AAD-2004, a potent spin trapping molecule and microsomal prostaglandin E synthase-1 inhibitor, shows safety and efficacy in a mouse model of ALS

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Abstract

While free radicals and inflammation constitute major routes of neuronal injury occurring in neurodegenerative diseases, neither antioxidants nor nonsteroidal anti-inflammatory drugs (NSAIDs) have shown significant efficacy in human clinical trials. To explore the possibility that concurrent blockade of free radicals and PGE_2-mediated inflammation might constitute a safe and effective therapeutic approach to certain neurodegenerative diseases, we have developed 2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobezoic acid (AAD-2004) as a derivative of aspirin. AAD-2004 completely removed free radicals at 50 nM as a potent spin trapping molecule and inhibited microsomal prostaglandin E synthase-1 (mPGES-1) with an IC_{50} of 230 nM. Oral administration of AAD-2004 blocked free radical formation, PGE_2 formation, and microglial activation in the spinal motor neurons of $SOD1^{G93A}$ mice. As a consequence, AAD-2004 reduced autophagosome formation, axonopathy, and motor neuron degeneration, improving motor function and increasing life span. In these assays, AAD-2004 was superior to ibuprofen or riluzole. Gastric bleeding was not induced by AAD-2004 even at a dose 400-fold higher than that required to obtain maximal therapeutic efficacy in $SOD1^{G93A}$ mice. Targeting both mPGES-1 and free radicals may be a promising approach to reduce neurodegeneration in ALS and possibly other neurodegenerative diseases.

Introduction

Extensive evidence supports the central role of free radicals in the pathogenesis of amyotrophic lateral sclerosis (ALS) as well as other neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Elevated oxidative products of protein, DNA, and lipid have been reported in the brain, spinal cord, and cerebrospinal fluid (CSF) in subjects with ALS [1-5]. Transgenic ($SOD1^{G93A}$) mice that overexpress mutant SOD1 in familial ALS show motor neuron degeneration, movement deficit, and decreased survival rates [6]. In $SOD1^{G93A}$ mice, oxidative stress is induced in spinal cord regions known to undergo the pathological changes in ALS [7-8]. Excess accumulation of pro-oxidants such as iron is also observed and expected to cause neurodegeneration in familial as well as sporadic forms of ALS [9-10]. A causative role for oxidative stress in the neurodegenerative pathology is supported by experimental findings that administration of antioxidants reduces neurological deficits apparent in $SOD1^{G93A}$ mice [8,11-12]. However, two clinical trials using oral supplementation of vitamin E at either 500 mg/d for 12 months or 1500 mg/d for 18 months produced no beneficial effect on mortality in ALS patients [13-14]. The therapeutic potential of anti-oxidants such as vitamin E, N-acetylcysteine and Coenzyme Q_{10} that were advanced to clinical trial for ALS was limited by side effects and poor BBB permeability [15-17]. Anti-oxidants that are capable of effectively and safely removing free radicals in the nervous system are needed to conduct a proof of concept study for ALS patients.

Inflammation constitutes an additional contributor to the neurodegenerative events observed in ALS. Inflammatory responses such as PGE_2, tumor necrosis factor-alpha (TNF-α), and C-reactive protein are significantly elevated in serum and CSF of patients with ALS [18-20]. In particular, COX-2, the inducible isoform of cyclooxygenase (COX), is induced in neurons, microglia, astrocytes, and endothelial cells in both $SOD1^{G93A}$ mice and patients with ALS [21-22]. COX-2 is thought to mediate inflammation and neuronal injury in the spinal cord of $SOD1^{G93A}$ mice through the generation of PGE_2 [23]. Celecoxib, a selective COX-2 inhibitor,

reduced levels of PGE_2 and neuronal death in the spinal cord and prolonged survival in $SOD1^{G93A}$ mice [24]. However, chronic treatment with the maximum dosage of celecoxib (800 mg/d) for 12 months improved neither motor function nor survival in ALS patients [25]. As levels of PGE_2, a surrogate marker for the pharmacological action of celecoxib, were not reduced in the cerebrospinal fluid of ALS patients treated with celecoxib, it remained to be resolved if selective COX-2 inhibitors would attenuate PGE_2, neurodegeneration, and neurological deficits in ALS patients in the absence of adverse gastric and cardiovascular effects.

While antioxidants or anti-inflammatory drugs (e.g. COX inhibitors) have reduced disease progression in $SOD1^{G93A}$ mice, a combination approach targeting both free radicals and inflammation may synergistically improve motor function and survival. In the $SOD1^{G93A}$ mice, combined treatment of the selective COX-2 inhibitors, celecoxib or rofecoxib, with creatine, a mitochondrial transition pore stabilizer shown to reduce oxidative stress in $SOD1^{G93A}$ mice [26], resulted in additive neuroproection and survival compared to the COX-2 inhibitors alone [27]. A phase II clinical trial of combination therapy for ALS patients revealed that the combination of celecoxib and creatine produced slower deterioration in the ALS Functional Rating Scale-Revised than the historical controls or the combination of minocycline and creatine [28]. Thus, concurrent blockade of free radicals and PGE_2-mediated inflammation may provide better therapeutic outcome than monotherapy for intervention of neurodegenerative process and neurological deficits in ALS patients. We have investigated the premise that a single agent combining the anti-inflammatory attributes of aspirin with powerful anti-oxidant efficacy would constitute an effective disease modifying therapeutic for ALS, based on the additive/synergistic neuroprotective effects of these two actions. We took a structural lead from sulfasalazine, and developed synthetic derivatives conjugated to 5-aminosalicylate that prevent free radical formation, as well as inflammation without causing gastric damage. AAD-2004 was chosen as a final drug candidate, based upon safety and efficacy profile

through multiple in vitro and in vivo screening processes.

Result

AAD-2004 blocks free radical neurotoxicity as a potent spin trapping molecule.

Mixed cortical cell cultures containing neurons and glia produced reactive oxygen species (ROS) within 4 h and widespread neuronal death over 24 h after continuous exposure to 50 μM Fe^{2+}, a transition metal ion catalyzing hydroxyl radicals from H_2O_2. Concurrent addition of 1 μM AAD-2004 blocked Fe^{2+}-induced ROS production and neuronal death (Fig. 1a). The efficacy and potency of AAD-2004 were compared to those of antioxidants that were included in clinical trials for treatment of neurodegenerative diseases. Vitamin E, a free radical scavenger, attenuated Fe^{2+} neurotoxicity in a dose-dependent manner (IC_{50} = 22.03 μM). Estrogen and melatonin revealed an IC_{50} of 2.41 μM and 311.6 μM in reducing Fe^{2+} neurotoxicity, respectively. However, administration of acetyl-L-carnitine up to 1 mM slightly reduced Fe^{2+}-induced neuronal death. In contrast, AAD-2004 showed IC_{50} of 0.097 μM and all but completely blocked Fe^{2+} neurotoxicity even at 0.3 μM (Fig. 1b), suggesting that AAD-2004 has better efficacy and potency against free radical neurotoxicity than the other antioxidants examined. AAD-2004 also protected against free radical injury by DL-buthionine-[S,R]-sulfoximine, a glutathione-depleting agent, and sodium nitroprusside, a nitric oxide donor (Unpublished data). As salicylate and acetyl salicylate (aspirin) can scavenge free radicals at millimolar concentrations [29-30], the antioxidant action of AAD-2004 may be attributable to direct scavenging of free radicals. Salicylate, aspirin, and sulfasalazine slightly reduced levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical that is widely used to analyze radical scavenging activity. Compared to the limited scavenging action of the salicylates, AAD-2004 rapidly reacted with DPPH with potency higher than vitamin E, suggesting that AAD-2004 is a potent free radical scavenger (Fig. 1c). The free radical scavenging action of AAD-2004 was further examined using the spectroscopic technique of

electron spin resonance (ESR). 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), a spin trapping agent, reacted with hydroxyl radicals, producing the ESR spectra of DMPO-OH (Fig. 1d). The addition of AAD-2004 reduced levels of DMPO-OH in a dose-dependent manner. The ESR spectra of DMPO-OH were almost completely blocked in the presence of AAD-2004 as low as 50 nM, demonstrating that AAD-2004 is a potent spin trapping molecule.

AAD-2004 is an mPGES-1 inhibitor and does not cause gastric damage.

AAD-2004 was derived from aspirin and thus expected to prevent inflammation as a COX inhibitor. The IC_{50} values of AAD-2004 for ovine COX-1 and COX-2 were 334.75 μM and 21.47 μM, respectively (Fig. 2a). However, AAD-2004 prevented PGE_2 production following exposure of BV2 cells to lipopolysaccharide (LPS) with IC_{50} of 1.4 μM (Fig. 2b). This implies that AAD-2004 inhibits PGE_2 production through another target besides cyclooxygenases. We examined the possibility that AAD-2004 would inhibit mPGES-1, an isomerase converting COX-derived PGH_2 to PGE_2 [31]. Addition of PGH_2 in extracts of LPS-treated BV2 cells resulted in increased PGE_2 production in the presence of excess indomethacin, a dual COX-1/COX-2 inhibitor, compared to those of control BV2 cells, suggesting that the bacterial endotoxin induces mPGES-1-mediated PGE_2 production as previously reported [32]. AAD-2004 inhibited the conversion of PGH_2 to PGE_2 by mPGES-1 with an IC_{50} of 0.23 μM (Fig. 2c). Oral administration of 200 mg/kg ibuprofen and 300 mg/kg aspirin caused severe gastric damage 24 h later (Fig. 2d). Celecoxib, a selective COX-2 inhibitor, also produced mild gastric damage at an extremely high dose of 1000 mg/kg. However, oral administration of 1000 mg/kg AAD-2004 did not damage gastric mucosal membrane. Interestingly, aspirin-induced gastric damage was prevented by co-administration of trolox, a vitamin E analogue. This implies that AAD-2004 prevents inflammation with reduced gastric risk possibly due to selectivity to mPGES-1 and spin-trapping property.

AAD-2004 blocks oxidative stress and inflammation in $SOD1^{G93A}$ mice.

As previously reported [8], $SOD1^{G93A}$ mice revealed marked oxidative stress in motor neurons of the lumbar spinal cord at 10 weeks of age as evident by increased immunoreactivity to nitrotyrosine and 8-OHdG (Fig. 3a). Administration of AAD-2004 (*i.p.*, b.i.d) from 8 weeks of age alleviated motor function deficits and increased survival in $SOD1^{G93A}$ mice. Maximal effects were observed from doses of 2.5 mg/kg (supplementary fig. 1). In mice, the oral administration of 2.5 mg/kg AAD-2004 showed an area under the curve (AUC) of 7.7 μg.h/mL which was 2-fold higher than the intraperitoneal administration of 2.5 mg/kg AAD-2004 (Unpublished data). Thus, the pharmacological effects of AAD-2004 were examined by the oral administration of 2.5 mg/kg (b.i.d.) in $SOD1^{G93A}$ mice. The administration of AAD-2004 from 8 weeks of age significantly blocked elevated levels of nitrotyrosine and 8-OHdG in the lumbar spinal cord of $SOD1^{G93A}$ mice at 10 weeks of age (Fig. 3a,b). The number of microglia immunoreactive to Iba-1 (ionized calcium-binding adaptor molecule-1), a marker of activated microglia/macrophage, and Iba-1 expression were increased in the ventral horn of the lumber spinal cord of $SOD1^{G93A}$ mice at 16 weeks of age compared to the wild type (Fig. 4a). The presence of Iba-1 positive microglia was prevented by AAD-2004 as evidenced by a decrease in immunoreactivity in spinal cord sections as well as Western blots (Fig. 4a-c). In addition, immunohistofluorescence studies revealed that the expression of mPGES-1 was increased throughout the lumbar ventral horn of $SOD1^{G93A}$ mice at 16 weeks of age (Fig. 4d-f). PGE_2 levels were significantly increased in the lumbar spinal cord and also in plasma of $SOD1^{G93A}$ mice, which was significantly reduced following administration of AAD-2004 (Fig. 4g). As the maximum plasma concentration of AAD-2004 is approximately 8.1 μM following the oral administration of 2.5 mg/kg in $SOD1^{G93A}$ mice (unpublished data), AAD-2004 is expected to prevent inflammation in the lumbar spinal cord of $SOD1^{G93A}$ mice through blockade of mPGES-1.

AAD-2004 prevents motor neuron degeneration, axonal damage, and autophagosome

formation in the lumbar spinal cord of SOD1^{G93A} mice.

Widespread motor neuron degeneration was observed in the ventral horn of the lumbar spinal cord in 16-week-old SOD1^{G93A} mice. The administration of 2.5 mg/kg AAD-2004 beginning 8 weeks of age significantly prevented the loss of spinal motor neurons in the SOD1^{G93A} mice compared to vehicle treatment (Fig. 5a, b). Immunohistochemistry with the tau-5 antibody further demonstrated degradation of cell bodies and axons originating from the motor neurons (Fig. 5c). Such degenerative changes were significantly ameliorated by the administration of AAD-2004 (Fig. 5c, d). However, the axonopathy was not prevented by ibuprofen or riluzole, a disease-modifying neuroprotectant known to reduce glutamate neurotoxicity and used as the only approved treatment for ALS. The conversion of LC3-I to LC3-II, microtubule-associated protein 1 light chain 3-II, known as a marker for autophagosome formation, was induced in the lumbar spinal cord of 16-week-old SOD1^{G93A} mice as previously reported [33]. The conversion to LC3-II was not observed in SOD1^{G93A} mice treated with AAD-2004 (Fig. 5e, f). In addition, administration of AAD-2004 also blocked the abnormal aggregation of mutant SOD1 observed in the lumbar spinal cord of SOD1^{G93A} mice (Fig. 5g).

AAD-2004 shows better beneficial effects than ibuprofen or riluzole in SOD1^{G93A} mice.

Finally, we carried out a study comparing the functional efficacy of AAD-2004 with that of riluzole or ibuprofen, a nonselective COX inhibitor that inhibited microglial activation and PGE_2 production in the lumbar spinal cord of SOD1^{G93A} mice (unpublished data). As reported [34], SOD1^{G93A} mice that orally received a maximally effective dose of riluzole revealed significant improvement in motor function and survival (Fig. 6). Administration of 25 mg/kg ibuprofen improved motor function and extended life span in SOD1^{G93A} mice comparable to riluzole. SOD1^{G93A} mice treated with 2.5 mg/kg AAD-2004 showed significantly better motor function and survival relative to riluzole or ibuprofen. The onset of Rotarod deficits was

significantly delayed by 12% and 15.6% in the riluzole and ibuprofen -treated groups, respectively, as compared with the control group. The disease onset was further delayed by 36% in $SOD1^{G93A}$ mice treated with AAD-2004 (Fig. 6e). Survival was extended by 8.2%, 9.4%, and 21% in the riluzole, ibuprofen, or AAD-2004-treated groups. While there was no difference in the disease onset and survival between the riluzole and ibuprofen groups, the AAD-2004 group significantly improved motor performance and survival compared to the riluzole or ibuprofen groups.

Discussion

AAD-2004, a dual function drug derived from aspirin and sulfasalazine, has been developed to protect against both free radicals and PGE_2-mediated inflammation associated with certain forms of neurodegeneration in the central nervous system (CNS). AAD-2004 is a potent spin trapping molecule and mPGES-1 inhibitor effective at nanomolar concentrations. Administration of AAD-2004 improves motor function and survival in $SOD1^{G93A}$ mice with a maximally effective dose of 2.5 mg/kg while no gastric damage was observed following oral administration of doses as high as 1000 mg/kg. AAD-2004 blocks oxidative stress and inflammation through inhibition of mPGES-1-mediated PGE_2 production in $SOD1^{G93A}$ mice, which results in blockade of neuronal death, axonopathy, and autophagosome formation normally observed in the lumbar spinal cord of these mice. As a consequence, blockade of oxidative stress and mPGES-1-mediated inflammation significantly extended disease onset and survival compared to riluzole and ibuprofen.

Salicylate (2-hydroxybenzoate) can react with hydroxyl radical to produce catecol, 2,3-dihydroxybenzoate, and 2,5-duhydroxybenzoate that act as a free radical trap [40]. However, salicylate weakly reacts with DDPH and does not reduce Fe^{2+}-induced free radical injury up to 1 mM, suggesting that salicylate is a poor anti-oxidant. Interestingly, sulfasalazine and 5-

aminosalicylate prevented Fe^{2+}-induced free radical neurotoxicity at ~ 30 μM [41]. The antioxidant effects of sulfasalazine and 5-aminosalicylate appear to be related with p-amine relative to the hydroxyl group of salicylate that increases stability of the peroxyl radical [30]. Furthermore, the anti-oxidant potency and efficacy of AAD-2004 were remarkably increased with the electron-rich moiety (4-trifluoromethylpheny group) linked to p-amine that favors reaction with hydroxyl radical.

In light of anti-inflammatory actions of salicylates as inhibitors of cyclooxygenases, we reasoned that AAD-2004 would inhibit COX-2. AAD-2004 was indeed a direct COX-2 selective inhibitor with IC_{50} of 21.47 μM, but reduced LPS-induced PGE_2 production with IC_{50} of 1.4 μM in BV2 cells. This led us to examine mPGES-1, an inducible terminal isomerase catalyzing PGE_2 biosynthesis, as a potential target of AAD-2004. AAD-2004 reduced activity of mPGES-1 with IC_{50} of 0.23 μM in extracts of LPS-treated BV2 cells. Thus, AAD-2004 is expected to selectively reduce PGE_2 production as an mPGES-1 inhibitor at nanomolar concentrations while it prevents production of PGI_2 as well as PGE_2 as a moderate COX-2 selective inhibitor at high doses (≥20 μM).

mPGES-1 mediates inflammatory responses in the CNS as well as peripheral inflammation [32]. Expression of mPGES-1 was sparsely detectable in normal brain but markedly increased in brain endothelial cells and the paraventricular nucleus of the hypothalamus during fever, arthritis, and burn injury in rodents [42-45]. Genetic deletion of *mPGES-1* was shown to reduce levels of PGE_2 in the CSF and fever following exposure to peripheral LPS injection [46], suggesting that mPGES-1-dependent PGE_2 production is a mediator of CNS inflammation. Increased expression of mPGES-1 was also observed in neurons, astrocytes, and microglia as well as endothelial cells in postmortem brain of AD [47]. We found that levels of PGE_2 and mPGES-1 were significantly increased in the lumbar spinal cord of $SOD1^{G93A}$ mice. The latter was observed in neurons, astrocytes, microglia, and endothelial cells in the ventral horn undergoing widespread neuronal death and inflammation

in SOD1^{G93A} mice (Unpublished data). The plasma concentration profiles of AAD-2004 after single or 4-week oral administration of 2.5 mg/kg show a maximal concentration of ~ 2.7 μg/ml (~ 8 μM) within 30 min after the final dosing and blood-brain barrier (BBB) permeability of AAD-2004 is 3 – 5 % in mouse and rat. Therefore, it can be suggested that AAD-2004 prevents inflammation in SOD1^{G93A} mice primarily by inhibiting mPGES-1-mdiated PGE_2 production in the spinal cord.

As an mPGES-1 inhibitor selectively lowering PGE_2 production, AAD-2004 appears to show better safety than conventional NSAIDs including selective COX-2 inhibitors that cause the risk of cardiovascular infarction and thrombosis by preventing production of vascular prostacyclin (PGI_2) as well as adverse gastrointestinal events[48]. The pharmacological property of AAD-2004 as a spin trapping molecule provides an additional safety profile. This is supported by recent reports demonstrating that anti-oxidants or free radical scavengers such as vitamin E, melatonin, DL-alpha-tocopherol, and L carnitine protect against NSAIDs-induced gastric injuries in rats [36, 49-51]. In line with this, oral administration of trolox dramatically attenuates aspirin-induced gastric bleeding in rat. Thus, the dual pharmacological properties of AAD-2004 are appropriate for intervention of chronic PGE_2-mediated inflammation and free radical production in the CNS with reduced adverse effects.

Although either anti-oxidants or NSAIDs improve motor function and prolonged life span in SOD1^{G93A} mice, none of them have shown significant benefits in the translational clinical studies for ALS patients [52-53]. Such unsatisfactory outcomes may be attributable to low numbers of patients and short duration of the trials, but may also be associated with low permeability of anti-oxidants through the BBB and adverse effects of NSAIDs that limit pharmacological action of anti-oxidants or NSAIDs in the CNS [54-55]. It is of note that combined treatment of celecoxib and creatine improves motor function in a randomized clinical trial phase II of ALS patients as well as SOD1^{G93A} mice [29], suggesting better efficacy of combined antioxidant and NSAID therapy than monotherapy. AAD-2004 blocked free

radical production and PGE_2-mediated inflammatory responses induced in the spinal cord of $SOD1^{G93A}$ mice. Compared to beneficial effects of ibuprofen or riluzole in $SOD1^{G93A}$ mice, AAD-2004 significantly improved survival and onset of motor function deficit approximately up to 2 to 3-fold. Thus, concurrent blockade of free radicals and mPGES-1-mediated PGE_2 production with AAD-2004 has potential to improve neurological function and survival in ALS patients with a better safety profile compared to NSAIDs.

AAD-2004 and ibuprofen attenuate motor neuron death in the ventral horn of the lumbar spinal cord as riluzole or other COX-2 inhibitors do in $SOD1^{G93A}$ mice [25]. The loss of ventral root axons correlates well with motor function deficit and appears before motor neuron death in $SOD1^{G93A}$ mice and ALS patients [56]. While neither riluzole nor ibuprofen attenuated degeneration of ventral root axons, AAD-2004 significantly protected the axons in $SOD1^{G93A}$ mice. Impaired autophagy has been proposed as a cause of progressive dystrophy and degeneration of axons [37-38, 57]. Administration of AAD-2004 prevented levels of LC3-II and SOD1 aggregates that were increased in the ventral horn of lumbar spinal cord in $SOD1^{G93A}$ mice. AAD-2004 prevents abnormal protein aggregates and autophagosome formation possibly by blocking free radicals and PGE_2-mediated inflammation. In support of this, iron or mitochondrial reactive oxygen species are shown to induce autophagy and autophagic cell death [58-59]. By inhibiting abnormal protein aggregation and axonopathy, AAD-2004 produces better motor function and survival in $SOD1^{G93A}$ mice than COX-2 inhibitors or riluzole.

Undoubtedly, free radicals and PGE_2-dependant inflammation contribute to progression of neuronal damage and neurological deficit in ALS. Neither antioxidants nor NSAIDs, however, showed significant efficacy in ALS patients due to poor BBB permeability and drug-related adverse effects at therapeutic doses. AAD-2004 blocks free radical production and inflammation in vitro and in $SOD1^{G93A}$ mice by scavenging free radicals as a spin trapping molecule and preventing PGE_2 production as an mPGES-1 inhibitor. With the dual

pharmacological actions, AAD-2004 did not cause gastric damage at a dose 400-fold higher than efficacy doses in $SOD1^{G93A}$ mice, prevented protein aggregation and axonopathy, and improved neurological function and survival better than NSAIDs or riluzole. A phase I study of AAD-2004 in healthy human volunteers has demonstrated a safety profile that AAD-2004 does not produce serious adverse events at doses higher than the therapeutic target dose determined in $SOD1^{G93A}$ mice (unpublished data). The present findings support the need for a novel medication that exhibits concurrent blockade of free radicals and mPGES-1 as a means to combat devastating neuronal cell loss in ALS and also has implications for the treatment of other neurodegenerative diseases including AD and PD.

Methods

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

B.J.G designed and directed the study.; J.H.S. and J.K.L. performed the *in vivo* experiments.; Y.A.L. performed free radical neurotoxicity experiments *in vitro.*; Y.B.L, W.C., and J.H.S. designed and performed mPGES-1 activity assay *in vitro*.; Y.A.L. and J.H.S. performed COX activity assay *in vitro*.; D.S.I., J.H.L., and S.Y.B. performed gastric damage experiments.; S.J.S., S.M.P., and S.Y.B prepared for experiments.; J.H.S., S.J.S., S.M.P., and S.Y.B. discussed the experimental data.; J.E.S. discussed the manuscript; J.H.S. and B.J.G. wrote the manuscript.

Online Methods

Electron spin resonance (ESR) spin trapping assay

Hydroxyl radicals were generated using a Fenton reaction system and reacted with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), a spin trapping agent. The ESR spectra of the resultant DMPO-OH adducts were measured as described [60]. In brief, DMPO-OH adducts

were produced by reacting 50 μM $FeSO_4$, 1 mM H_2O_2, and 1 mM DMPO in a PBS (pH 7.4) for 2.5 m. The ESR spectra were recorded using an ESR spectrometer (JEOL, JES-TE300, Tokyo Japan) set at the following conditions: microwave power 1.01 mW, modulation frequency 100 kHz, modulation amplitute 5.0 mT, response time 30 s, received gain 4.0 x100, scan constant 200 s, and cell temperature 24°C.

mPGES-1 activity assay

mPGES-1 activity in cell lysates was analyzed by measuring the conversion of PGH_2 to PGE_2 as previously reported [35]. mPGES-1 activities in cell lysates were measured by assessment of the conversion of PGH_2 to PGE_2. The cells were scraped from the dishes and disrupted by sonication (10 s, three times, at 1 m intervals) in 250 μL 0.2 M Tris-HCL, pH8.0. After centrifugation of the sonicates at 15000 rpm for 10 m at 4 °C, the supernatant fluids were used as the enzyme source. An aliquot of each lysate (90 μg protein equivalents) was incubated with 2 μg PGH_2 for 30 s at 24 °C in 100 μM 0.1 M Tris-HCL, pH 8.0, containing 2 mM glutathione and 14 μM indomethacin. After terminating the reaction by the addition of 100 mM $FeCl_2$, PGE_2 contents in the supernatant fluids were quantified using an EIA kit (Cayman Chemical Cat. No. 514010 for PGE_2,).

Mice and treatment regimens

Animal care and treatment were in compliance with a protocol approved by the institutional animal care committee. $SOD1^{G93A}$ mice carrying the G93A human SOD1 mutation were obtained from the Jackson Laboratory (Bar Harbor, ME). Male G93A transgenic mice were crossbred with B6SJLF1/J hybrid females as previously described [6]. Mice were orally, peritoneally, or dietary treated with AAD-2004, ibuprofen, or riluzole beginning at 8 weeks of age. Nontransgenic litter mates were used as controls.

Evaluation of motor function

Motor function was evaluated by analysis of Rotarod and PaGE as previously reported [8]. **(Supplementary Methods)**

Stereological analysis of motor neuron survival

The whole lumbar spinal segment (L1~L5) were coronally cut into thickness of 40 μm using Cryocut Microtome (Leica Microsystems, Wetzlar). Neuronal death was analyzed by staining every 10th section with 0.5% cresyl violet and counting viable motor neurons larger than 20 μm in the ventral lumbar region. To estimate the total number of motor neuron, the optical fractionator method was used. **(Supplementary methods)**

Preparation of 2-hydroxy-5-[2-(4-trifluoromethylphenyl)ethylamino]benzoic acid (AAD-2004)

AAD-2004 was synthesized at Zhejian Avilive Laboratories Ac., Ltd. (China). In brief, methyl 2-hydroxy-5-[2-(4-trifluoromethylphenyl) ethylamino] benzoate was produced by condensation of methyl 5-aminosalicylate and 2-(4-trifluoromethylphenyl) ethyl methanesulfonate in the presence of triethylamine and then hydrolyzed.

Statistical analysis

All data performed on cell cultures and animals are expressed as the mean ± S.E.M. An independent-samples t-test was used to compare two samples. Analysis of ANOVA and the Student Newman-Keuls test were used for multiple comparisons. Survival data are analyzed by means of Kaplan-Meier survival curve. All analyses were calculated using the SPSS version 12.0 from windows. Statistical significance was set at $p < 0.05$

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Figure legend

Figure 1 AAD-2004 is a potent spin trapping molecule.

(a) Upper panel: Fluorescence photomicrographs showing 2',7'-dichlorofluorescein (DCF), the oxidation product of 2',7'-dichlorodihydrofluorescein (DCDHF), in cortical cell cultures (DIV 12 – 14) after 4 h exposure to a sham control or 50 μM Fe^{2+}, alone (Fe^{2+}) or with 1 μM AAD-2004. Bottom panel: Phase contrast photomicrographs of cortical cell cultures after 16 h exposure. Note Fe^{2+}-induced neuronal cell degeneration (arrows) sensitive to AAD-2004. (b) Cortical cell cultures (DIV 12 - 14) were exposed to 50 μM Fe^{2+} for 24 h, alone or with the indicated doses of AAD-2004, vitamin E, melatonin, estrogen, or acetyl-L-carnitine. Neuronal death was analyzed 24 h later by measuring LDH efflux into the bathing media (n = 4 – 24 culture wells per condition). (c) Free radical scavenging action was analyzed by measuring the reduction of DPPH with addition of AAD-2004, sulfasalazine, salicylic acid, aspirin, or vitamin E (n = 3 per condition). (d) The ESR spectra of DMPO-OH adducts were obtained from the reaction of hydroxyl radicals and DMPO, alone (Control) or with addition of AAD-2004.

Figure 2 AAD-2004 inhibits mPGES-1 activity without producing gastric damage.

(a) IC_{50} values of AAD-2004 against COX-1 and COX-2 determined using a colorimetric ovine COX inhibitor screening assay. (b,c) BV2 cells were exposed to 1 μg/ml LPS for 24 h, alone or in the presence of AAD-2004. (b) Levels of PGE_2 were analyzed by an enzyme immunoassay and scaled to LPS-treated group (=100%) (n = 8 cultures for each condition). (c) mPGES-1 activity was analyzed by measuring PGE_2 converted from PGH_2 in BV2 cell lysates added with vehicle (=100%) or AAD-2004 (n = 4 cultures for each condition). (d) Representative photomicrographs of rat stomach 24 h after the oral administration of vehicle, 200 mg/kg ibuprofen, 1000 mg/kg celecoxib, 1000 mg/kg AAD-2004, 300 mg/kg aspirin, or

300 mg/kg aspirin plus 100 mg/kg trolox.

Figure 3 AAD-2004 blocks oxidative stress in $SOD1^{G93A}$ mice.

(a) Fluorescent and bright-field photomicrographs of lumbar ventral sections immunolabeled with an antibody against nitrotyrosine (top panel; scale bar, 20 μm) or 8-OHdG (bottom panel; scale bar, 50 μm) in wild type or $SOD1^{G93A}$ mice treated with saline (vehicle) or 2.5 mg/kg AAD-2004 (*p.o.*, b.i.d) for 2 weeks starting from 8 weeks of age. (b) Levels of nitrotyrosine and 8-OHdG were analyzed by measuring immunofluorescence intensity of nitrotyrosine in the lumbar motor neurons (n = 20 sections from 5 mice per condition), and using an enzyme immunoassay of 8-OHdG in the lumbar spinal cord (n = 5 mice for each condition). * Significant difference from wild type; # significant difference from vehicle, at $p < 0.05$.

Figure 4 AAD-2004 prevents microglia activation and PGE_2 production in $SOD1^{G93A}$ mice.

(a) Bright-field photomicrographs of lumbar ventral sections stained with an Iba-1 antibody, a marker of activated microglia/macrophage, in wild type or $SOD1^{G93A}$ mice treated with saline (vehicle) or 2.5 mg/kg AAD-2004 for 8 weeks starting from 8 weeks of age (scale bar, 100 μm) (b,c) Western blot analysis of Iba-1 in lumbar spinal cord of wild type and $SOD1^{G93A}$ mice. Levels of Iba-1 were measured and scaled to those of actin (n = 5 mice for each condition) (d) Fluorescent photomicrographs of lumbar ventral sections immunolabeled with an mPGES-1 antibody from wild type and $SOD1^{G93A}$ mice (scale bar, 20 μm) (e,f) Western blot analysis of mPGES-1 in lumbar spinal cord from wild type and $SOD1^{G93A}$ mice. Levels of mPGES-1 were scaled to actin (n = 5 mice for each condition) (g) Levels of PGE_2 were analyzed in lumbar spinal cord and plasma from wild type and $SOD1^{G93A}$ mice treated with vehicle or AAD-2004. * Significant difference from wild type; #, significant difference from vehicle, at $p < 0.05$.

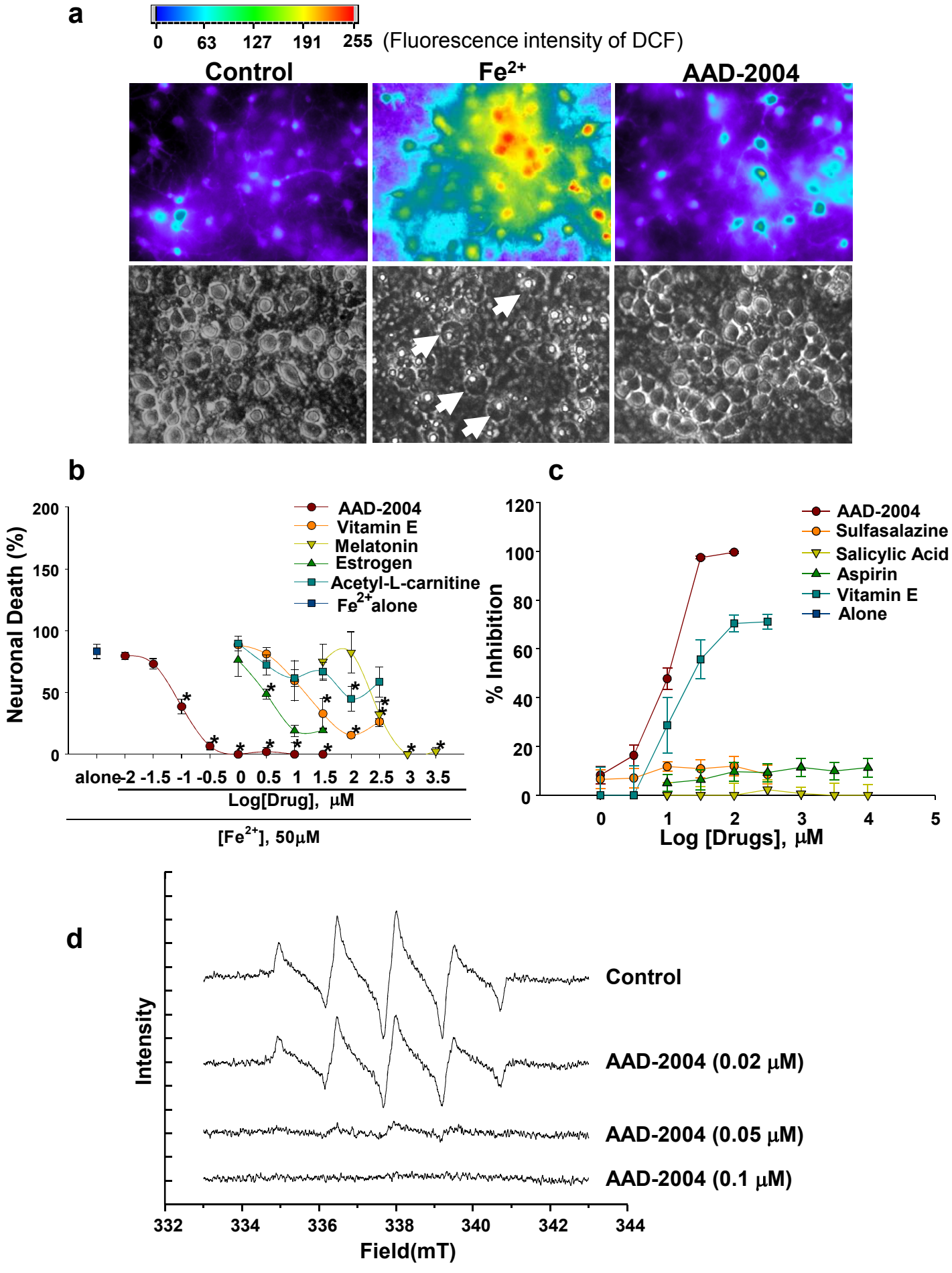
Figure 5 AAD-2004 prevents motor neuron degeneration, axonopathy, and autophagosome formation in SOD1^{G93A} mice.

(a,b) (a) Bright-field photomicrographs of the lumbar ventral horn stained with cresyl violet in wild type and SOD1^{G93A} mice following 8-week administration of vehicle or 2.5 mg/kg AAD-2004 from 8 weeks of age (scale bar, 20 μm) (b) The number of viable motor neurons was stereologically analyzed (n = 5~6 mice per group) (c) Fluorescent photomicrographs of the lumbar ventral horn immunolabeled with a tau-5 antibody in wild type and SOD1^{G93A} mice following 8-week administration of vehicle, 2.5 mg/kg AAD-2004 (*p.o.*, b.i.d), or 25 mg/kg ibuprofen (*i.p.*,b.i.d) from 8 weeks of age (scale bar, 100 μm) (d) Western blot analysis of tau-5 in lumbar spinal cord from wild type and SOD1^{G93A} mice treated with vehicle, AAD-2004, ibuprofen, or riluzole (50 mg/kg/d in diet). Levels of tau-5 were measured and scaled to those of actin (n = 5 for each condition) (e,f) Western blot analysis of LC3-I and LC3-II in lumbar spinal cord from wild type and SOD1^{G93A} mice treated with vehicle or AAD-2004. Levels of LC3-II were scaled to those of actin (n = 5 for each condition) (g) Western blot analysis of mutant hSOD1 aggregates in the lumbar spinal cord from wild type (WT) and SOD1^{G93A} mice treated with vehicle (veh) or AAD-2004 (AAD). * Significant difference from wild type; $^{\#}$, significant difference from vehicle, at $p < 0.05$.

Figure 6 AAD-2004 shows better motor function and survival than ibuprofen or riluzole in SOD1^{G93A} mice

SOD1^{G93A} mice received vehicle or 25 mg/kg ibuprofen, 2.5 mg/kg AAD-2004, or 50 mg/kg riluzole from 8 weeks of age. (a,b) Motor function was analyzed using Rotarod test (a) and PaGE test (b) at indicated points of age (n = 14 per group). (c,d) Cumulative probability of onset of Rotarod deficits (c) and survival (d). (e) Onset of Rotarod deficits and mortality of SOD1^{G93A} mice. $^{a}p<0.05$ compared with vehicle group; $^{b}p<0.05$ compared with AAD-2004

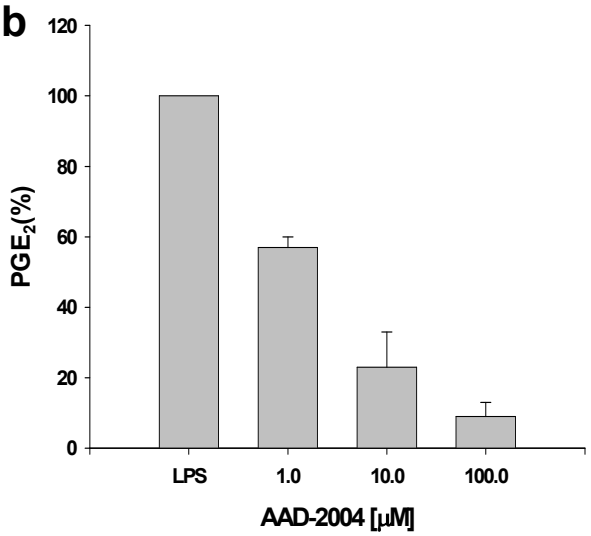
and with ibuprofen or riluzole.


a
0
63
127
191
255
(Fluorescence intensity of DCF)
Control
Fe2+
AAD-2004
b
Neuronal Death (%)
200
150
100
50
0
AAD-2004
Vitamin E
Melatonin
Estrogen
Acetyl-L-carnitine
Fe2+alone
alone -2 -1.5 -1 -0.5 0 0.5 1 1.5 2 2.5 3 3.5
Log[Drug], μM
[Fe2+], 50μM
c
% Inhibition
120
100
80
60
40
20
0
AAD-2004
Sulfasalazine
Salicylic Acid
Aspirin
Vitamin E
Alone
0 1 2 3 4 5
Log [Drugs], μM
d
Intensity
Control
AAD-2004 (0.02 μM)
AAD-2004 (0.05 μM)
AAD-2004 (0.1 μM)
332 334 336 338 340 342 344
Field(mT)

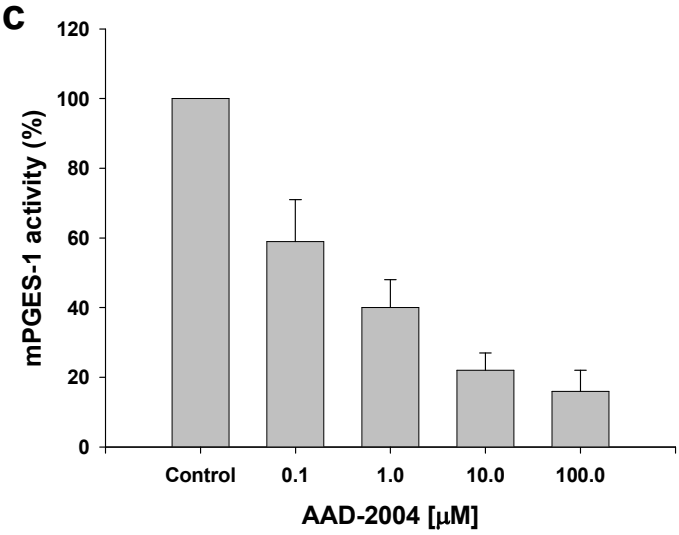
a

IC_{50} (μM)	COX-1	COX-2	COX-1/COX-2
AAD-2004	334.75± 131.4	21.47 ± 10.9	15.6

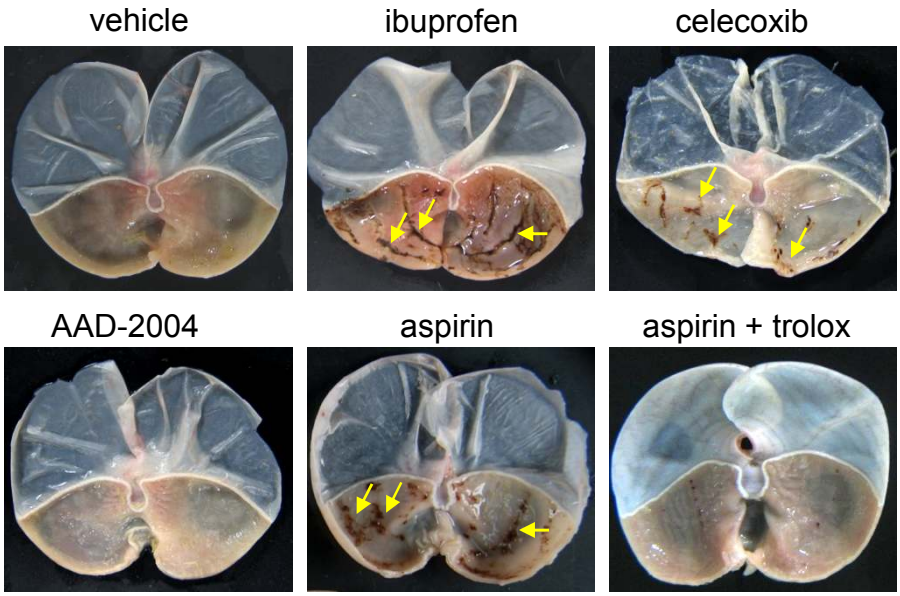
b

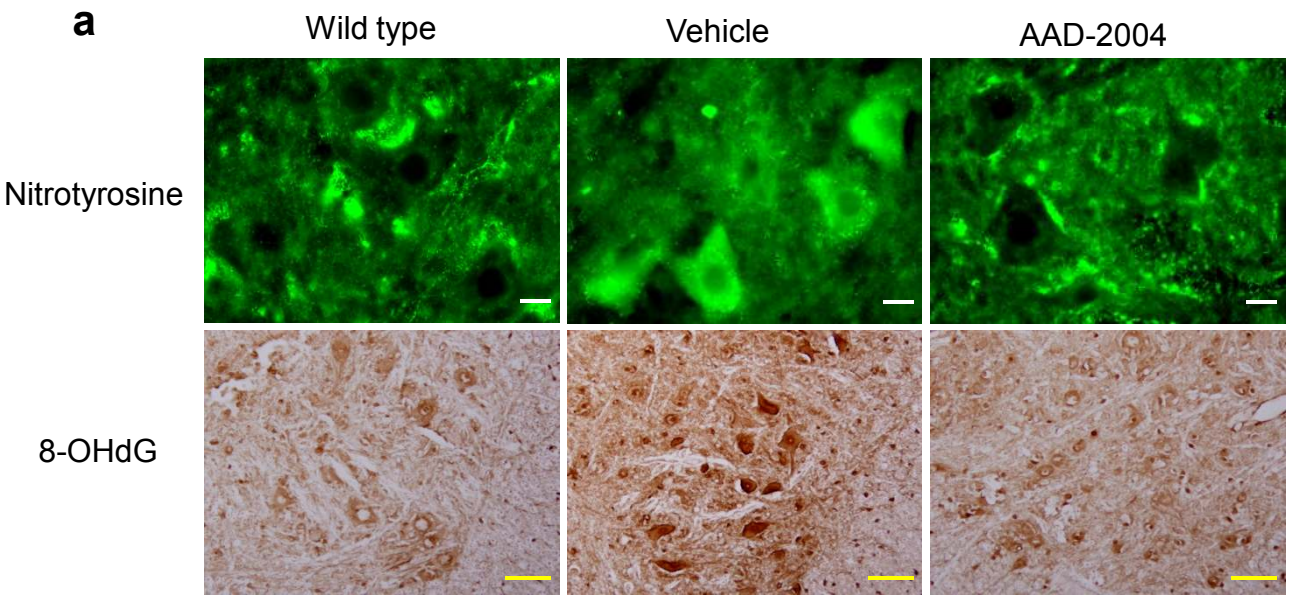


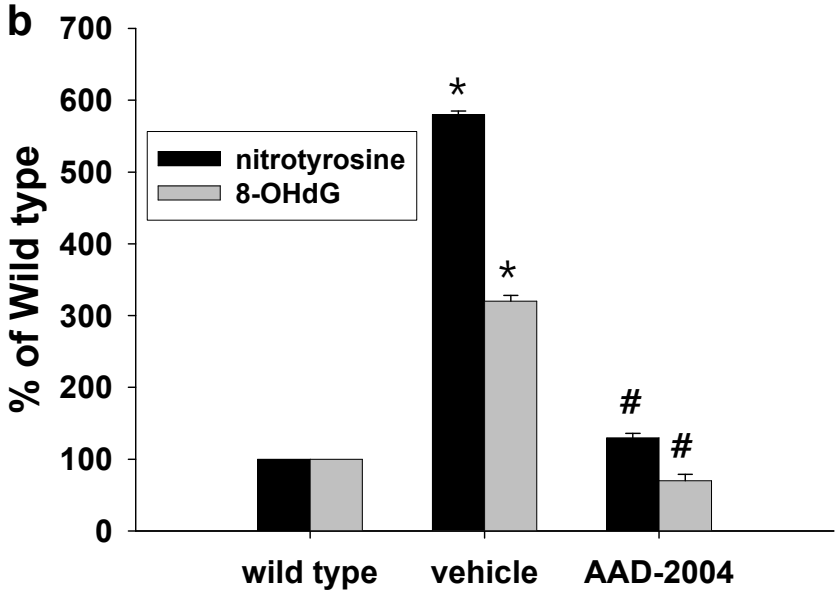
c

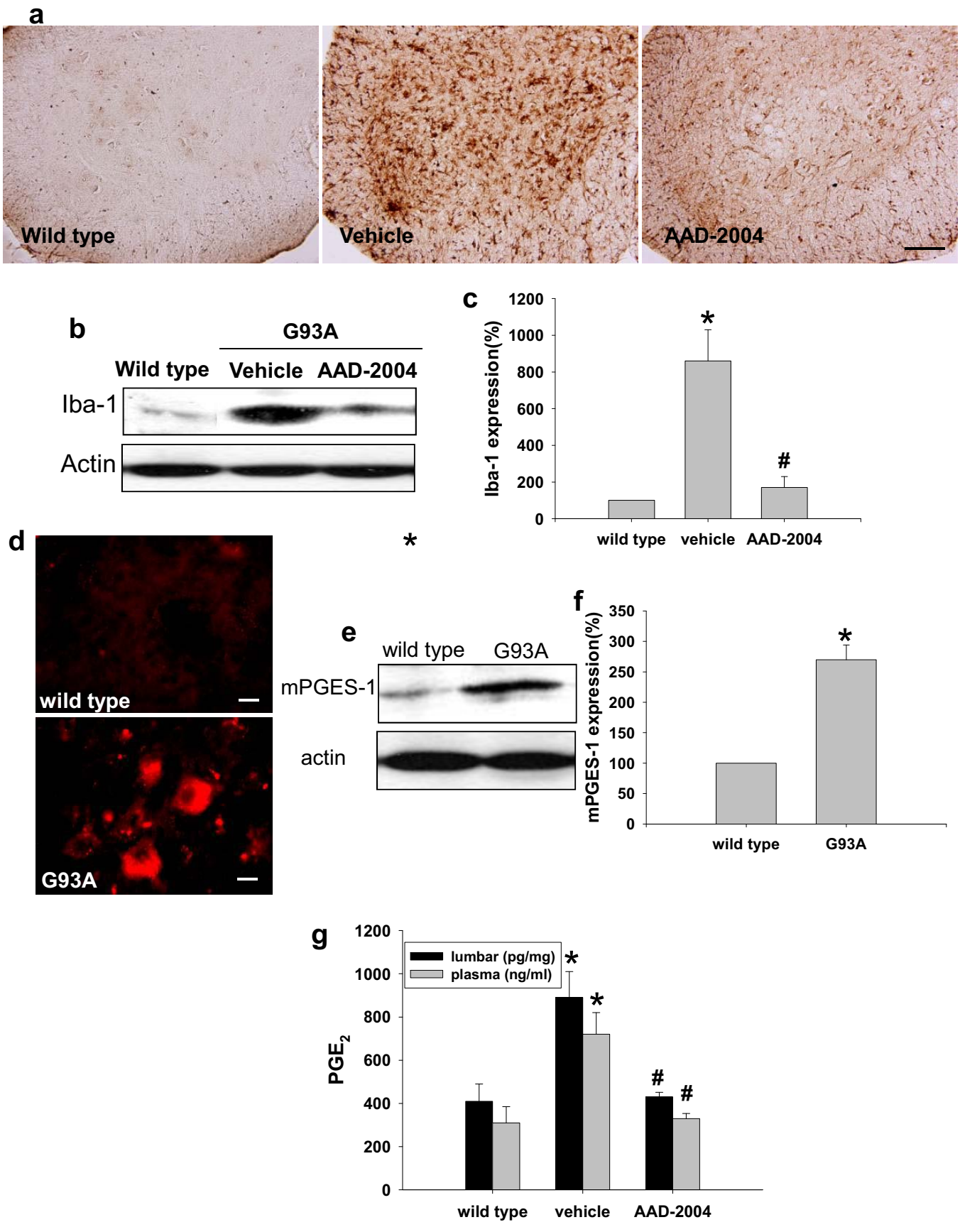


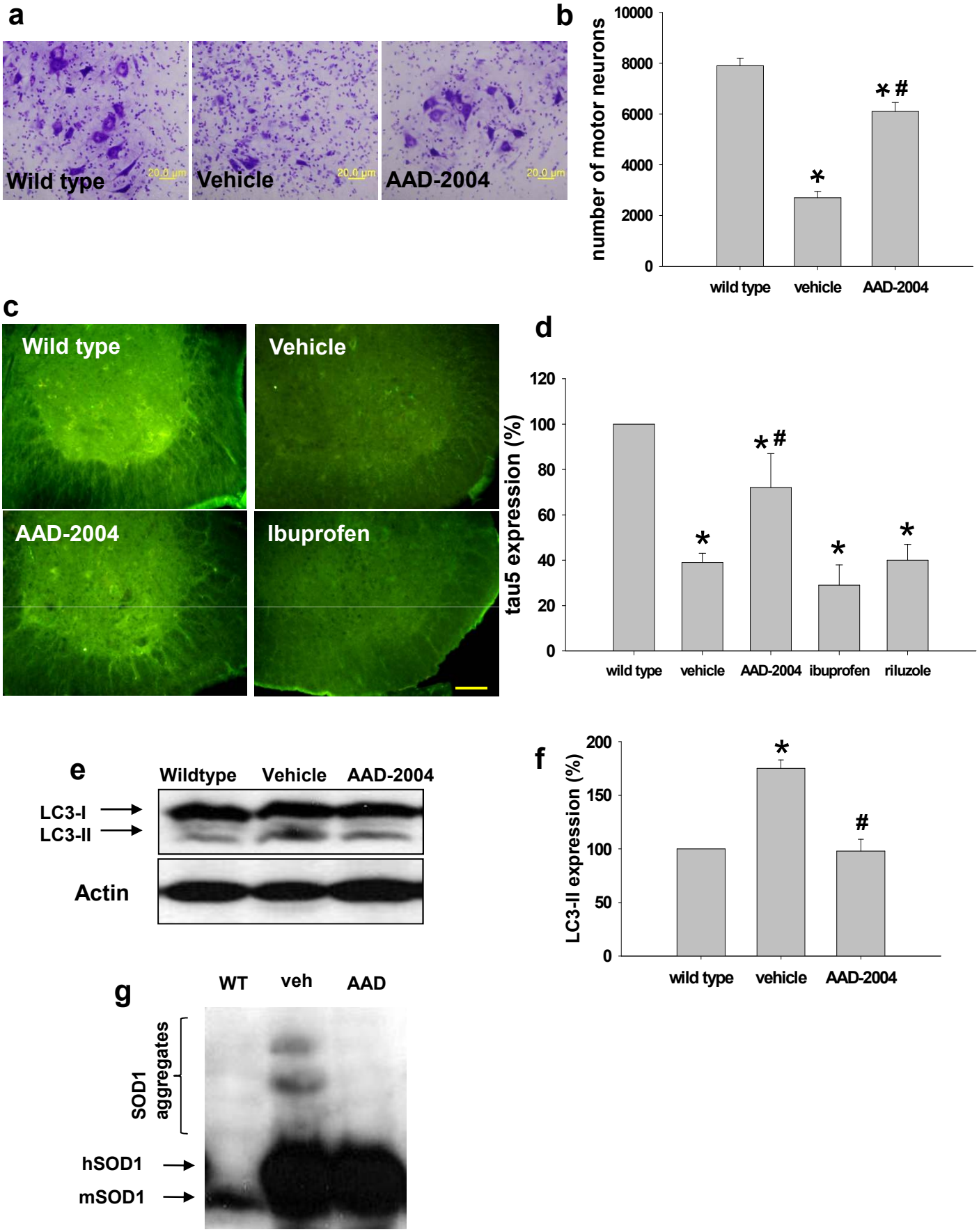
d

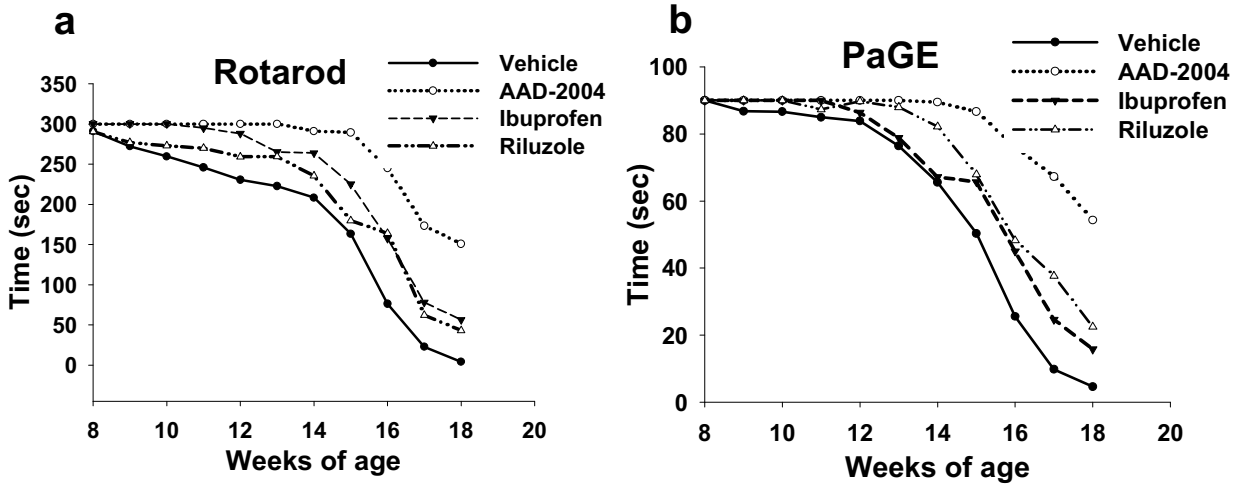


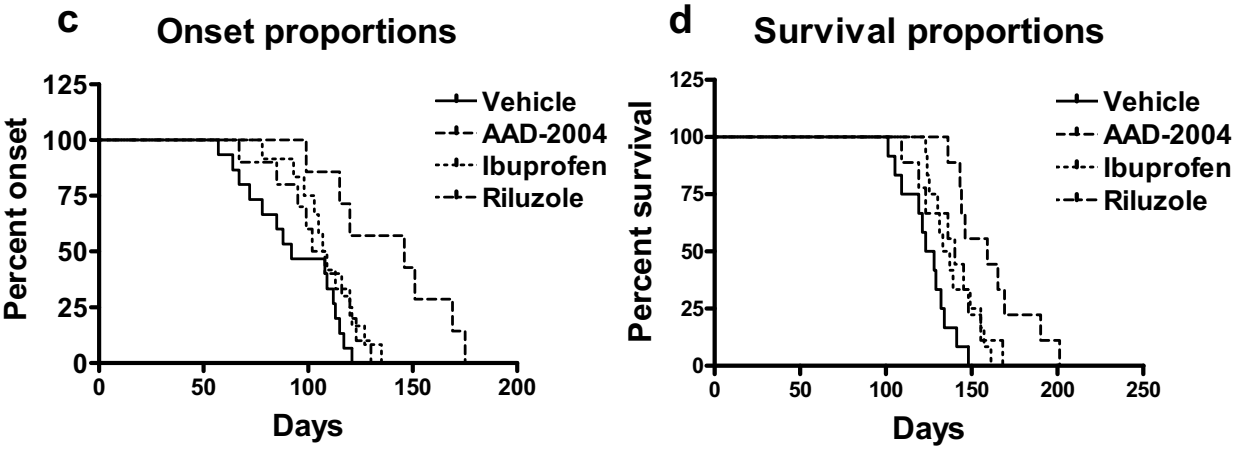

a
Wild type
Vehicle
AAD-2004
Nitrotyrosine
8-OHdG


b
% of Wild type
700
600
500
400
300
200
100
0
nitrotyrosine
8-OHdG
*
*
#
#
wild type
vehicle
AAD-2004


a
Wild type
Vehicle
AAD-2004
b
G93A
Wild type
Vehicle
AAD-2004
Iba-1
Actin
c
Iba-1 expression(%)
1200
1000
800
600
400
200
0
*
#
wild type
vehicle
AAD-2004
d
*
wild type
G93A
e
wild type
G93A
mPGES-1
actin
f
mPGES-1 expression(%)
350
300
250
200
150
100
50
0
*
wild type
G93A
g
lumbar (pg/mg)
plasma (ng/ml)
PGE2
1200
1000
800
600
400
200
0
*
*
#
#
wild type
vehicle
AAD-2004


a
Wild type
Vehicle
AAD-2004
20.0 µm
b
number of motor neurons
10000
8000
6000
4000
2000
0
*
*#
wild type
vehicle
AAD-2004
c
Wild type
Vehicle
AAD-2004
Ibuprofen
d
tau5 expression (%)
120
100
80
60
40
20
0
*
*#
*
*
wild type
vehicle
AAD-2004
ibuprofen
riluzole
e
Wildtype
Vehicle
AAD-2004
LC3-I
LC3-II
Actin
f
LC3-II expression (%)
200
150
100
50
0
*
#
wild type
vehicle
AAD-2004
g
WT
veh
AAD
SOD1 aggregates
hSOD1
mSOD1





e

	Vehicle	AAD-2004	Ibuprofen	Riluzole
Onset	94.96 ± 3.67	129.15 ± 6.50 [a,b]	109.79 ± 3.86 [a]	106.36 ± 4.3 4.34 [a]
Mortality	127.58 ± 2.05	154.38 ± 5.91 [a,b]	139.14 ± 3.35 [a]	138.00 ± 3.9 3.96 [a]