties including increased reactive oxygen species formation, abnormal mitochondrial membrane potential, increased vulnerability to toxins/stress conditions, and even the formation of insoluble protein aggregates that contain mutated SOD1 were detected in these cells, making them a suitable model for in vitro ALS studies.[23] The potential cytoprotective effect of the tested compounds on the viability of these cells following 24 h pre-incubation with the ER stress inducer tunicamycin was studied. Figure 1A shows that only compound **14** was able to prevent the cytotoxic effect of tunicamycin in both ^{hWTSOD1}NSC-34 and ^{hSOD1G93A}NSC-34 motor neuron-like cells. Importantly, compound **14** did not demonstrate a significant protective cellular effect



Scheme 2. *Reagents and conditions*: a) 3% aq. H_2O_2, AcOH, 6 h, 80 °C; b) acetic anhydride, 4 h, reflux; c) MeONa, MeOH, 4 h, RT; d) glutaric anhydride, dry THF, 30 min, RT; e) 3-hydroxy-*N*,*N*-dimethylpropan-1-amine oxide (**S-1**)/3-amino-*N*,*N*-dimethylpropan-1-amine oxide (**S-2**)/taurine/3-carboxy-*N*,*N*-dimethylpropan-1-amine oxide (**S-3**)/4-(4-methoxyphenyl)butyric acid, PyBOP, Et_3N, DMF, 10 h, RT.

in rat L6 myotubes (pretreated with tunicamycin) which do not express human SOD1 (data not shown).

We note that even small structural changes may affect the biochemical parameters (e.g., cell permeation, intracellular disposition, stability, binding to misfolded proteins) of the synthesized compounds. For example, the ester bond derivative **14** was found to be active, whereas compound **15**, in which this bond is replaced by the more stable amide bond, was found to be inactive. Compound **14** was found to be stable under physiological conditions (37 °C, pH 7.4) for 24 h (Supporting Information Figure S1) Furthermore, its active concentration was found to be 20-fold lower (50 μM versus 1 mM) than that of the chaperone alone (TMAO). Taken together, these data support the hypothesis that the cytoprotective effect was caused by compound **14** and not by its potential hydrolysis product (TMAO), which was demonstrated to be cytoprotective by itself.[12]

Based on these results, only compound **14** was further investigated. The dose–response and time course effect of this compound were tested in ^{hSOD1G93A}NSC-34 motor neuron-like cells as shown respectively in Figure 1 B,C. At concentrations below 25 μM, **14** did not show any significant effect on cell viability. The maximal effect was obtained at a concentration of 150 μM. An interesting result obtained from this experiment was that only a relatively long incubation (24–48 h) with **14** had a cytoprotective effect. Both shorter (5–12 h) and longer incubation periods (72 h) were not beneficial for cell viability. These data suggest that intracellular accumulation of **14** is needed for maximizing its biological effect, but degradation probably occurs during long incubations of the compound, thus lowering its activity.

We did not succeed in isolating sufficient amounts of the motor neurons from the ^{hSOD1G93A}transgenic mice to test the effect of **14** in this primary cell culture. Thus, the neuroprotective effect of **14** was validated in primary astrocytes, which were isolated from spinal cords of the ^{hSOD1G93A}transgenic mice as described in the Experimental Section below. It has been shown that primary astrocytes can be used as a model system to investigate ALS molecular mechanisms and to evaluate potential therapies for ALS.[24]

Similar to the activity in ^{hSOD1G93A}NSC-34 motor neuron-like cells, compound **14** protected ^{hSOD1G93A}primary mouse astrocytes from ER stress which was induced by tunicamycin when the cells were incubated with the compound for 48 h (Figure 1 D). In contrast to what was observed in the ^{hSOD1G93A}NSC-34 motor neuron cell model, the compound was unable to demonstrate a cytoprotective effect after 24 h incubation. This result suggests that the cytoprotective intracellular concentra-

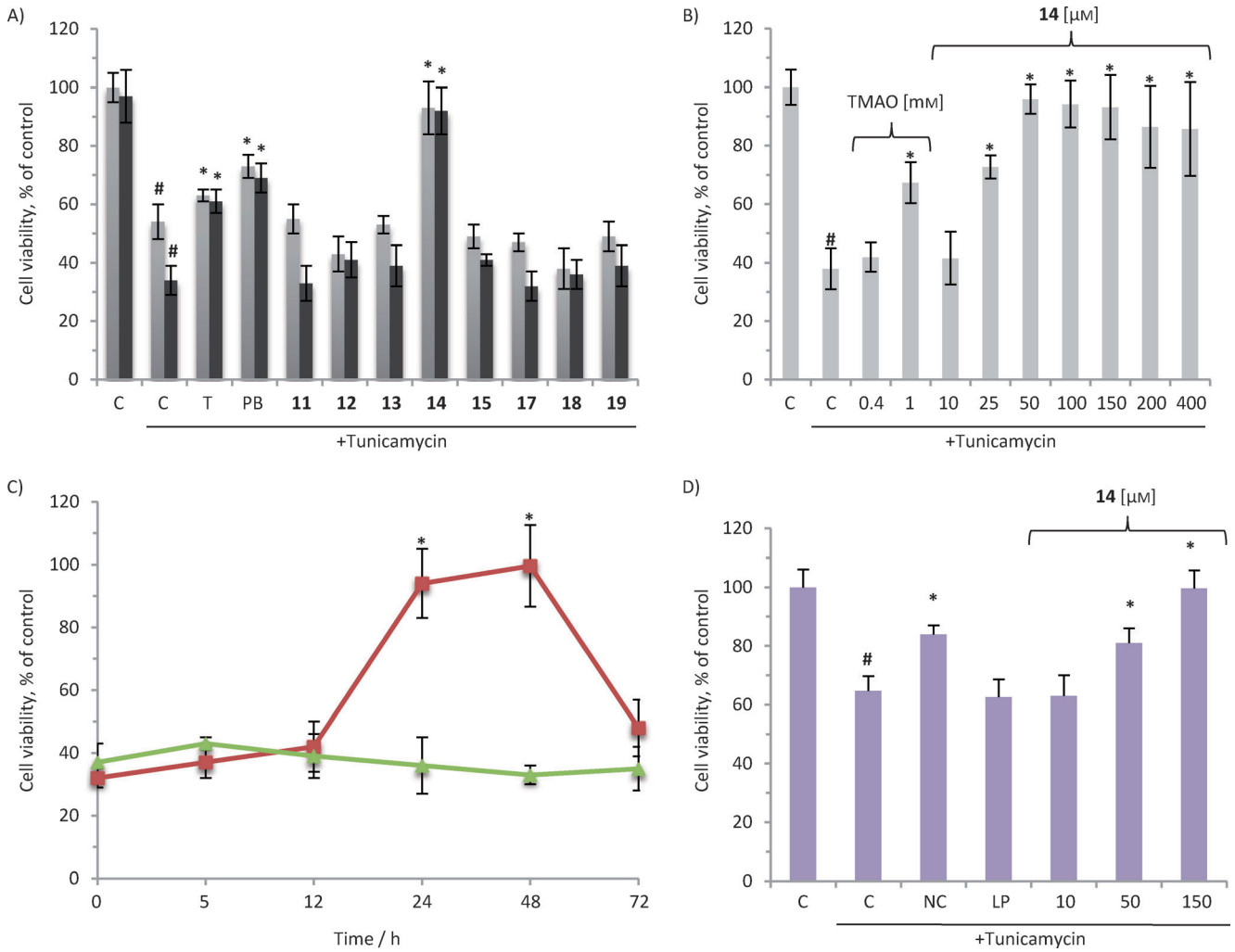


Figure 1. In vitro biological evaluation of compound **14**. A) Preliminary screening of synthesized compounds. ^{hWTSOD1} and ^{hSOD1G93A}NSC-34 motor neuron-like cells (grey and black columns, respectively) were grown as described in the Experimental Section. Cells were initially exposed to 0.75 μM tunicamycin for 12 h and then exposed to trimethylamino *N*-oxide (TMAO) for 24 h (T, 1 mM), *p*-phenylbutyric acid (PB, 500 μM) or to 50 μM of the test compound for 24 h. Following exposure, a standard MTT analysis was conducted. B) Dose–response analysis of the effect of **14**. ^{hSOD1G93A}NSC-34 motor neuron-like cells were grown and treated with tunicamycin as described for panel A. Cells were exposed to increasing concentrations of **14** and TMAO as indicated. After 24 h, MTT analysis was conducted as described in the Experimental Section. C) Time course analysis of the effect of **14**. Cells were treated as described in panel A. Compound **14** (50 μM) was added to cultures at indicated time points (■). MTT analysis was conducted as described in the Experimental Section for each indicated time for the treated and non-treated cells (▲). D) Dose–response effect of **14** in primary ^{hSOD1G93A}mice astrocytes. The primary cultures of mice astrocytes were obtained as described in the Experimental Section. The cultures were pre-incubated with naked chaperone (3-hydroxy-*N*,*N*-dimethylproban-1-amine oxide, 200 μM, NC), **10** (linker with dimethylpyridine moiety, 200 μM, LP) and increasing concentrations of **14** for 48 h. Afterward, the cells were exposed to tunicamycin (1 μM) for an additional 24 h. MTT analysis was conducted as described in the Experimental Section. Significant difference between untreated control cells and tunicamycin-treated cells is indicated by #, and between tunicamycin-treated control and cells exposed to test compound by *; $p \leq 0.05$ mean ± SE ($n = 6$).

tion of **14** is not identical in different cell types or that the rate of accumulation is cell dependent.

Precipitation of aggregated protein into inclusion bodies is a common cytopathological hallmark of the majority of neurodegenerative diseases.[25] Mutated SOD1 can aggregate into high-molecular-weight insoluble protein complexes.[5] Such SDS-resistant species are detectable in western blots of SOD1 in lysates obtained from transfected cells, spinal cord extracts from transgenic mice expressing mutant SOD1, and in autopsy samples of ALS patients. We therefore studied the effect of **14** on the formation of SDS-resistant high-molecular-weight SOD1 (the aggregate-like species) in ^{hSOD1G93A}NSC-34 motor neuron-like cells under ER stress conditions. Figure 2 A,B show that **14** dose-dependently (50–150 μM) reduces the appearance of misfolded mutated SDS-resistant high-molecular-weight SOD1 relative to control cells, most likely due to its chaperoning activity. TMAO also reduced the level of misfolded SOD1, but at a much higher concentration (1 mM).

In addition, the effect of **14** on the level of two well-known ER stress markers—binding immunoglobulin protein (BiP) and C/EBP homologous protein (CHOP)[26]—was investigated as shown in Figure 2 A,B. Misfolded and aggregated proteins cause ER stress, which lead to increased intracellular levels of both proteins. The levels of both markers were significantly

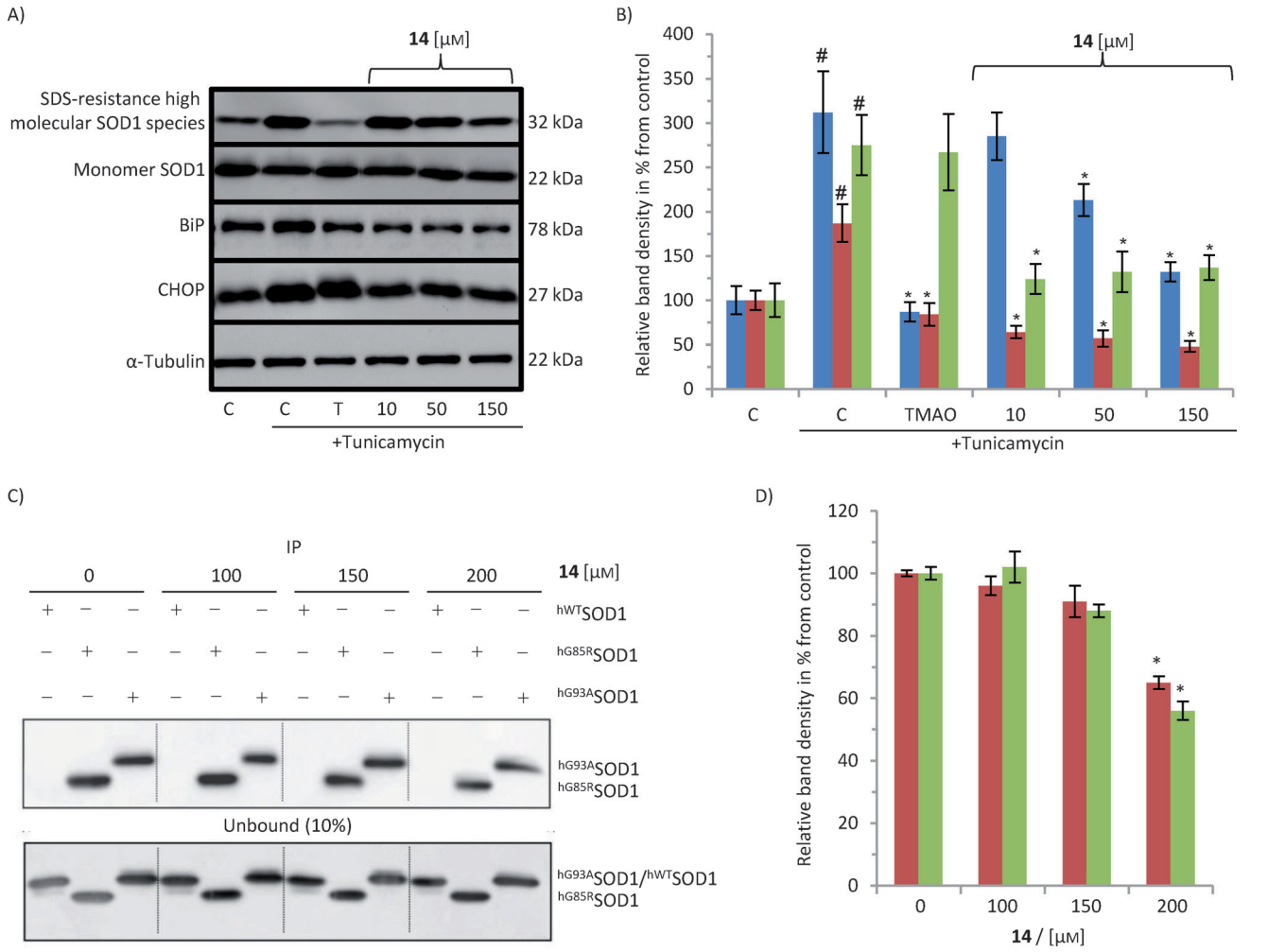


Figure 2. In vitro biological evaluation of compound **14**. A) Western blot analysis. ^{hSOD1G93A}NSC-34 motor neuron-like cells were treated as described in Figure 1 A. Subsequently, cell lysates were produced as described in the Supporting Information. Standard SDS-PAGE (10%) was conducted. Western blots were obtained by using commercially available antibodies as described in the Supporting Information. Experiments were run $n = 4$ times, and representative gels are shown. B) Western blot bands density calculations. The density of the signal of SDS-resistance high-molecular-weight SOD1 species (■) was normalized according to the amount of normal size monomer SOD1 band. The signal of BiP (■) and CHOP (■) were normalized according to the density of α-tubulin; $^*p \leq 0.05$, mean ± SE ($n = 4$). C) Effect of compound **14** on the level of misfolded mutant SOD1. Recombinant proteins were incubated for 20 min with increasing concentrations of compound **14**. After incubation, the immunoprecipitation (IP) procedure was performed, and it was followed by PAGE–western blot analysis as described in the Experimental Section. D) IP band density calculations. The densities of the signals of ^{hG93A}SOD1 (■) and ^{hG85R}SOD1 (■) were normalized according to the density of the unbound SOD1 band. The control (not treated by **14**) band density was taken as 100% for each SOD1 mutant. The "naked" chaperone, trimethylamino *N*-oxide (TMAO), 3-hydroxy-*N*,*N*-dimethylproban-1-amine oxide, and **10** (linker with dimethylpyridine moiety), were not active at 200 μM (data not shown). $^*p \leq 0.05$, mean ± SE ($n = 3$).

lower in cells treated with **14** at 10 μM than in untreated cells. Increasing concentrations of **14** did not significantly decrease any of the tested ER stress markers. Importantly, the cell-protective and anti-aggregation effects of the compound likely follow different mechanisms of action, and higher concentrations of **14** are probably needed for neuroprotection (see above). Interestingly, TMAO treatment, which was used as a positive control, decreased the level of BiP and failed to decrease the amount of CHOP.

Furthermore, the ability of compound **14** to directly reduce the amount of misfolded human recombinant mutated SOD1 was investigated. Two mutated human SOD1 recombinant proteins were chosen for this analysis: G93A and G85R. The G85R mutation in SOD1 is associated with a rapid progression of ALS in humans which is accompanied by the accumulation of the protein in astrocytes and motor neurons.[27] Using an immunoprecipitation assay with an antibody that specifically recognizes misfolded SOD1 only, compound **14** (at 200 μM) was found to decrease the levels of both SOD1 mutants by 35–40% (Figure 2C,D).

The potential anti-aggregation effect of compound **14** was further tested in transfected $^{hWTSOD1\text{-}EGFP}$ and $^{hSOD1A4V\text{-}EGFP}$HEK293T cells. This mutation is common in many fALS patients and is associated with a clinically severe disease manifestation.[28] As shown in Figure 3, **14** (200 μM) decreased the expression levels and aggregation of ^{hA4V}SOD1 as well as ^{hWT}SOD1 in these cells. The reduced expression level of ^{hA4V}SOD1-EGFP as well as ^{hWT}SOD1-EGFP suggests enhanced degradation rates for both

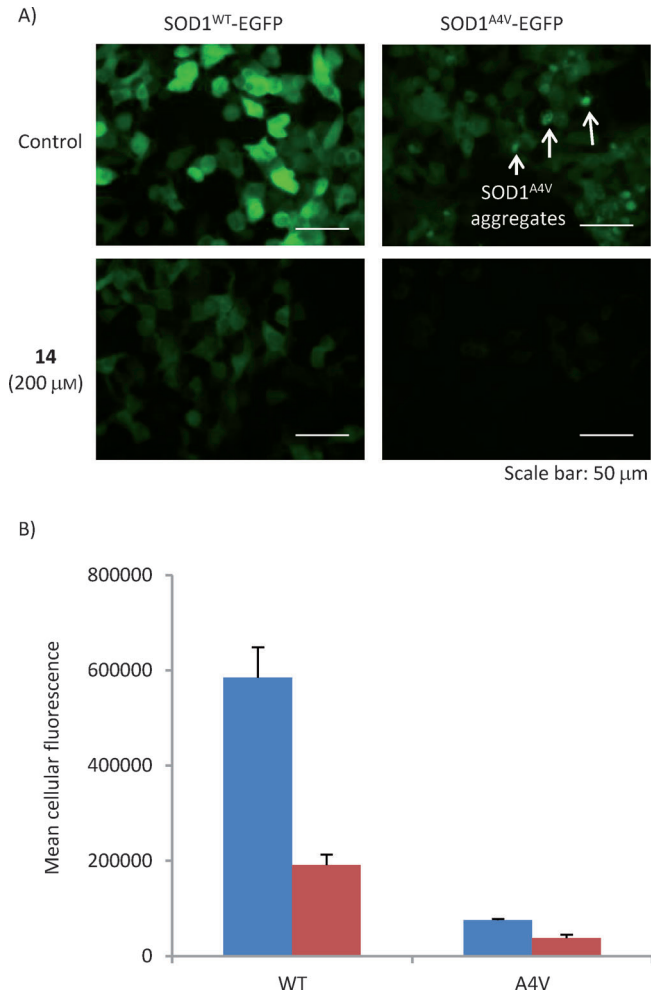


Figure 3. Anti-aggregation effect of **14** in transfected ^{hWT}SOD1-EGFP and ^{hA4V}SOD1-EGFP HEK293T cells. A) The fluorescence microscopic images of the transfected HEK293T cells expressing EGFP fusion of SOD1 variants. The images of transfected HEK293T cells expressing EGFP fusion of ^{hWT}SOD1 and ^{hA4V}SOD1 in the absence or the presence of 200 μM of **14** were taken at two days post-transfection (white arrows: intracellular SOD1 mutant aggregates; scale bar: 50 μm). B) The calculated data of the cellular fluorescence. The mean cellular fluorescence of the transfected HEK293T cells expressing EGFP fusion of ^{hWT}SOD1 and ^{hA4V}SOD1 in the absence (■) or presence (■) of **14**. Values represent the mean cellular fluorescence with error bars denoting standard deviations ($n=3$). To determine whether the addition of **14** affects the mean cellular fluorescence, two-sided Student's *t*-tests were applied to the data (*$p<0.05$). The "naked" chaperone, trimethylamino *N*-oxide (TMAO), and lipophilic domain with the linker (**10**) did not significantly affect the cellular fluorescence.

SOD1 variants. Because the addition of **14** did not significantly decrease the HEK293T cell growth rate, inhibition of protein synthesis may not be the cause of the reduced expression level. Therefore, the apparent reduction of intracellular aggregates of ^{hA4V}SOD1 in the presence of **14** is likely due to the promoted degradation of the entire human SOD1 species.[29] To further probe this point, we tested the ability of compound **14** to activate the proteasome. Given the large number of misfolded/aggregated proteins implicated in ALS, the activation of one of the most important disposal mechanisms of the cell (even in a nonspecific manner) is a viable approach to combating the disease. In line with this expectation, a small-molecule proteasome activator was recently reported by Trippier et al., 5-(((2,4-dichloro-6-methylphenyl)thio)methyl)-1,2-dihydro-3*H*-pyrazol-3-one, which demonstrated biological activity in ALS mice.[30] Using a commercially available proteasome activity assay kit, we performed the experiment and determined that compound **14** is not a proteasome activator even at supra-pharmacological concentrations (Supporting Information Figure S2).

Despite its relatively high active concentration in vitro, compound **14** was tested in vivo. For the in vivo evaluation we used ^{hSOD1G93A}transgenic mice, which are commonly used as an ALS model.[31] This model of ALS mice is also widely used for ALS drug screening.[31] Compound **14** or a saline control were injected daily (10 mg kg^{-1} i.p., soluble in saline) from day 40 (from birth) to day 125 of the experiment. Female and male mice were treated separately. As shown in Figure 4 A,B, chronic treatment with **14** slowed the progression of body weight loss in ^{hSOD1G93A}transgenic mice. The loss of skeletal muscle mass (resulting in total body weight loss) is one of the hallmarks of the disease.[1] Thus, the ability of the compound to slow this process may translate into a significant effect in ALS patients. Treatment with **14** also delayed the onset and slowed the progression of the loss of neurological functions (extension reflex of both hind paws) in both groups of ALS mice, starting from day 90 of treatment (Figure 4 C,D). The effect was especially dramatic in females. From day 120, the control group showed a significant paralysis of the lower legs relative to almost normal lower leg movement ability in treated female mice (see the movie file in the Supporting Information). Following withdrawal of the treatment on day 130 (due to ethical considerations for prevention of misery to the animals), no statistically significant differences in life span were observed in the treated mice, and both groups died by around the 155th day (Figure 5). Indeed, in many studies using this specific model, the effect of the studied compounds was evident in the motor symptoms and was not reflected in the general survival rate.[31]

Many of the symptoms arising during the course of the disease are untreatable so far, and all efforts should be made to improve the quality of life, helping to maintain the patient's autonomy for as long as possible.[32] Thus, the results of the present study support the use of **14** as a novel drug candidate for the delay of symptom progression, prevention of deterioration of motor functions, and the improvement in the quality of life of ALS patients, even though the compound was not shown to prolong survival rate. Moreover, the mechanism of action of compound **14** might also be useful in the development of universal drugs for the treatment of disorders that are related to general protein misfolding and aggregation.

Conclusions

Herein we report a novel compound, 3-((5-((4,6-dimethylpyridin-2-yl)methoxy)-5-oxopentanoyl)oxy)-*N*,*N*-dimethylpropan-1-amine oxide (**14**), which demonstrated biological effect in ALS mice. The molecule was designed as a lipophilic derivative of a known chemical chaperone (TMAO). The compound showed

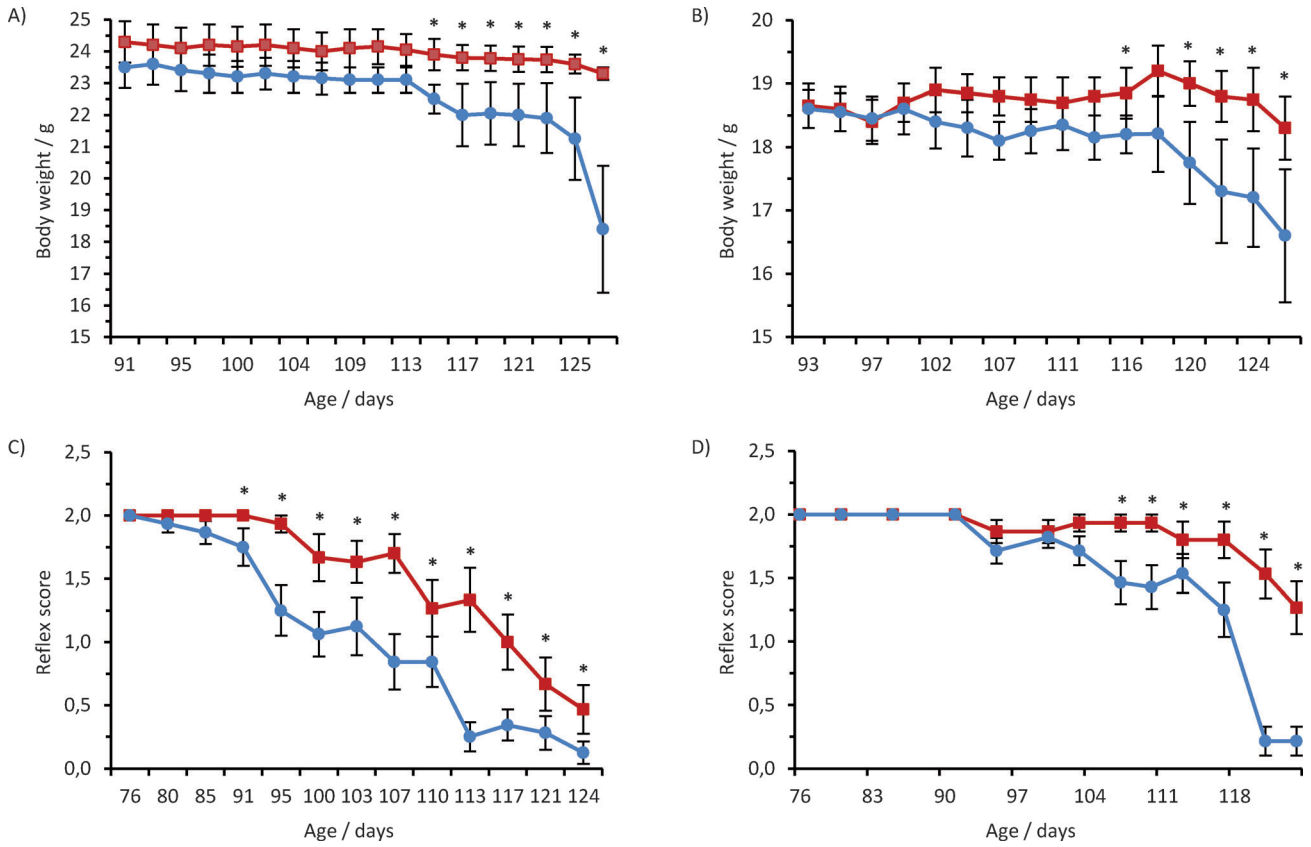


Figure 4. In vivo evaluation of compound **14**. Chronic treatment with **14** prevent loss of the body weight and neurological function in ^{hSOD1G93A}transgenic mice. Mice were daily treated i.p. with 10 mg kg^{-1} of **14** (■) beginning from day 40 and continuing until day 130; the untreated control group (●) is shown for comparison. Body weight was determined every other day until day 125 in A) male and B) female mice. The effect of **14** on neurological function was measured from day 90. Mice were tested every other day by measuring reflex of both hind paws in C) males and D) females. Significant difference between treated and control mice is indicated by *; ANOVA, $p < 0.001$ mean ± SD ($n = 15$).

prevention of body mass loss and an improvement in neurological functions. The in vitro study revealed several characteristics of its mechanism of action. Compound **14** significantly decreased the formation of misfolded mutated SOD1 (G93A and G85R, recombinant proteins). Evidence presented in this study indicate that this compound is able to prevent ER-stress-induced apoptosis of NSC-34 motor neuron-like cells and primary mouse astrocytes (both types of cells were transfected with mutated SOD1 G93A, which leads to fALS in humans). In addition, **14** decreased the levels of known ER stress markers (CHOP and BiP) in NSC-34 motor neuron-like cells. Finally, this novel derivative of TMAO decreased the formation of misfolded mutant SOD1 aggregates in NSC-34 motor neuron-like cells (G93A) and in HEK293T cells transfected by another related to ALS in humans SOD1 mutant (A4V). Future detailed characterization of the mechanism of action of compound **14** in vivo is needed. Also, the compound may not be specifically selective for ALS-induced protein misfolding. Therefore, we plan to test the compound in other types of neurodegenerative disease models, such as mouse models of Alzheimer's, Parkinson's, and Huntington's disease.

Experimental Section

Materials

All chemical reagents, solvents, and acids were purchased from Sigma–Aldrich, Acros Organic, Alfa Aesar, Bio-Lab Ltd., Merck, or IU-CHEM Ltd., and were all used as received. Anhydrous THF was obtained by distillation from a boiled blue colored mix containing sodium (1% *w/v*) and benzophenone (0.2% *w/v*). Column chromatography was performed on silica gel 60 (230–400 mesh; Merck). Analytical and preparative HPLC (Young Lin Instruments, Anyang, Korea) were performed on LUNA C_{18} preparative (10 μm, 100× 30 mm) or analytical (5 μm, 250×4.6 mm) columns, both from Phenomenex Inc. (Torrance, CA, USA). HPLC purification was carried out with an increasing linear gradient of CH_3CN in H_2O. Purity of the synthesized compounds was confirmed by HPLC analysis (Supporting Information Table S1). Analytical TLC was carried out on pre-coated silica gel 60 F_{254} (Merck) sheets using UV absorption and iodine physical adsorption for visualization. Mass spectra were recorded on a Finnigan Model 400 instrument using a QToF microspectrometer (Micromass, Milford, MA, USA), using electrospray ionization (ESI) in the positive ion mode. Data were processed using mass LynX ver. 4.1 calculation and de-convolution software (Waters Corp., Milford, MA, USA). High-resolution mass spectra (HRMS) were obtained using an LTQ Orbitrap XL (Thermo Scientific, Waltham, MA, USA). Melting points were measured with a Fisher–

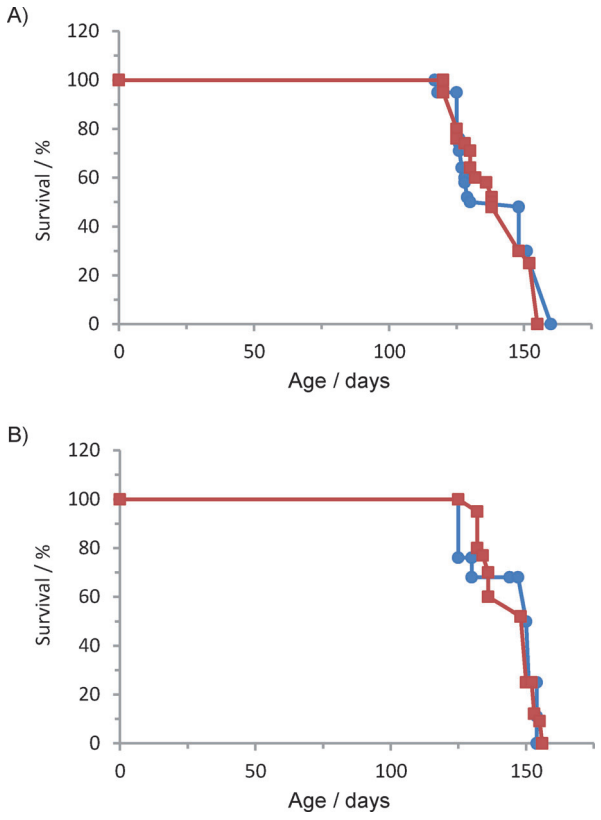


Figure 5. Survival curves following the treatment with compound **14** (■); the untreated control group (●) is shown for comparison. A) Effect of **14** on the survival rate in male ^{hSOD1G93A}transgenic mice. B) Effect of **14** on the survival rate in female ^{hSOD1G93A}transgenic mice. The experiment was conducted as described in the Experimental Section. Significant difference between treated and untreated mice is indicated by *; $p \leq 0.05$ mean ± SD ($n = 15$).

Johns melting point apparatus (Waltham, MA, USA). Hank's balanced salt solution (HBSS) was purchased from Life Technologies (Carlsbad, CA, USA). β-Mercaptoethanol, PMSF, sodium orthovanadate, sodium-β-glycerophosphate, sodium dodecyl sulfate (SDS), and sodium pyrophosphate decahydrate were purchased from Alfa Aesar (Karlsruhe, Germany). EGTA was from AppliChem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated and normal fetal calf serum (FCS), L-glutamine, penicillin/streptomycin, nystatin, neomycine-G418 and trypsin were purchased from Biological Industries (Beth-Haemek, Israel). Bromphenol blue, *N,N,N,N*-tetramethylethylenediamine, ammonium persulfate, fat-free milk, acrylamide solution for gel preparation, and protein molecular weight markers were purchased from Bio-Rad (Hercules, CA, USA). BSA, methylthiazolyl blue (MT) was from Chem-Impex International (Wood Dale, IL, USA). Bradford reagent, DMSO, DNAse, DTT, EGTA, glycerol, IGEPAL, NaCl, PBS tablets, protease inhibitor cocktail for mammalian cells, *para*-phenylbutyric acid, PBS tablets, Tris·HCl, tunicamycin, trimethylamino *N*-oxide (TMAO), Triton X-100, and NaF were purchased from Sigma–Aldrich Chemicals (Rehovot, Israel). Hygromycin B was purchased from Molecula (Shaftesbury, UK). The following antibodies were used: rabbit polyclonal anti-SOD1 antiserum (Santa Cruz Biotechnology, TX, USA); CHOP, monoclonal antibody which was produced based on a synthetic peptide corresponding to residues surrounding Leu 159 of the human CHOP protein which also recognizes the mouse protein. Anti-Bip rabbit mAb was produced by immunizing rabbits with a synthetic peptide corresponding to residues surrounding Gly 584 of the human protein, which recognizes the mouse protein as well. These two antibodies and anti-α-tubulin 1 were purchased from Cell Signaling (Danvers, MA, USA). B8H10 antibody was supplied by MediMabs, (Montreal, QC, Canada). The proteasome activity assay kit was purchased from Abcam (Cambridge, UK).

Chemistry

General procedure for the preparation of S-1 and S-2: To a stirred solution of commercially available 3-(dimethylamino)propan-1-ol/3-(dimethylamino)propan-1-amine (2.62 mmol), 3% aq. H_2O_2 (5 mL) was added dropwise, and the solution was stirred for 72 h at room temperature. The water was removed by lyophilization, and the resulting crystalline mass was dissolved in 20 mL MeOH and filtered. MeOH was evaporated under reduced pressure to obtain an *N*-oxide derivative.

3-Hydroxy-*N*,*N*-dimethylpropan-1-amine oxide (S-1): The title compound was obtained starting from 3-(dimethylamino)propan-1-ol (colorless syrup, 93%): ^{1}H NMR (300 MHz, D_2O): $\delta = 2.06$ (m, 2H), 3.45 (s, 6H), 3.43 (m, 2H), 3.7 (t, $J = 6$ Hz, 2H), 4.79 ppm (s, 1H); ^{13}C NMR (75.49 MHz, D_2O): $\delta = 25.78$, 57.15, 58.54, 67.74 ppm; MS (ES, 30 eV) *m/z* (%): 120 (100) $[M+H]^+$, 142 (5) $[M+Na]^+$.

3-Amino-*N*,*N*-dimethylpropan-1-amine oxide (S-2): The title compound was obtained starting from 3-(dimethylamino)propan-1-amine (yellow oil, 91%): ^{1}H NMR (600 MHz, D_2O): $\delta = 1.79$ (m, 2H), 2.56 (t, $J = 7.8$ Hz, 2H), 3.00 (s, 6H), 3.17 ppm (m, 2H); ^{13}C NMR (150.9 MHz, D_2O): $\delta = 25.2$, 37.65, 57.19, 67.93 ppm; MS (ES, 30 eV) *m/z* (%): 119 (100) $[M+H]^+$.

General procedure for the preparation of S-3: To a stirred solution of commercially available 4-(dimethylamino)butyric acid hydrochloride (29.8 mmol), a solution of NaOH (10% *w/v*) was added to reach pH 14. Subsequently, 3% aq. H_2O_2 (200 mL) were added dropwise, and the reaction mix was stirred for 72 h at room temperature. The reaction was quenched by the addition of 1 M HCl to reach pH 3 and extracted with EtOAc (3×50 mL). The combined organic phases were dried over Mg_2SO_4, and the solvent was removed under reduced pressure to obtain an *N*-oxide derivative.

3-Carboxy-*N*,*N*-dimethylpropan-1-amine oxide (S-3): The title compound was obtained starting from 4-(dimethylamino)butyric acid hydrochloride (white oil, 90%): ^{1}H NMR (400 MHz, D_2O): $\delta =$ 2.08 (m, 2H), 2.32 (t, $J = 7.08$ Hz, 2H), 3.37 (s,6H), 3.51 ppm (m, 2H); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 19.17$, 48.60, 55.94, 68.53, 179.65 ppm; MS (ES, 30 eV) *m/z* (%): 148 (100) $[M+H]^+$, 170 (16) $[M+Na]^+$.

General procedure for the preparation of 3 and 4: To a stirred solution of commercially available 2,3,5-collidine/2,4,6-collidine (76.8 mmol), AcOH (85 mL) and 30% aq. H_2O_2 solution (33 mL) were added. The mixture was heated at 80 °C for 6 h. The reaction progress was followed by TLC. After cooling, the mixture was poured into H_2O (50 mL) and neutralized by the addition of a saturated $NaHCO_3$ solution. The aqueous layer was extracted with CH_2Cl_2 (3×200 mL); the combined organic phases were dried over Mg_2SO_4, and the solvent was removed under reduced pressure.

2,3,5-Trimethylpyridine 1-oxide (3):[33] The title compound was obtained starting from 2,3,5-collidine (yellow oil, 57%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.32$ (s, 3H), 2.38 (s, 3H), 2.53 (s, 3H), 7.16 (s, H), 8.21 (s, H), AcOH 2.1 ppm (s, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 13.02$, 17.31, 18.93, 131.41, 134.34, 136.81, 145.45, AcOH 20.72,

173.46 ppm; MS (ES, 30 eV) *m/z* (%): 138 (100) $[M+H]^+$, 160 (18) $[M+Na]^+$.

2,4,6-Trimethylpyridine 1-oxide (4): The title compound was obtained starting from 2,4,6-collidine (yellow oil, 61%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=2.09$ (s, 3H), 2.29 (s, 6H), 6.76 ppm (s, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=17.18$, 19.21, 124.00, 135.81, 147.21 ppm; MS (EI, 30 eV) *m/z* (%): 137 (82) $[M]^+$.

General procedure for the preparation of 5 and 6: The compounds were synthesized using the Boekelheide rearrangement.[34] Solution of **3**/**4** (19 mmol) was added to acetic anhydride (40 mL) and was heated at reflux for 4 h. After cooling, excess acetic anhydride was evaporated under reduced pressure (high-vacuum oil pump) to obtain black crude. The resulting syrup was purified by silica gel column (petroleum ether/EtOAc/MeOH 10:30:60).

(3,5-Dimethylpyridin-2-yl)methyl acetate (5): The title compound was obtained starting from **3** (yellow oil, 69.5%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=1.99$ (s, 3H), 2.18, 2.22 (s, 6H), 5.08 (s, 2H), 7.20 (s, H), 8.16 ppm (s, H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=17.23$, 20.14, 56.02, 131.22, 132.37, 138.21, 146.70, 149.91, 169.95 ppm; MS (ES, 30 eV) *m/z* (%): 180 (100) $[M+H]^+$, 202 (16) $[M+Na]^+$.

(4,6-Dimethylpyridin-2-yl)methyl acetate (6): The title compound was obtained starting from **4** (yellow oil, 71%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=2.10$ (s, 3H), 2.29 (s, 3H), 2.48 (s, 3H), 5.14 (s, 2H), 6.91 (s, H), 6.98 (s, H), AcOH 2.03 (s, 3H), 10.21 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 75.49 MHz): $\delta=20.87$, 20.99, 23.04, 65.95, 120.56, 124.17, 149.52, 154.22, 157.63, 170.87, AcOH 20.99, 176.52 ppm; MS (ES, 30 eV) *m/z* (%): 180 (100) $[M+H]^+$, 202 (47) $[M+Na]^+$.

General procedure for the preparation of 7 and 8: The compounds were obtained using a modification of the procedure reported by Ellervik and Magnusson.[35] Compound **5**/**6** (18 mmol) was dissolved in MeOH (170 mL), and 1 M $NaOCH_3$ in MeOH (3 mL) was added. The mixture was stirred for 3 h at room temperature. Excess MeOH was evaporated under reduced pressure. The reaction was quenched by the addition of H_2O (50 mL) and extracted with EtOAc (3×50 mL). The combined organic phase was washed with brine and dried over Mg_2SO_4, and the solvent was removed under reduced pressure. The resulting syrup was purified by a silica gel column (petroleum ether/EtOAc/MeOH 10:30:60).

(3,5-Dimethylpyridin-2-yl)methanol (7): The title compound was obtained starting from **5** (yellow oil, 47.3%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=2.11$ (s, 3H), 2.22 (s, 3H), 4.57 (s, 2H), 7.20 (s, H), 8.12 ppm (s, H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=16.32$, 17.77, 61.42, 128.97, 131.45, 138.43, 145.28, 153.43 ppm; MS (ES, 30 eV) *m/z* (%): 138 (100) $[M+H]^+$, 160 (4) $[M+Na]^+$.

(4,6-Dimethylpyridin-2-yl)methanol (8): The title compound was obtained starting from **6** (yellow solid, 49%, mp: 43 °C): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=2.30$ (s, 3H), 2.50 (s, 3H), 4.66 (s, 2H), 6.84 (s, H), 6.87 ppm (s, H); ^{13}C NMR ($CDCl_3$, 75.49 MHz): $\delta=21.00$, 24.02, 64.00, 118.56, 122.95, 148.28, 157.17, 158.35 ppm; MS (ES, 30 eV) *m/z* (%): 138 (100) $[M+H]^+$.

General procedure for the preparation of 9, 10, and 16: Compound **7**/**8**/**S-10** (14 mmol) was dissolved in dry THF (15 mL). Subsequently, glutaric anhydride (14 mmol) was dissolved in dry THF (2 mL) and was added dropwise to the reaction solution while stirring at room temperature for 1 h. The reaction was quenched by the addition of H_2O (50 mL) and extracted with EtOAc (3×150 mL). The combined organic phase was washed with brine and dried over Mg_2SO_4, and the solvent was removed under reduced pressure. The resulting syrup was purified by silica gel column (*n*-hexane/EtOAc 50:50).

5-((3,5-Dimethylpyridin-2-yl)methoxy)-5-oxopentanoic acid (9):[36] The title compound was obtained starting from **7** (white solid, 47%, mp: 87 °C): ^{1}H NMR (300 MHz, D_2O): $\delta=1.89$ (quint, $J=7.5$ Hz, 2H), 2.27 (t, $J=7.5$ Hz, 2H), 2.44 (s, 3H), 2.45 (s, 3H), 2.54 (t, $J=7.5$ Hz, 2H), 5.39 (s, 2H), 8.06 (s, 1H), 8.33 ppm (s, 1H); ^{13}C NMR (100.6 MHz, [D_6]acetone): $\delta=17.50$, 17.61, 20.80, 33.05, 33.31, 65.57, 132.66, 133.75, 139.48, 147.12, 151.18, 172.86, 174.32 ppm; MS (ES, 30 eV) *m/z* (%): 252 (100) $[M+H]^+$.

5-((4,6-Dimethylpyridin-2-yl)methoxy)-5-oxopentanoic acid (10): The title compound was obtained starting from **8** (white solid, 49%, mp: 64 °C): ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta=1.77$ (quint, $J=7.40$ Hz, 2H), 2.26 (t, $J=7.40$ Hz, 2H), 2.27 (s, 3H), 2.40 (s, 3H), 2.44 (t, $J=7.40$ Hz, 2H), 5.05 (s, 2H), 7.00 (s, 1H), 7.01 ppm (s, 1H); ^{13}C NMR (100.6 MHz, [D_6]DMSO): $\delta=20.06$, 20.37, 23.66, 32.65, 32.89, 65.99, 119.18, 122.97, 147.78, 154.79, 157.23, 172.32, 174.06 ppm; MS (ES, 30 eV) *m/z* (%): 252 (100) $[M+H]^+$, 174 (3) $[M+Na]^+$.

5-((3-(1*H*-Imidazol-1-yl)propyl)amino)-5-oxopentanoic acid (16): The title compound was obtained starting from **S-10** (white solid, 64%, mp: 95 °C): ^{1}H NMR (300 MHz, D_2O): $\delta=1.72$ (quint, $J=7.75$ Hz, 2H), 2.01 (quint, $J=6.63$ Hz, 2H), 2.12 (m, 4H), 3.13 (t, $J=6.63$ Hz, 2H), 4.04 (t, $J=6.62$ Hz, 2H), 7.26 (s, 1H), 7.34 (s, 1H), 8.36 ppm (s, 1H); ^{13}C NMR (75.49 MHz, D_2O): $\delta=22.58$, 29.21, 35.63, 36.27, 36.88, 46.42, 121.52, 122.29, 135.79, 170.51, 176.89 ppm; MS (ES, 30 eV) *m/z* (%): 240 (100) $[M+H]^+$, 262 (3) $[M+Na]^+$.

General procedure for the preparation of (11–15, 17–19): To a suspension of acid derivative (1.6 mmol) in DMF (3 mL), Et_3N (1 mL) and PyBOP (1.8 mmol) were added. The mixture was stirred at room temperature for 30 min. Then, R-OH/R-NH_2 (1.6 mmol) dissolved in DMF (2 mL) was added to the reaction. The reaction mixture was stirred for 18 h. After that, DMF was evaporated under high pressure to obtain a yellow oil. The product was purified by HPLC.

3-(5-((3,5-Dimethylpyridin-2-yl)methoxy)-5-oxopentanoyl)-*N*,*N*-dimethylpropan-1-amine oxide (11): The title compound was obtained starting from **9** (yellow oil, 39%): ^{1}H NMR (400 MHz, [D_6]acetone): $\delta=1.90$ (quint, $J=7.40$ Hz, 2H), 2.24 (s, 3H), 2.25 (quint, $J=6.62$ Hz, 2H), 2.32 (s, 3H), 2.39 (t, $J=6.46$ Hz, 2H), 2.43 (t, $J=7.10$ Hz, 2H), 3.22 (s, 6H), 3.45 (m, 2H), 4.19 (t, $J=6.26$ Hz, 2H), 5.17 (s, 2H), 7.40 (s, 1H), 8.20 ppm (s, 1H); ^{13}C NMR (100.6 MHz, [D_6]acetone): $\delta=17.92$, 18.01, 21.16, 24.10, 33.67, 58.68, 62.90, 66.48, 68.18, 133.88, 139.41, 147.88, 151.81, 162.77, 173.16, 173.33 ppm; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{18}H_{28}N_2O_5$: 353.20710, found: 353.20746; MS (ES, 30 eV) *m/z* (%): 353 (46) $[M+H]^+$.

3-(5-((4,6-Dimethylpyridin-2-yl)methoxy)-5-oxopentanoyl)-*N*,*N*-dimethylpropan-1-amine oxide (14): The title compound was obtained starting from **10** (yellow oil, 33%): ^{1}H NMR (400 MHz, [D_6]acetone): $\delta=1.90$ (quint, $J=7.14$ Hz, 2H), 2.25 (m, 2H), 2.31 (s, 3H), 2.42 (s, 3H), 2.44 (t, $J=7.33$ Hz, 2H), 2.51 (t, $J=7.44$ Hz, 2H), 3.16 (s, 6H), 3.38 (m, 2H), 4.21 (t, $J=6.32$ Hz, 2H), 5.11 (s, 2H), 6.99 (s, 1H), 7.04 ppm (s, 1H); ^{13}C NMR (100.6 MHz, [D_6]acetone): $\delta=$ 20.96, 23.97, 33.60, 58.71, 62.81, 67.16, 68.10, 119.93, 123.67, 148.79, 156.24, 158.52, 173.03, 173.18 ppm; MS (ES, 30 eV) *m/z* (%): 353 (100) $[M+H]^+$, 375 (4) $[M+Na]^+$; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{18}H_{28}N_2O_5$: 353.20710, found: 353.20758.

3-(5-((3,5-Dimethylpyridin-2-yl)methoxy)-5-oxopentanamido)-*N*,*N*-dimethylpropan-1-amine oxide (12): The title compound was

obtained starting from **9** (yellow oil, 46%): ^{1}H NMR (400 MHz, D_2O): $\delta = 1.87$ (quint, $J = 7.03$ Hz, 2H), 2.02 (m, 2H), 2.25 (t, $J = 7.45$ Hz, 2H), 2.48 (t, $J = 7.45$ Hz, 2H), 3.30 (s, 6H), 3.24 (t, $J = 6.78$ Hz, 2H), 3.46 (m, 2H), 5.30 (s, 2H), 7.91 (s, 1H), 8.25 ppm (s, 1H); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 17.98$, 18.58, 21.93, 24.34, 34.10, 36.13, 37.66, 58.00, 63.66, 68.93, 136.58, 138.02, 142.77, 146.75, 147.82, 176.23, 177.42 ppm; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{18}H_{29}N_3O_4$: 352.22308, found: 352.22372; MS (ES, 30 eV) *m/z* (%): 352 (100) $[M+H]^+$.

3-(5-((4,6-Dimethylpyridin-2-yl)methoxy)-5-oxopentanamido)-*N*,*N*-dimethylpropan-1-amine oxide (15): The title compound was obtained starting from **10** (yellow oil, 42%): ^{1}H NMR (400 MHz, D_2O): $\delta = 1.91$ (quint, $J = 7.31$ Hz, 2H), 2.02 (m, 2H), 2.29 (t, $J = 7.45$ Hz, 2H), 2.31 (s, 3H), 2.44 (s, 3H), 2.49 (t, $J = 7.45$ Hz, 2H), 3.18 (s, 6H), 3.26 (t, $J = 6.61$ Hz, 2H), 3.34 (m, 2H), 5.09 (s, 2H), 7.09 (s, 1H), 7.12 ppm (s, 1H); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 19.65$, 20.00, 21.49, 22.49, 32.31, 35.82, 56.79, 65.46, 67.32, 120.44, 124.00, 151.06, 152.24, 156.84, 174.51, 175.24 ppm; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{18}H_{29}N_3O_4$: 352.22308, found: 352.22314; MS (ES, 30 eV) *m/z* (%): 352 (38) $[M+H]^+$.

2-(5-((3,5-Dimethylpyridin-2-yl)methoxy)-5-oxopentanamido)ethanesulfonic acid (13): The title compound was obtained starting from **9** (yellow oil, 54%): ^{1}H NMR (300 MHz, D_2O): $\delta = 1.81$ (quint, $J = 7.11$ Hz, 2H), 2.19 (t, $J = 7.11$ Hz, 2H), 2.26 (s, 3H), 2.28 (s, 3H), 2.44 (t, $J = 7.11$ Hz, 2H), 2.95 (t, $J = 6.67$ Hz, 2H), 3.44 (t, $J = 6.67$ Hz, 2H), 5.20 (s, 2H), 7.70 (s, 1H), 8.15 (s, 1H), Et_3N (1.19, t, $J = 7.33$ Hz, 6H), (3.11 ppm, q, $J = 7.33$ Hz, 4H); ^{13}C NMR (75.49 MHz, D_2O): $\delta =$ 16.89, 17.35, 20.63, 34.87, 35.28, 49.88, 63.47, 134.44, 135.97, 143.30, 143.62, 147.63, 147.63, 175.86, Et_3N (8.48, 46.92 ppm); ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{15}H_{22}N_2O_5S$: 359.12713, found: 359.12753; MS (ES−, 30 eV) *m/z* (%): 357 (37) $[M-H]^-$.

3-(5-((3-(1*H*-Imidazol-1-yl)propyl)amino)-5-oxopentanamido)-*N*,*N*-dimethylpropan-1-amine oxide (17): The title compound was obtained starting from **16** (yellow oil, 74%): ^{1}H NMR (400 MHz, D_2O): $\delta = 1.77$ (quint, $J = 7.54$ Hz, 2H), 1.96 (m, 4H), 2.16 (m, 4H), 3.07 (m, 2H), 3.23 (m, 4H), 4.00 (t, $J = 6.79$ Hz, 2H), 6.95 (s, 1H), 7.09 (s, 1H), 7.61 ppm (s, 1H); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 21.12$, 22.52, 28.82, 29.69, 34.34, 35.87, 35.99, 43.96, 56.90, 67.36, 119.54, 127.15, 137.35, 175.18, 175.36 ppm; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{16}H_{29}N_5O_3$: 340.23432, found: 340.23477; MS (ES, 30 eV) *m/z* (%): 340 (53) $[M+H]^+$, 362 (4) $[M+Na]^+$.

4-((3-(1*H*-Imidazol-1-yl)propyl)amino)-*N*,*N*-dimethyl-4-oxobutan-1-amine oxide (18): The title compound was obtained starting from **S-10** (yellow oil, 68%): ^{1}H NMR (300 MHz, D_2O): $\delta = 1.99$ (m, 2H), 2.24 (m, 4H), 3.03 (m, 2H), 3.19 (s, 6H), 3.32 (m, 2H), 4.33 (t, $J = 7.15$ Hz, 2H), 7.42 (s, 1H), 7.50 (s, 1H), 8.66 ppm (s, 1H); ^{13}C NMR (75.49 MHz, D_2O): $\delta = 20.23$, 27.75, 34.07, 36.68, 46.20, 57.16, 69.89, 121.32, 121.74, 135.32, 181.48 ppm; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{12}H_{22}N_4O_2$: 255.18155, found: 255.18199; MS (ES, 30 eV) *m/z* (%): 255 (100) $[M+H]^+$, 277 (13) $[M+Na]^+$.

***N*-(3-(1*H*-Imidazol-1-yl)propyl)-4-(4-methoxyphenyl)butanamide (19)**: The title compound was obtained starting from 4-(4-methoxyphenyl)butyric acid (white solid, 54%, mp: 58 °C): ^{1}H NMR (400 MHz, D_2O): $\delta = 1.84$ (quint, $J = 7.59$ Hz, 2H), 2.01 (m, 2H), 2.18 (m, 2H), 2.55 (t, $J = 7.59$ Hz, 2H), 3.11 (t, $J = 6.04$ Hz, 2H), 3.77 (s, 3H), 4.17 (t, $J = 6.04$ Hz, 2H), 6.91 (d, $J = 8.62$ Hz, 2H), 7.18 (d, $J = 8.62$ Hz, 2H), 7.40 (s, 1H), 7.44 (s, 1H), 8.59 ppm (s, 1H); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 28.25$, 30.37, 34.98, 36.61, 37.40, 48.20, 56.92, 115.53, 121.83, 123.11, 131.26, 136.00, 136.22, 158.58, 178.28 ppm; ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{17}H_{23}N_3O_2$: 302.18630, found: 302.18665.

Biology

Animals

Mice overexpressing SOD1^{G93A} were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The ^{G93A}SOD1 transgenic mouse model was developed and characterized as a model for ALS by Gurney et al.[22] The animals were housed in standard conditions: constant temperature (22 ± 1 °C), humidity (relative, 40%), and a 12 h light/dark cycle and were allowed free access to food and water. Male mice with hemizygous background were bred with control C57Bl females so that each litter would generate hemizygous ^{G93A}SOD1 transgenic mice and littermate controls. Newborn mice were genotyped by PCR analysis using the following primers. IL2 primers: CTA GGC CAC AGA ATT GAA AGA TCT and GTA GGT GGA AAT TCT AGC ATC ATC C and the ^{hG93A}SOD1 primers: CAT CAG CCC TAA TCC ATC TGA and CGC GAC TAA CAA TCA AAG TGA. Genomic DNA was extracted from tail biopsies using the D-Tail DNA extraction kit (Syntezza Bioscience, Jerusalem, Israel). The experiments were performed in accordance with local and international regulations, every effort was made to reduce the number of animals used and to minimize their suffering. Primary astrocyte cultures were obtained from littermates which were positive for the transgene. The Tel Aviv University is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) internationally accredited institution.

Cell cultures

Human ^{G93A}SOD1 transfected motor neuron-like cells (NSC-34): ^{hG93ASOD1}NSC-34 and ^{hWTSOD1}NSC-34 cells were kindly provided by Prof. Nava Zisapel (Tel Aviv University, Israel). Cells were grown in DMEM (22.5 mM glucose) supplemented with 15% heat inactivated FCS, 1 mM glutamine, and antibiotics (100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 200 μg mL^{-1} hygromicine and 700 μg mL^{-1} neomycin-G418) at 37 °C in a 5% CO_2 humidified atmosphere.

^{hWTSOD1}HEK293T and ^{hSOD1A4V}HEK293T cells: HEK293T cells were maintained at 37 °C and 5% CO_2 in DMEM/High Glucose (Thermo Scientific) supplemented with fetal bovine serum (FBS; 10% *v/v*), streptomycin sulfate (100 μg mL^{-1}), and penicillin (100 U mL^{-1}).

Mice astrocyte primary cultures

Astrocyte primary cultures were prepared from the cortex of newborn mice as previously described by Apricò et al., with minor changes.[37] The cortex tissue was dissected from newborn mice (postnatal days 1–3) and the meninges were removed. Spinal cords were isolated from newborn mice as described in the isolation protocol. Purified tissues were mechanically dissociated in cold PBS and digested by incubation with trypsin in PBS (1:5 *v/v*) for 10 min at 37 °C. The reaction was stopped by the addition of complete DMEM, supplemented with 10% FBS, 100 μg mL^{-1} streptomycin, 100 U mL^{-1} penicillin, 12.5 U mL^{-1} nystatin, 2 mM L-glutamine and 50 μg mL^{-1} DNAse. At this point, the tissue was mechanically dissociated once more to ensure full dissociation into single cells. The cells were washed with complete medium once and centrifuged at 1100 rpm for 7 min. The supernatant was removed and the pellet was re-suspended in complete medium. The cells were plated at a density of 10^4 cells cm^{-2}. Cell cultures were grown in complete medium and maintained at 37 °C and 5% CO_2.

The cells were allowed to proliferate until confluence was achieved; at this point microglia cells were eliminated by shaking at 250 rpm for 18 h on a horizontal orbital shaker followed by removal of the microglia-containing medium. After a few hours the cells were trypsinized and re-plated. Astrocyte cultures from this passage (P1) were used for all studies. Cells were seeded in 96-well plates at a concentration of 10000 cells per well and allowed to attach. All experiments were done in serum-free medium. Cell viability was measured by Alamar blue 10% (AbD Serotec, Kidlington, UK). The results were read at λ 590 nm using a Fluostar device.

MTT cell viability measurement

This assay measures the reduction of a tetrazolium component in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide] into an insoluble formazan product by the mitochondria of viable cells. Cells were incubated with MTT (2 mg mL^{-1}) in growth medium for 30 min at 37C°. The medium was then aspirated, and DMSO was added to solubilize the colored crystals and absorbance at 570 nm was measured in an ELISA reader. The amount of color produced is directly proportional to the number of viable cells.

Western blot protocols

Western blot of SOD1 and the SDS resistance mutant human SOD1 dimer (32–35 kDa): Cells were treated as described for the MTT preparation. After that, whole-cell lysates were prepared in the following manner: the lysis buffer contained 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 150 mM NaCl, 50 mM NaF, 10 mM sodium glycerophosphate, 5 mM sodium pyrophosphate, and 1 mM PMSF, supplemented with 0.1% (*v*/*v*) NP-40, 0.1% (*v*/*v*) β-mercaptoethanol, and protease inhibitor cocktail (1:100 dilution). The cells were washed with ice-cold PBS, and 1 mL lysis buffer was then added and incubated at 4 °C for 40 min. The resulting cell lysates were centrifuged at 8700 *g* for 30 min at 4 °C, and the resulting supernatant fractions were separated and kept at −20 °C until use. Protein content in the supernatant was determined according to the Bradford assay, using a BSA standard dissolved in the same buffer. Aliquots (5–60 μg of protein) were mixed with the sample buffer [62.5 mM Tris·HCl, pH 6.8, 2% (*w*/*v*) SDS, 10% (*v*/*v*) glycerol, 50 mM DTT, and 0.01% (*w*/*v*) bromophenol blue], heated at 95 °C for 5 min. The proteins were separated on 10% SDS-PAGE and western blot analyses were performed using rabbit polyclonal anti-SOD1 antiserum (Santa Cruz) followed by an HRP-conjugated anti-rabbit IgG secondary antibody before visualization with ECL plus solution using the Image Quant program (GE Biosciences, Pittsburgh, PA, USA); 25 μg total protein was loaded in each lane.

Western blot of ER stress markers in ^{hG93A}SOD1 transfected motor neuron-like cells (NSC-34): A cell lysis and standard western blot–PAGE analysis were conducted. The CHOP and BiP antibodies were used according to the manufacturer's instructions. A total of 25 μg protein was loaded in each lane. The total protein concentration in the samples was determined by the Bradford method.

SOD1-EGFP transfection, fluorescence microscopic analysis, flow cytomeric analysis

Transfection of HEK293T cells was performed as previously reported.[29] Cells were seeded on six-well plates one day prior to transfection. Once the cells grew to 80–90% confluence, they were transfected with 3.5 μg of the appropriate plasmid via the calcium phosphate precipitation method. All samples were treated in triplicate unless otherwise mentioned. For transfected HEK293T cells treated with **14**, compound **14** (200 μM) was added to cell culture medium three times during the transfection, when the medium was replaced.

Fluorescence microscopy was performed as previously reported.[29] Two days post-transfection, the transfected HEK293T cells were examined by fluorescence microscopy using a VistaVision inverted fluorescence microscope (VWR, Radnor, PA, USA). Images were captured using a DC-2C digital camera. The fluorescence excitation wavelength range was between 420 and 485 nm and the emission wavelength was 515 nm. Flow cytometric analysis of cellular fluorescence was performed as previously reported.[29] Two days post-transfection, the transfected HEK293T cells were trypsinized, washed twice with 1× PBS and re-suspended in 500 μL 1× PBS. The fluorescence intensities of the HEK293T cells expressing SOD1 variant-EGFP fusion protein were measured using a C6 flow cytometer (BD Biosciences, San Jose, CA, USA). The excitation wavelength was 488 nm and the fluorescence emission was detected at 585 nm. Only GFP-positive cells were used to calculate the mean cellular fluorescence. Each sample was prepared in triplicate, and cellular fluorescence indicates mean cellular fluorescence unless otherwise noted.

Immunoprecipitation

Recombinant ^{hWT}SOD1, ^{hG93A}SOD1, and ^{hG85R}SOD1 were expressed in sf9 cells and purified using hydrophobic interaction chromatography (HIC) and ion-exchange chromatography (IEX), as described previously.[38] Proteins (4 μg) were solubilized in immunoprecipitation (IP) buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 plus protease inhibitors] and incubated overnight with B8H10 (MediMabs, Montreal, QC, Canada) antibodies previously cross-linked to Dynabeads protein G (Invitogen, Waltham, MA, USA) with dimethyl pimelimidate (Pierce, Waltham, MA, USA) according to the manufacturer's instructions. The beads were magnetically isolated and washed three times with IP buffer. Samples were eluted with boiling in 2× sample buffer. For immunoblotting, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with goat anti-SOD1 (C-17; SCB) antibodies.

Proteasome activation

The test was conducted using a proteasome activity assay kit according to the manufacturer's instructions (Abcam, Cambridge, UK). Lysates of the INS-1E cells were used as a source of the proteasome.[39]

Measurement of mice neurological functions

As disease progresses, SOD1 mice gradually begin to lose their ability to extend their legs. From day 90 the mice were evaluated weekly for their hind-limb reflex by a blinded observation. Each mice was given the following score: 0 for full extension of both hind limbs, 1 for extension of the legs of no more than 50% of the capacity, and 2 for no extension in both hind limbs.

Statistical analysis

In vitro results are given as mean ±SEM. Statistical significance ($p < 0.05$) was calculated among experimental groups using the two-tailed Student's t-test. The QuickCalcs online service (Graph-

Pad Software: www.graphpad.com/quickcalcs/ttest1.cfm was used for statistical evaluations. Statistical significance for the in vivo study was determined by ANOVA (Supporting Information Table S2). The Kaplan–Mayer test was used to determine the statistical significance for the survival test. Significance was considered if $p < 0.05$.

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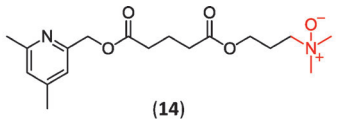
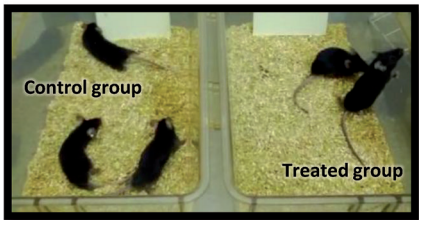
Low concentration efficacy: Compound **14** demonstrated biological effects in ALS mice, showing the prevention of body mass loss and improvements in neurological function. In vitro studies revealed that this lead compound significantly decreases the formation of misfolded mutated superoxide dismutase 1 (SOD1) and prevents ER-stress-induced apoptosis. In addition, **14** decreased the levels of known ER stress markers and decreased the formation of misfolded mutant SOD1 aggregates.



(**14**)



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A Chemical Chaperone-Based Drug Candidate is Effective in a Mouse Model of Amyotrophic Lateral Sclerosis (ALS)