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Tertiary amine pyrazolones and their salts as inhibitors of mutant superoxide dismutase 1-dependent protein aggregation for the treatment of amyotrophic lateral sclerosis

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Abstract

Pyrazolone derivatives have previously been found to be inhibitors of Cu/Zn superoxide dismutase 1 (SOD1)-dependent protein aggregation, which extended survival of an amyotrophic lateral sclerosis (ALS) mouse model. On the basis of ADME analysis, we describe herein a new series of tertiary amine-containing pyrazolones and their structure-activity relationships. Further conversion to the conjugate salts greatly improved their solubility. Phosphate compound **17** exhibited numerous benefits both to cellular activity and to CNS-related drug-like properties in vitro and in vivo, including microsomal stability, tolerated toxicity, and blood-brain barrier permeation. These results indicate that tertiary amine pyrazolones comprise a valuable class of ALS drug candidates.

Introduction

Well known by its rapid and fatal neuronal degeneration, amyotrophic lateral sclerosis (ALS) is characterized by the progressive loss of upper and/or lower neurons in the motor circuitry, including cortex, brainstem, and ventral spinal cord.¹ The prevalence of this disease is lower than 5 per 100,000 worldwide, and only about three-fold higher than its

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Notes

The authors declare no competing financial interest.

Supporting Information

NMR spectra for compounds **6–22**, primary cortical neurons protection assay of compounds **2** and **6**, and analysis of pharmacokinetic parameters for compounds **13** and **17**. This material is available free of charge via the Internet at <http://pubs.acs.org>

incidence, because ALS has the most rapid average progression to death among neurodegeneration diseases: less than 3–5 years after diagnosis.² Despite relatively low patient numbers³ there is a disproportionately high societal cost of care for ALS patients who become immobilized in late-stage disease⁴. The only FDA-approved drug, riluzole, provides no significant symptom alleviation and only a small, 2–3 month, lifespan extension.⁵

Along with the unprecedented mechanistic investigation of ALS in the past decade, 22 genes have been found to be closely associated with the disease,⁶ and pathophysiological studies have already provided a useful indication for possible therapeutic treatments.⁷ However, to overcome the past failures in the search for effective treatments, we still face several principal challenges:⁸ (1) the complexity of familial and sporadic ALS onsets divides the patients into different pathological subsets and may require personalized medicine based on the underlying molecular causes; (2) compared to the determination of a cohort of susceptible genes and their mutations,⁹ no prominent target(s) has been identified to directly correlate with the disease, greatly restricting the development of a drug screening platform; (3) preclinical considerations of central nervous system (CNS) drugs demand that the potential hits not only have good efficacy on animal models, but also fit excellent pharmacokinetic and toxicological characteristics, such as ADME properties and blood brain barrier (BBB) permeability.

Mutant Cu/Zn superoxide dismutase 1 (SOD1) provides an insight to the understanding of ALS pathology;¹⁰ subsequent studies of this mutation have shown it to affect a series of biological malfunctions during ALS progression,¹¹ resulting in the ultimate neuronal toxicity of motor neurons in both familial and sporadic ALS.^{12–13} Although the effects on the lifespan of SOD1 ALS mouse models does not parallel the results in humans,¹⁴ the more rapid disease progression in the ALS animal model supports rapid and efficient drug testing, and thus SOD1 mediated protein misfolding- and aggregation-related cellular and animal models are accepted as the major criteria before moving potential candidates into clinical trials.¹⁵ Therefore, based on an assay using PC12 cells expressing G^{93A}SOD1,¹⁶ we carried out a high-throughput screen and identified several neuron-protection scaffolds based on mitigating protein aggregation and toxicity.¹⁷ Among them, the arylsulfanylpirazolone (ASP) derivatives¹⁸ showed good in vitro potency and median survival time in the G^{93A}ALS model, and after an extensive SAR investigation, the corresponding aryloxanylpirazolones (e.g., **1**, Figure 1) exhibited enhanced potency and stability.¹⁹ Continuing efforts from our lab, by modification to a series of arylazanylpirazolones (e.g., **2**, Figure 1),²⁰ have demonstrated that the tautomer of the pyrazolone ring may be the active pharmacophore and may also contribute to enhancing proteasomal activation in neuron cells.²¹ To further improve the potency and drug-like properties of pyrazolone compounds, we describe here tertiary amine pyrazolones, which exhibited excellent pharmacokinetic and toxicological characteristics as CNS drug candidates (Figure 1).

Results and Discussion

Chemistry

The general synthetic strategy to the tertiary amine pyrazolone derivatives is summarized in Scheme 1. The initial step was a reductive amination of substituted benzaldehydes and

various aliphatic amines. The secondary amines (**3**) were then converted to α -aminoacetate intermediates **4**, which were condensed with the enolate of ethyl acetate to provide γ -amino- β -ketoesters **5** in moderate to high yields. These intermediates were treated with hydrazine to form tertiary amine pyrazolones **6–12** in high yields. Furthermore, by treating the free amine with different acids in organic solvents, the corresponding salts (**13–18**) were successfully afforded in quantitative yields. Treatment of 1-iodo-3,5-dichlorobenzene with acetic acid and allyl alcohol with a palladium catalyst produced the one-carbon homologated acid (**21a**) and the two-carbon homologated aldehyde (**22a**), respectively. Acid **21a** was further converted to the one-carbon homologated aldehyde (**21b**) by borane reduction and Dess-Martin oxidation.

In vitro activity of tertiary amine pyrazolone 6 and HCl salt 13—Our previous studies^{19,20} showed that the in vitro pharmacokinetic properties of compound **1** and its isosteric successor **2** are consistent with standard minimal criteria for preclinical advancement.²² For example, both have sub-micromolar potency, good microsomal stability, and a high maximum tolerated dose. However, in follow-up evaluations, we found that aryloxanylpirazolone **1** has relatively low first-pass clearance, and arylazanylpirazolone **2** has unsatisfactory Caco-2 permeability. Therefore, a tertiary amine fragment was incorporated into the linker between the pyrazolone and the aryl group on the basis of principles of optimal brain exposure in CNS drug molecule design.²³ To characterize the new linker, tertiary amine pyrazolone **6** and its hydrochloride salt (**13**) were initially synthesized and evaluated.

As shown in Figure 2, the two compounds exhibited similar activities in the SOD1 aggregation and cytotoxicity protection assay. Microsomal stability in vitro (Table 1) and Caco-2 permeability (Table 2) are associated with hepatic metabolic stability/first-pass clearance and gut permeability, respectively.²⁴ Because of some of the pharmacokinetic deficits of previous scaffolds the cationic pyrazolone (**13**) is compared with **1** and **2** in Table 2; the introduction of the amine salt led to a boost in the in vitro microsomal stability. The sizeable increase in human and mouse plasma half-life for **13** relative to **1** and doubling of the mouse plasma half-life of **13** relative to **2** are significant. Although the Caco-2 cell permeability and efflux ratio are best for **1**, those parameters for **13** are still excellent and considerably better than those for **2**. The low efflux ratio (< 2) for **13** suggests that efflux proteins have not significantly acted on this scaffold, indicating favorable penetration through the epithelium of the GI tract and potentially the blood–brain barrier (Table 2).²⁵ Compound **13** also exhibited high plasma stability ($t_{1/2} > 180$ min) and moderate human plasma protein binding (94%). On the basis of these favorable results, we synthesized a series of tertiary amine pyrazolone analogues with different substituents and salts.

Structure-activity relationship of tertiary amine pyrazolone analogues—All of the analogues exhibited viability (EC_{50}) in the cytotoxicity protection assay as previously described¹⁷ (Supporting Information, Table S1). In general, the potency of the tertiary amine pyrazolone analogues (Figure 3) is comparable to that of the arylazanylpirazolone analogues. Detailed in our previous investigation of aryloxanylpirazolones¹⁹ and arylazanylpirazolone,²⁰ the dichloro phenyl moiety was the most effective aromatic

substitution pattern and was kept in the tertiary amine series. However, 3,5-dichloro-substitution in this series shows slightly better activity than the 2,4-dichloro-substitution in the arylazanylpirazolones. The potencies of the *N*-substituted compounds indicate a decrease with an increase in substituent size from the ethyl to benzyl group. An investigation of the linker length between the aryl moiety and the tertiary amine showed that one carbon is the optimal linker length; longer homologated derivatives **21** and **22** reduce the activity, which is consistent with our previous observation in the arylazanylpirazolone series.²⁰ These results show that the one-atom elongation of the linker between the nitrogen atom and the phenyl ring is tolerable and this allows the formation of stable amine salts.

Since positively charged amines are favorable for BBB penetration, increasing basicity of CNS drugs is a good approach for enhanced BBB penetration.²⁶ On the basis of the cytotoxicity protection assay and synthetic availability, compound **6** was selected for further evaluation. In the cytotoxicity protection assay with cortical neurons, which is essential prior to an animal study,²⁷ compound **6** paralleled the result with its arylazanylpirazolone counterpart (**2**), having a recovery of 100% neuronal activity at 10 μ M (Figure S1, Supporting Information). Table 3 summarizes the aqueous solubility and activity in the cytotoxicity protection assay for a variety of salts of **6**. It was found that the most desirable salts were those formed from stronger acids ($pK_a < 4$); those made from weaker acids ($pK_a > 4$), such as acetic acid and benzoic acid, did not produce the corresponding salts in organic solvents. All of the salts had greatly improved water solubility and exhibited similar activities to the parent tertiary amine pyrazolone. In general, the salts from inorganic acids had better solubility than the salts made from organic acids; methanesulfonate **16** was abandoned because of its high hygroscopicity in air.

In vivo ADMET of tertiary amine pyrazolone salts—Safety is one of the most important drug properties in pharmaceutical development; the maximum tolerated dose (MTD) provides an estimation of the safety window for in vivo studies. The tolerated dose ranges for selected tertiary amine pyrazolone salts were determined in wild-type mice by increasing the dose b.i.d. As shown in Table 4, tartrate salt **14** displayed a four-fold lower MTD than the hydrochloride (**13**), sulfate (**16**), and phosphate (**17**) salts. The hydrochloride and sulfate salts caused irritation to the mice as evidenced by the observation that the mice began to chew at the injection point once the dose reached 160 mg/kg. The phosphate salt did not show that adverse effect and had a rather high MTD of 640–1280 mg/kg.

On the basis of the above results, we selected compounds **13** and **17** to determine in vivo pharmacokinetics and BBB permeation. Mice were administered 300 mg/kg of **13** and **17** intraperitoneally and sacrificed at various time points (0, 3, 6, 12, and 24 h; Figure 4). The in vivo steady-state level of **13** and **17** from plasma was determined by ESI mass spectrometry. The analytical results with blood and brain samples showed that **13** and **17** peaked at 1–3 h in brain with the highest concentration at 2.470 and 12.957 μ g/g, respectively. The elimination curves roughly followed first-order kinetics with clearance rates of 43175 and 10038 mL/h/kg, respectively, in brain, (see Table 5 and Supporting Information for pharmacokinetic parameters). The in vivo plasma half-life of compound **13** is four-fold greater than in vitro microsomal results ($T_{1/2}$ 51 min); in vitro predictions, while useful in

early compound optimization, are generally accepted as serving only as a rough guide to in vivo pharmacology.²⁸ On the basis of clearance and plasma half-life, the brain half-life is estimated to be 1.5 h for both compounds **13** and **17**. We speculate that the relatively more rapid clearance in brain is the result of efflux from the brain compartment. Since the volume of blood plasma ($d = 1.025 \text{ g/mL}$) is approximately equal to the weight of blood plasma,²⁹ the blood–brain barrier penetration ratio can be calculated from the ratio of $AUC_{\text{brain}}/AUC_{\text{blood}}$. Both compounds have a brain/plasma ratio similar to that of prescribed CNS drugs ($B/P > 0.3$).³⁰ Phosphate salt **17** has a B/P ratio of 0.6, 17% higher than that of the hydrochloride salt (**13**), but this B/P ratio difference between **13** and **17** is within experimental error, and these salts are likely to have similar brain distribution. Therefore, a sufficient amount of tertiary amine pyrazolone can be delivered to brain to maintain the protection level obtained in the in vitro cell assay.

Conclusion

A new series of tertiary amine pyrazolone analogues having a basic amine linker, have been designed and synthesized. The impetus for this design originated from an attempt to circumvent the ADME disadvantages of the earlier arylazanyl pyrazolone analogues by taking advantage of a basic moiety to improve BBB permeation. The tertiary amine pyrazolone scaffolds exhibited superior properties for potential neuronal activity and in metabolic studies, such as microsomal stability, plasma stability, and Caco-2 permeability. Conversion of the amine to the corresponding salt forms was critical to gain excellent solubility without increasing cytotoxicity. On the basis of the MTD, phosphate salt **17** has no visible adverse effects on mice, even at a dose of 640–1280 mg/kg, and the B/P ratio of 0.6 indicates that a sufficient effective concentration accumulates in brain tissue. Recent experiments with a photolabile analogue of **17** suggest interaction with a heat shock protein, consistent with the expected in vivo activity (unpublished results). Therefore, the results described here demonstrate that the tertiary amine pyrazolone scaffold has high potential as a class of orally bioavailable, brain permeable ALS drug candidates.

Experimental Section

General chemical methods

All reactions were carried out with magnetic stirring and were monitored by thin-layer chromatography on precoated silica gel 60 F254 plates. Column chromatography was performed with silica gel 60 (230–400 mesh). Proton and carbon NMR spectra were recorded in deuterated solvents on a Bruker Ag500 (500 MHz) spectrometer. The chemical shifts were reported in δ (ppm) (^1H NMR: DMSO- d_6 , δ 2.50 ppm; D_2O 4.81 ppm; ^{13}C NMR: δ DMSO- d_6 , δ 39.52 ppm). The following abbreviations were used to define the multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet. Electrospray mass spectra (ESIMS) were obtained using an Agilent 1100 MSD with methanol as the solvent in the positive ion mode. The C, H, and N microanalyses were performed at Atlantic Microlab, Inc. (Norcross, GA) by combustion using automatic analyzers, and all of the compounds analyzed showed >95% purity. All reagents purchased from Sigma-Aldrich, Alfa Aesar, and TCI were used without further purification unless stated otherwise. Anhydrous solvents

(THF and DMF) were distilled prior to use; other solvents (MeOH, EtOH, and EtOAc) were analytical pure grade.

General procedure A for reductive amination of benzaldehydes and primary amines

—To a solution of methanol (5 mL) and benzaldehyde (2 mmol) was added the primary amine (2.4 mmol). After being stirred at room temperature for 15 min, the solution was cooled to 0 °C prior to adding sodium borohydride (2 mmol) portionwise. The resulting solution was stirred at room temp for another 1 h. The reaction was neutralized with the addition of water (2 mL), and the methanol was evaporated under vacuum. The resulting aqueous phase was extracted with CH₂Cl₂ (20 mL × 2). The combined organic layers are evaporated to afford the crude product as colorless oils that were directly used in the next step.

General procedure B for the reaction of secondary amines 3 and ethyl bromoacetate

—To a solution of K₂CO₃ (200 mol%) and secondary amines **3** (1.5 mmol) in DMF (5 mL) was added ethyl bromoacetate (150 mol %). The reaction mixture was stirred at room temperature for 16 h. The reaction solution was diluted with ethyl acetate (20 mL), washed twice with water (20 mL) to remove DMF, and washed with brine. The collected organic layers were combined, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel chromatography, eluting with a mixture of ethyl acetate and hexane (5% to 10% ethyl acetate) to afford the products as colorless oils in high yields (80–95%).

General procedure C for the synthesis of β-ketoesters 5

—Ethyl acetate (110 mol %) was added to a THF (5 mL/mmol) solution of LiHMDS (1 N in THF, 120 mol%) at –78 °C and stirred for 60 min. A THF (1 mL/mmol) solution of β-aminoacetate (1.0 equiv) was added dropwise to the reaction mixture at –78 °C. After the resulting solution was stirred at –78 °C for another 2 h, the reaction mixture was quenched with saturated NH₄Cl. The aqueous layer was extracted with ethyl acetate and washed twice with water and brine. The collected organic layers were combined, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel chromatography, eluting with a mixture of ethyl acetate and hexane (10% to 30% ethyl acetate) to afford the products as colorless to pale yellow oils in yields of 40%–80%.

General procedure D for the synthesis of pyrazolones from β-ketoesters

—To a solution of β-ketoesters (1 equiv) in EtOH (5 mL/mmol) was added anhydrous hydrazine (200 mol%). The resulting solution was stirred at room temp overnight. After evaporating the volatiles, the reaction residue was purified by silica gel chromatography, eluting with a mixture of MeOH and dichloromethane (2% to 10% MeOH) to afford the products as white to pink solids, which were then recrystallized from dichloromethane/hexane to give pure products as white solids in yields ranging from 60% to 75%.

General procedure E for the preparation of pyrazolone salts 13–18

—Pyrazolone **6** (0.2 mmol) was dissolved in polar solvents (2 mL; **13** and **15** in THF; **14** and **16** in EtOH; **17** and **18** in EtOAc). The equivalent corresponding acids (for the hydrochloride salt, 1 N HCl in dioxane was used) were added, and the resulting mixtures were sonicated for 10 min.

For compounds **13–16**, the salts were prepared after removal of the volatiles by rotary evaporation and dried on a vacuum pump overnight. For compounds **17** and **18**, the white precipitates were filtered, washed with EtOAc, and dissolved in MeOH. After removal of the volatiles by rotary evaporation, the salts were dried under vacuum overnight.

5-(((3,5-Dichlorobenzyl)(methyl)amino)methyl)-1H-pyrazol-3(2H)-one (6): The title compound was prepared according to general procedures A–D. ¹H NMR (DMSO-d₆, 500 MHz): δ = 9.38 (br s, 1H), 7.48 (t, J = 2.0 Hz, 1H), 7.42 (s, 2H), 5.39 (s, 1H), 3.46 (m, 4H), 2.07 (s, 3H); ¹³C NMR (DMSO-d₆, 125 MHz): δ = 161.1, 143.7, 140.0, 133.9X2, 127.1X2, 126.6, 89.7, 59.1, 52.1, 41.5 ppm; MS (ESI): m/z 286.1 [M+H]⁺; CHN calculated for C₁₂H₁₃Cl₂N₃O: C, 50.37; H, 4.58; N, 14.68; found: C, 50.57; H, 4.69; N, 14.68.

5-(((3,5-Dichlorobenzyl)(ethyl)amino)methyl)-1H-pyrazol-3(2H)-one (7): The title compound was prepared according to general procedures A–D. ¹H NMR (DMSO-d₆, 500 MHz): δ = 11.47 (br s, 1H), 9.61 (br s, 1H), 7.45–7.40 (m, 3H), 5.37 (s, 1H), 3.52–3.36 (m, 4H), 2.38 (t, J = 7.0 Hz, 2H), 0.98 (t, J = 7.0 Hz, 3H); ¹³C NMR (DMSO-d₆, 125 MHz): δ = 144.6, 133.9X2, 126.9X2, 126.4, 89.3, 55.7, 48.4, 46.6, 11.7 ppm; MS (ESI): m/z 300.1 [M+H]⁺; CHN calculated for C₁₃H₁₅Cl₂N₃O: C, 52.01; H, 5.04; N, 14.00; found: C, 52.15; H, 5.04; N, 14.00.

5-(((3,5-Dichlorobenzyl)(propyl)amino)methyl)-1H-pyrazol-3(2H)-one (8): The title compound was prepared according to general procedures A–D. ¹H NMR (DMSO-d₆, 500 MHz): δ = 11.49 (br s, 1H), 9.39 (br s, 1H), 7.46 (s, 1H), 7.41 (s, 2H), 5.36 (s, 1H), 3.52–3.46 (m, 4H), 2.28 (t, J = 7.0 Hz, 2H), 1.45–1.40 (m, 2H), 0.79 (t, J = 7.5 Hz, 3H); ¹³C NMR (DMSO-d₆, 125 MHz): δ = 144.6, 133.8X2, 126.9X2, 126.4, 56.3, 54.7, 19.6, 11.7 ppm; MS (ESI): m/z 313.1 [M+H]⁺; CHN calculated for C₁₄H₁₇Cl₂N₃O: C, 53.52; H, 5.45; N, 13.37; found: C, 53.53; H, 5.71; N, 13.12.

5-(((3,5-Dichlorobenzyl)(isopropyl)amino)methyl)-1H-pyrazol-3(2H)-one (9): The title compound was prepared according to general procedures A–D. ¹H NMR (DMSO-d₆, 500 MHz): δ = 11.45 (br s, 1H), 9.51 (br s, 1H), 7.43–7.42 (m, 3H), 5.34 (s, 1H), 3.51 (s, 2H), 3.40 (s, 2H), 2.78 (m, 1H), 0.98 (d, J = 6.5 Hz, 6H); ¹³C NMR (DMSO-d₆, 125 MHz): δ = 145.6, 133.8X2, 126.7X2, 126.3, 51.7, 49.2, 40.0, 17.6X2 ppm; MS (ESI): m/z 313.1 [M+H]⁺; CHN calculated for C₁₄H₁₇Cl₂N₃O: C, 53.52; H, 5.45; N, 13.37; found: C, 53.32; H, 5.47; N, 13.00.

5-((Cyclopropyl(3,5-dichlorobenzyl)amino)methyl)-1H-pyrazol-3(2H)-one (10): The title compound was prepared according to general procedures A–D. ¹H NMR (DMSO-d₆, 500 MHz): δ = 11.41 (br s, 1H), 9.59 (br s, 1H), 7.45 (d, J = 5.0 Hz, 1H), 7.30 (d, J = 6.0 Hz, 2H), 5.35 (s, 1H), 3.63 (s, 2H), 3.52 (s, 2H), 1.82 (s, 1H), 0.38 (s, 2H), 0.26 (s, 2H); ¹³C NMR (DMSO-d₆, 125 MHz): δ = 143.7, 133.7X2, 127.4X2, 126.5, 56.7, 49.1, 36.0, 7.2X2 ppm; MS (ESI): m/z 311.1 [M+H]⁺; CHN calculated for C₁₄H₁₅Cl₂N₃O: C, 53.86; H, 4.84; N, 13.46; found: C, 54.04; H, 5.02; N, 13.28.

5-((Benzyl(3,5-dichlorobenzyl)amino)methyl)-1H-pyrazol-3(2H)-one (11): The title compound was prepared according to general procedures A–D. ¹H NMR (DMSO-d₆, 500

MHz): δ = 11.62 (br s, 1H), 9.58 (br s, 1H), 7.45-7.24 (m, 8H), 5.42 (s, 1H), 3.50-3.49 (m, 4H), 3.43 (s, 2H); ^{13}C NMR (DMSO- d_6 , 125 MHz): δ = 159.2, 143.9, 138.5, 134.0X2, 128.6X2, 128.3X2, 127.1X2, 126.9, 126.6, 57.0, 55.6, 49.0 ppm; MS (ESI): m/z 361.1 [$\text{M} + \text{H}$] $^+$; CHN calculated for $\text{C}_{18}\text{H}_{17}\text{Cl}_2\text{N}_3\text{O}$: C, 59.68; H, 4.73; N, 11.60; found: C, 59.68; H, 4.85; N, 11.66.

5-(((2,4-Dichlorobenzyl)(propyl)amino)methyl)-1H-pyrazol-3(2H)-one (12): The title compound was prepared according to general procedures A–D. ^1H NMR (DMSO- d_6 , 500 MHz): δ = 11.45 (br s, 1H), 9.51 (br s, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.55 (s, 1H), 7.42-7.41 (m, 1H), 5.36 (s, 1H), 3.57 (s, 2H), 3.50 (s, 2H), 2.31 (t, J = 7.0 Hz, 2H), 1.45-1.41 (m, 2H), 0.77 (t, J = 7.0 Hz, 3H); ^{13}C NMR (DMSO- d_6 , 125 MHz): δ = 136.3, 136.7, 131.8, 131.6, 128.5, 127.2, 89.5, 54.9, 54.1, 48.8, 19.7, 11.7 ppm; MS (ESI): m/z 313.1 [$\text{M} + \text{H}$] $^+$; CHN calculated for $\text{C}_{14}\text{H}_{17}\text{Cl}_2\text{N}_3\text{O}$: C, 53.52; H, 5.45; N, 13.37; found: C, 53.64; H, 5.41; N, 13.38.

N-(3,5-Dichlorobenzyl)-N-methyl-1-(5-oxo-2,5-dihydro-1H-pyrazol-3-yl)methanaminium chloride (13): The title compound was prepared according to general procedure E from compound **6**. ^1H NMR (D_2O , 500 MHz): δ = 7.57 (s, 1H), 7.39 (s, 2H), 4.30 (m, 4H), 2.78 (s, 3H); ^{13}C NMR (D_2O , 125 MHz): δ = 158.4, 137.1, 135.2X2, 131.9, 130.0, 129.2X2, 58.2, 51.4, 48.7, 39.6 ppm; MS (ESI): m/z 286.1 [$\text{M} + \text{H}$] $^+$; HPLC: 97.3% pure.

N-(3,5-Dichlorobenzyl)-N-methyl-1-(5-oxo-2,5-dihydro-1H-pyrazol-3-yl)methanaminium L-tartrate (14): The title compound was prepared according to general procedure E from compound **6**. ^1H NMR (DMSO- d_6 , 500 MHz): δ = 7.48 (s, 1H), 7.42 (s, 2H), 5.39 (s, 1H), 4.29 (s, 2H), 3.49-3.44 (m, 4H), 2.08 (s, 3H); ^{13}C NMR (DMSO- d_6 , 125 MHz): δ = 173.2X2, 160.4, 143.4, 140.9, 133.9X2, 127.3X2, 126.7, 89.3, 72.2X2, 59.0, 52.4, 41.4 ppm; MS (ESI): m/z 286.1 [$\text{M} + \text{H}$] $^+$.

N-(3,5-Dichlorobenzyl)-N-methyl-1-(5-oxo-2,5-dihydro-1H-pyrazol-3-yl)methanaminium citrate (15): The title compound was prepared according to general procedure E from compound **6**. ^1H NMR (DMSO- d_6 , 500 MHz): δ = 7.50 (s, 1H), 7.44 (d, J = 1.5 Hz, 2H), 5.41 (s, 1H), 3.55 (s, 2H), 3.52 (s, 2H), 2.73 (d, J = 15.5 Hz, 2H), 2.63 (d, J = 15.5 Hz, 2H), 2.13 (s, 3H); ^{13}C NMR (DMSO- d_6 , 125 MHz): δ = 174.9, 171.4X2, 160.2, 142.7, 140.7, 134.0X2, 127.5X2, 126.9, 89.3, 72.3, 58.8, 52.3, 42.9X2, 41.2 ppm; MS (ESI): m/z 286.1 [$\text{M} + \text{H}$] $^+$.

Di-N-(3,5-dichlorobenzyl)-N-methyl-1-(5-oxo-2,5-dihydro-1H-pyrazol-3-yl)methanaminium sulfate (16): The title compound was prepared according to general procedure E from compound **6**. ^1H NMR (DMSO- d_6 , 500 MHz): δ = 7.76 (s, 1H), 7.67 (s, 2H), 5.59 (s, 1H), 4.15-3.75 (s, 4H), 2.61 (s, 3H); ^{13}C NMR (DMSO- d_6 , 125 MHz): δ = 134.3X2, 133.9, 130.2X2, 129.3, 89.3, 61.2, 51.8, 38.9 ppm; MS (ESI): m/z 286.1 [$\text{M} + \text{H}$] $^+$.

N-(3,5-Dichlorobenzyl)-N-methyl-1-(5-oxo-2,5-dihydro-1H-pyrazol-3-yl)methanaminium dibasicphosphate (17): The title compound was prepared according to general procedure E from compound **6**. ^1H NMR (DMSO- d_6 , 500 MHz): δ = 7.51 (s, 1H), 7.45 (s, 2H), 5.42 (s, 1H), 3.56-3.53 (m, 4H), 2.13 (s, 3H); ^{13}C NMR (DMSO- d_6 , 125

MHz): δ = 160.0, 142.4, 140.6, 134.0X2, 127.5X2, 126.9, 89.3, 58.7, 52.2, 41.0 ppm; MS (ESI): m/z 286.1 $[M+H]^+$; CHN calculated for $C_{12}H_{16}Cl_2N_3O_5P(+0.11eq\ H_3PO_4)$: C, 36.49; H, 4.17; N, 10.64; found: C, 36.17; H, 4.08; N, 10.44.

2-(3,5-Dichlorophenyl)acetic acid (21a): A solution of 1-iodo-3,5-dichlorobenzene (816 mg, 3 mmol), $PdCl_2$ (53 mg, 0.3 mmol), NaOAc (1.25 g, 15.0 mmol) and AgOAc (752 mg, 4.5 mmol) in AcOH (10 mL) was refluxed at 130 °C with stirring for 16 h. After being cooled to room temp, the mixture was filtered through Celite and the filter cake was washed with EtOAc (20 mL $\times$ 2). The combined filtrate was washed with water (30 mL $\times$ 3) and brine (20 mL), dried over Na_2SO_4 , and evaporated. The residue was subjected to column chromatography using hexane/EtOAc (15/1-10/1) as eluent to afford the product as a colorless solid (310 mg, 50%). 1H NMR ($CDCl_3$, 500 MHz): δ = 7.30 (s, 1H), 7.19 (s, 2H), 3.61 (s, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 176.6, 136.2, 135.2, 128.1X2, 127.9X2, 40.4 ppm; MS (ESI): m/z 203.0 $[M-H]^-$.

2-(3,5-Dichlorophenyl)acetaldehyde (21b): To the reaction solution of **21a** (310 mg, 1.5 mmol) in anhydrous THF (8 mL) was added $BH_3 \cdot THF$ complex (6 mL, 1 M in THF, 6.0 mmol) dropwise at 0 °C. The solution was stirred at 0 °C for 1 h and then at ambient temperature for a further 2 h. The solution was cooled to 0 °C, and unreacted borane was quenched by the dropwise addition of water. When no further evolution of H_2 was observed, aqueous NaOH (5 mL of a 2 N aqueous solution, 10 mmol) was added to the resulting mixture. The solution was extracted with EtOAc (20 mL $\times$ 2), and the combined organic layers were washed with water (20 mL), brine (20 mL), dried with Na_2SO_4 , and concentrated to give the crude product without further purification.

To a solution of the crude product in anhydrous DCM (10 mL) was added Dess-Martin periodinane (760 mg, 1.8 mmol) at 0 °C. The solution was stirred at 0 °C for 10 min and then at ambient temperature for a further 1 h. The solution was partitioned between DCM and water. The aqueous layer was washed with DCM (10 mL), and the combined organic layers were washed with water (10 mL), brine (10 mL), dried with Na_2SO_4 , and concentrated. The residue was purified on silica gel using hexane/EtOAc (30/1-20/1) to get the product as a colorless solid (170 mg, 0.9 mmol, 60% for two steps). 1H NMR ($CDCl_3$, 500 MHz): δ = 9.75 (s, 1H), 7.32 (s, 1H), 7.12 (s, 2H), 3.68 (s, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 197.6, 135.6, 135.1, 128.3X2, 127.9X2, 49.7 ppm; MS (ESI): m/z 189.0 $[M+H]^+$.

5-(((3,5-Dichlorophenethyl)(methyl)amino)methyl)-1H-pyrazol-3(2H)-one (21): The title compound was prepared according to general procedures A–D from compound **21b**. 1H NMR ($DMSO-d_6$, 500 MHz): δ = 7.35 (s, 1H), 7.26 (s, 2H), 5.26 (s, 1H), 3.39 (s, 2H), 2.70 (t, J = 7.2 Hz, 2H), 2.49 (t, J = 7.2 Hz, 2H), 2.13 (s, 3H); ^{13}C NMR ($DMSO-d_6$, 125 MHz): δ = 160.5, 145.1, 140.6, 133.6X2, 127.5X2, 125.5, 89.4, 56.9, 51.9, 41.5, 31.9 ppm; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{13}H_{16}Cl_2N_3O$ 300.0670, found 300.0650; HPLC, 96.0 pure.

3-(3,5-Dichlorophenyl)propanal (22a): To a solution of 1-iodo-3,5-dichlorobenzene (816 mg, 3.0 mmol), $Pd(OAc)_2$ (20 mg, 0.09 mmol), LiCl (127 mg, 3.0 mmol), NaOAc (738 mg, 9.0 mmol) and $n-Bu_4NBr$ (2.29 g, 6.0 mmol) in DMF (15 mL) was added allyl alcohol (265 μ L, 3.9 mmol), and the resulting mixture was stirred at 40 °C for 16 h. After being cooled to

room temp, the mixture was filtered through Celite, and the filter cake was washed with EtOAc (20 mL $\times$ 2). The combined filtrate was washed with water (30 mL $\times$ 3), brine (20 mL), dried over Na₂SO₄, and evaporated. The residue was subjected to column chromatography using hexane/EtOAc (30/1-20/1) as eluent to afford the product as a colorless oil (400 mg, 66% yield). ¹H NMR (CDCl₃, 500 MHz): δ = 9.79 (s, 1H), 7.19 (s, 1H), 7.07 (s, 2H), 2.89 (t, J = 7.2 Hz, 2H), 2.79 (t, J = 7.2 Hz, 2H); ¹³C NMR (CDCl₃, 125 MHz): δ = 200.3, 143.9, 135.1, 127.0X2, 126.7X2, 44.7, 27.5 ppm; MS (ESI): m/z 203.0 [M + H]⁺.

5-(((3-(3,5-Dichlorophenyl)propyl)(methyl)amino)methyl)-1H-pyrazol-3(2H)-one (22):

The title compound was prepared according to general procedures A–D from compound **22a**. ¹H NMR (DMSO-d₆, 500 MHz): δ = 7.37 (s, 1H), 7.29 (s, 2H), 5.33 (s, 1H), 3.36 (s, 2H), 2.58 (t, J = 7.2 Hz, 2H), 2.26 (t, J = 7.2 Hz, 2H), 2.10 (s, 3H), 1.72-1.68 (m, 2H); ¹³C NMR (DMSO-d₆, 125 MHz): δ = 160.6, 146.7, 140.8, 133.7X2, 127.2X2, 125.4, 89.4, 55.3, 52.2, 41.7, 32.1, 28.1 ppm; HRMS (ESI): m/z [M+H]⁺ calcd for C₁₄H₁₈Cl₂N₃O 314.0827, found 314.0811; HPLC, 96.0 pure.

Mutant SOD1-induced cytotoxicity protection assay

Viability and EC₅₀ values were determined according to the previously reported assay procedure.¹⁷ PC12 cells were seeded at 15000 cells/well in 96-well plates and incubated 24 h prior to compound addition. Compounds were assayed in 12-point dose-response experiments to determine potency and efficacy. The highest compound concentration tested was 32 μ M, which was decreased by one-half with each subsequent dose. After 24 h incubation with the compounds, MG132 was added at a final concentration of 100 nM. MG132 is a well-characterized proteasome inhibitor, which would be expected to enhance the appearance of protein aggregation by blocking the proteosomal clearance of aggregated proteins. Cell viability was measured 48 h later using the fluorescent viability probe, Calcein-AM (Molecular Probes). Briefly, cells were washed twice with PBS, Calcein-AM was added at a final concentration of 1 μ M for 20 min at room temperature, and fluorescence intensity was read in a POLARstar fluorescence plate reader (BMG). Fluorescence data were coupled with compound structural data, then stored and analyzed using the CambridgeSoft Chemoffice Enterprise Ultra software package.

In vitro ADME assays

In vitro microsomal stability, aqueous solubility, Caco-2 permeability, and plasma stability assays were determined at Apredica, Inc (Watertown, MA) according to the previously reported procedure.¹⁸

In vivo MTD assays

Compounds **13** and **17** were each dissolved in PBS with 0.1% DMSO and sonicated 20 min in a water bath sonicator (Bransonic 1015R-MT, 70W output). The maximum tolerated dose of each compound was determined using 6 female and 6 male B6SJL mice by b.i.d. intraperitoneal injection of solubilized compound at escalating doses ranging from 20 mg/kg to 5120 mg/kg. Mice were observed hourly for behavioral changes following each dose. One

week following the final dose animals were sacrificed, tissues were collected and examined for abnormality, and blood samples were collected and analyzed.¹⁹

In vivo ADME assays

In vivo PK profiling and BBB permeation assays were performed by administering a 300 mg/kg bolus intraperitoneal injection of **13** (prepared in solution as above) into B6SJL mice. Blood and brain samples were collected at 0, 1, 3, 6, 12, and 24 h time points and flash frozen in liquid nitrogen, stored at –80 °C, and shipped to Apredica, Inc. for analysis. An untreated group (n = 6) was used as a negative control. The analysis was carried out as described in our previously reported procedure.^{18,31}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAP	arylazanyl pyrazolone
ADME	absorption, distribution, metabolism, excretion
ALS	amyotrophic lateral sclerosis
ASP	arylsulfanyl pyrazolone
BBB	blood brain barrier
CNS	central nervous system
FALS	familial ALS
PBS	phosphate buffered saline
PK	pharmacokinetics
SALS	sporadic ALS
SOD1	Cu/Zn superoxide dismutase

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Figure 1.
Evolution of pyrazolone derivatives as inhibitors against SOD1-dependent protein aggregation and toxicity

Figure 2.
Preliminary tertiary amine pyrazolone **6** and its hydrochloride salt **13**

Figure 3.

Activity of tertiary amine pyrazolone analogues with different *N*-substituents; EC_{50} for **6** is 0.48 μM

Figure 4.
Time-concentration profiles (ip) for compounds **13** and **17** in the blood and brain

Scheme 1.

Synthetic routes for tertiary amine pyrazolones and their salts.^a

^aReagents and conditions: (a) MeOH, room temp, 30 min; then NaBH₄, 0 °C, 1 h; (b) ethyl bromoacetate, K₂CO₃, DMF, room temp, 16 h; (c) ethyl acetate, LiHMDS, THF, -78 °C, 1 h; then 4, -78 °C-room temp, 2 h; (d) NH₂NH₂, EtOH, room temp, 16 h; (e) various acids, EtOAc or THF or EtOH, sonication, room temp, 1 h; (f) AcOH, AgOAc, PdCl₂, NaOAc, 130 °C, 16 h; (g) BH₃·THF, THF, 0 °C-room temp, 3 h; (h) Dess-Martin periodinane, DCM, 0 °C-room temp, 1 h; (i) allyl alcohol, Pd(OAc)₂, LiCl, NaOAc, *n*-Bu₄NBr, 40 °C, 1h.

Table 1In vitro microsomal stability of **1**, **2** and **13**^a

Cmpd	NADPH-dependent			NADPH-absent		
	CL _{int} ^b (mL min ⁻¹ kg ⁻¹)	T _{1/2} ^c (min)	CL _{int} ^b (mL min ⁻¹ kg ⁻¹)	T _{1/2} ^c (min)		
Human	1	25	93	13	173	
	2	0	>180	0	>180	
	13	5	>180	0	>180	
Mouse	1	64	36	21	111	
	2	93	25	0	>180	
	13	45	51	3	>180	

^aData were from Apredica, Inc.^bMicrosomal intrinsic clearance^cHalf-life

Table 2In vitro Caco-2 permeability of **1**, **2**, and **13**^a

Cmpd	P _{app} (A→B) ^b (10 ⁻⁶ cm/s)	P _{app} (B→A) ^b (10 ⁻⁶ cm/s)	Efflux ratio (B→A)/(A→B)
1	36.7	14.1	0.4
2	2.2	7.6	3.5
13	21.5	16.9	0.8

^aData from Apredica^b Apparent permeability

Table 3Activity and water solubility of salts from tertiary amine pyrazolone **6**

Compounds	X ⁻	EC ₅₀ (uM)	Solubility (mg/mL)
6		0.48	<1
13	Cl ⁻	0.51	>20
14	L-tartrate	0.60	5
15	Citrate	ND ^d	3
16	SO ₄ ²⁻	0.68	>20
17	H ₂ PO ₄ ⁻	0.45	>20
18^b	MeSO ₃ ⁻	ND ^d	>20
19^c	CH ₃ COO ⁻	-	-
20^c	PhCOO ⁻	-	-

^a PC12 cell assay; EC₅₀ value was determined according to our previous report²⁰.^b high hygroscopicity^c Not formed.^d Not determined.

Table 4

MTD results of selected tertiary amine pyrazolone salts.

Compounds	MTD (mg/Kg)	Compounds	MTD (mg/Kg)
13^a	640–1280	16^a	640–1280
14	160–320	17	640–1280

^a Mice displayed reduced feeding starting at a dose over 160 mg

Table 5

In vivo pharmacological parameters of compounds **13** and **17**.

Compounds	Plasma		Brain	
	C _{max} (µg/mL)	CL (mL/h/kg)	C _{max} (µg/g)	CL (mL/h/kg)
13	3.18	18.75	2.47	43.19
17	14.50	6.03	12.96	10.04

^a Calculated based on C_{max} and CL data.