Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 122 | 952–961 doi: 10.1111/j.1471-4159.2012.07771.x

ORIGINAL ARTICLE

Concurrent blockade of free radical and microsomal prostaglandin E synthase-1-mediated PGE_2 production improves safety and efficacy in a mouse model of amyotrophic lateral sclerosis

Jin Hee Shin,*,† Young Ae Lee,† Jae Keun Lee,‡ Yong Beom Lee,*,† Woong Cho,† Doo Soon Im,*,† Jin Hwan Lee,*,† Bok Sun Yun,† Joe E. Springer§ and Byoung Joo Gwag*,†,¶

*Department of Neuroscience, Ajou University School of Medicine, Suwon, South Korea

†GNT Pharma, Yongin, South Korea

‡School of Life Science and Biotechnology, Korea University, Seoul, South Korea

§Department of Physical Medicine and Rehabilitation, University of Kentucky, Lexington, Kentucky, USA

¶Department of Pharmacology, Ajou University School of Medicine, Suwon, South Korea

Abstract

While free radicals and inflammation constitute major routes of neuronal injury occurring in amyotrophic lateral sclerosis (ALS), neither antioxidants nor non-steroidal anti-inflammatory drugs have shown significant efficacy in human clinical trials. We examined the possibility that concurrent blockade of free radicals and prostaglandin E_2 (PGE_2)-mediated inflammation might constitute a safe and effective therapeutic approach to ALS. We have developed 2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic 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 PGE_2 synthase-1 (mPGES-1) activity in response to both lipopolysaccharide-treated BV2 cell with IC_{50} of 230 nM and recombinant human mPGES-1 protein with IC_{50} of 249 nM *in vitro*. In superoxide dismutase 1^{G93A} transgenic mouse model of ALS, AAD-2004 blocked free radical production, PGE_2 formation, and microglial activation in the spinal cords. 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 riluzole or ibuprofen. Gastric bleeding was not induced by AAD-2004 even at a dose 400-fold higher than that required to obtain maximal therapeutic efficacy in superoxide dismutase 1^{G93A}. Targeting both mPGES-1-mediated PGE_2 and free radicals may be a promising approach to reduce neurodegeneration in ALS and possibly other neurodegenerative diseases.

Keywords: ALS, inflammation, mPGES-1, oxidative stress, PGE_2, $SOD1^{G93A}$.

J. Neurochem. (2012) **122**, 952–961.

Read the Editorial Highlight for this article on page 869.

Extensive evidence supports the central role of free radicals in the pathogenesis of neurodegenerative diseases. Elevated oxidative products have been reported in the brain, spinal cord, and CSF in subjects with amyotrophic lateral sclerosis (ALS) (Bowling *et al.* 1993; Beal *et al.* 1997; Ferrante *et al.* 1997; Pedersen *et al.* 1998; Ihara *et al.* 2005). Transgenic mice ($SOD1^{G93A}$) that over-express mutant superoxide dismutase 1 (SOD1) in familial ALS show motor neuron degeneration, movement deficit, and decreased survival rates (Gurney *et al.* 1994). In $SOD1^{G93A}$, oxidative stress is

Received April 16, 2012; revised manuscript received April 19, 2012; accepted April 20, 2012.

Address correspondence and reprint requests to Byoung Joo Gwag, 4F Hyunwoo Building 381-1 Gomae-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-901, South Korea.
E-mail: bjgwag@naver.com

Abbreviations used: ALS, amyotrophic lateral sclerosis; BBB, blood–brain barrier; COX-1 or COX-2, cyclooxygenase- 1 or -2; ESR, electron spin resonance; mPGES-1, microsomal prostaglandin E synthase-1; NSAIDs, non-steroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2; PGH_2, prostaglandin H_2; SOD1, superoxide dismutase 1.

induced in spinal cord regions known to undergo the pathological changes in ALS (Liu *et al.* 1998; Shin *et al.* 2007). Administration of antioxidants reduces neurological deficit in $SOD1^{G93A}$ (Andreassen *et al.* 2000; Crow *et al.* 2005; Shin *et al.* 2007). However, clinical trials of vitamin E produced no beneficial effect in ALS patients (Desnuelle *et al.* 2001; Graf *et al.* 2005), possibly due to side effects and poor blood–brain barrier (BBB) permeability.

Inflammation constitutes an additional contributor of neurodegeneration. Inflammatory responses are elevated in serum and CSF of ALS patients (Ilzecka 2003; Babu *et al.* 2008; Keizman *et al.* 2009). COX-2, the inducible isoform of cyclooxygenase (COX), is induced in neurons, microglia, astrocytes, and endothelial cells in both $SOD1^{G93A}$ and ALS patients (Almer *et al.* 2001; Maihofner *et al.* 2003). COX-2 is thought to mediate inflammation and neuronal injury in the spinal cord of $SOD1^{G93A}$ (Yasojima *et al.* 2001). Celecoxib, a selective COX-2 inhibitor, reduced levels of prostaglandin E_2 (PGE_2) and neuronal death and prolonged survival in $SOD1^{G93A}$ (Drachman *et al.* 2002). However, treatment with celecoxib improved neither motor function nor survival in ALS patients (Cudkowicz *et al.* 2006). In this study, celecoxib did not reduce levels of PGE_2 in the CSF of ALS patients. Further study is not warranted due to the increased risk of myocardial infarction and stroke following long-term therapy with selective COX-2 inhibitors (Martinez-gonzalez and Badimon 2007).

A combination approach targeting both free radicals and PGE_2-mediated inflammation may synergistically improve motor function and survival. Unlike COX-2 inhibitors increasing the risk of cardiovascular toxicity, aspirin prevents PGE_2 production, platelet aggregation, and has been used for the prevention of cardiovascular disease as well as inflammatory diseases such as rheumatoid arthritis (Evans *et al.* 1968; Collier 1971; Reilly and Fitzgerald 1988; Shadick *et al.* 2010). We have investigated the premise that a single agent combining the anti-inflammatory attributes of aspirin or salicylate with powerful antioxidant 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 with improved safety. AAD-2004 was chosen as a final drug candidate, based on safety and efficacy profile through multiple *in vitro* and *in vivo* screening processes.

Materials and methods

See Appendix S1.

Mice and treatment regimens

Animal care and treatment were in compliance with a protocol approved by the institutional animal care committee of Ajou University School of Medicine and GNT Pharma. $SOD1^{G93A}$ mice carrying the G93A human SOD1 mutation were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Male G93A transgenic mice were crossbred with B6SJLF1/J hybrid females as previously described (Gurney *et al.* 1994). Mice were treated with 2.5 mg/kg AAD-2004 (p.o., twice a day), 25 mg/kg ibuprofen (i.p., twice a day), or 50 mg/kg riluzole in diet beginning at 8 weeks of age. Non-transgenic litter mates were used as controls. Both male and female mice were used for behavior tests and histological experiments.

Evaluation of motor function

Motor strength and coordination were evaluated twice a week using a Rotarod (Columbus Instruments, Columbus, OH, USA) and paw grip endurance. Details are given in Appendix S1.

Evaluation of disease onset and survival

Disease onset was defined as the first day that a mouse showed a motor function deficit on the rotarod test. The time of death was defined as the date on which $SOD1^{G93A}$ showed complete paralysis of body and could not roll over within 20 s of being placed on their side. Death follows within a few hours after such an extreme morbidity.

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, Germany). Neuronal death was analyzed by staining every tenth section with 0.5% cresyl violet and counting viable motor neurons larger than 20 μm in outlined areas of the ventral lumbar region using a 4× objective. To estimate the total number of motor neuron, the optical fractionator method was used. Details are given in Appendix S1.

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

AAD-2004 was synthesized at Zhejiang Avilive Laboratories (Hengdian Industrial Zone, 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 ± SEM. An independent-samples *t*-test was used to compare two samples. The ANOVA and the Student–Newman–Keuls test were used for multiple comparisons. Survival data were analyzed by means of Kaplan–Meier survival curve. All analyses were performed using the SPSS version 12.0 from windows. Statistical significance was set at $p < 0.05$.

Results

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

Cortical cell cultures containing neurons and glia produced reactive oxygen species 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 reactive oxygen species production and neuronal death (Fig. 1a). The efficacy and potency of AAD-2004 were compared with those of antioxidants that were included in clinical trials for treatment of neurodegenerative diseases. Vitamin E 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, 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. As salicylates can scavenge free radicals at millimolar concentration (Dinis *et al.* 1994), 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. AAD-2004 rapidly reacted with DPPH with potency higher than vitamin E (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. 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.

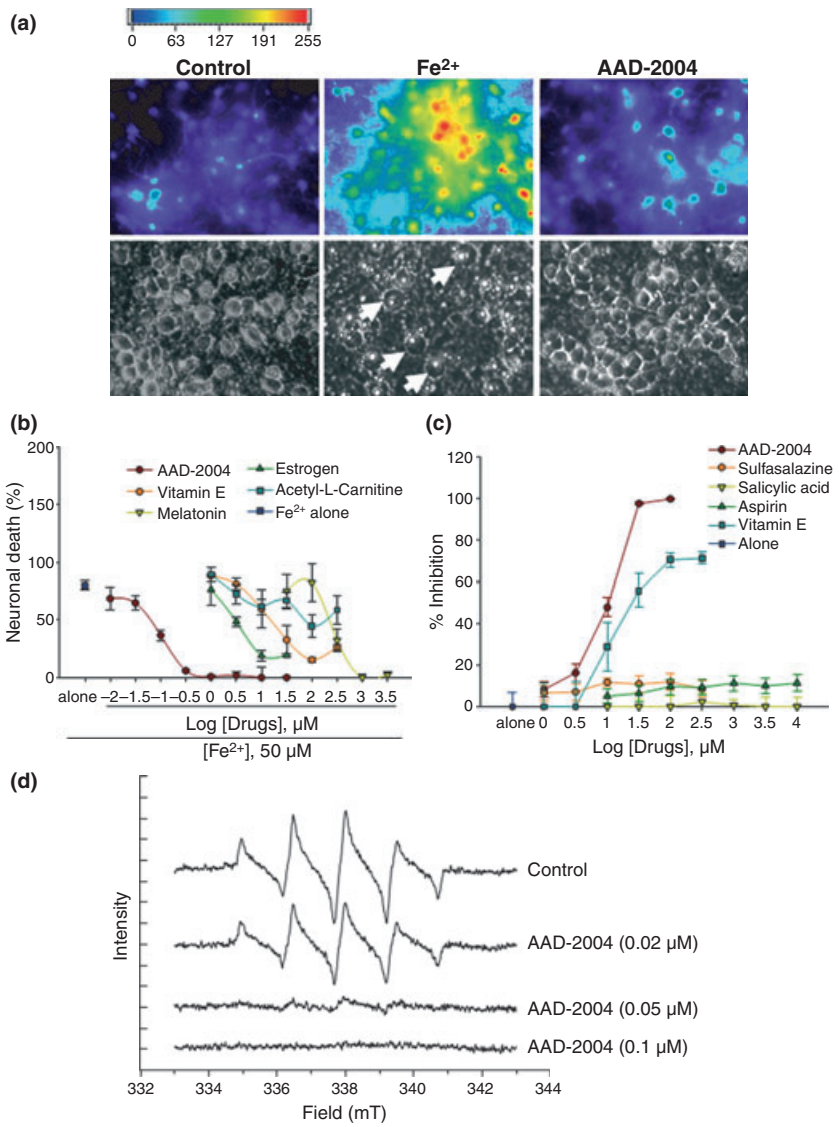

Fig. 1 AAD-2004 is a potent spin-trapping molecule. (a) Upper panel: fluorescence photomicrographs showing 2′,7′-dichlorofluorescein (DCF), the oxidation product of 2′,7′-dichlorodihydrofluorescein (DCDHF), in cortical cell cultures after 4 h exposure to a sham control or 50 μM Fe^{2+}, alone (Fe^{2+}) 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 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 (n = 4 culture wells per condition). (c) Free radical scavenging action was analyzed by measuring the reduction in 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.

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

AAD-2004 dose-dependently prevented PGE_2 production following exposure of BV2 cells to lipopolysaccharide (LPS) with IC_{50} of 1.4 μM (Fig. 2a). Additional experiments were performed if AAD-2004 would inhibit PGES, the terminal isomerase converting PGH_2 to PGE_2 (Samuelsson *et al.* 2007). 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 with those of control BV2 cells, suggesting that the bacterial endotoxin induces PGES-mediated PGE_2 production as previously reported (Ikeda-matsuo *et al.* 2005). AAD-2004 inhibited the conversion of PGH_2 to PGE_2 with an IC_{50} of 0.23 μM (Fig. 2b). Among three isoforms of PGES including cytosolic PGES (cPGES), microsomal PGES-2 (mPGES-2), and microsomal PGES-1(mPGES-1), levels of mPGES-1 were increased in BV2 cells exposed to LPS (Fig. 2c and d). AAD-2004 directly and dose-dependently prevented activity of recombinant human mPGES-1[(rh) mPGES-1] with IC_{50} of 0.249 μM (Fig. 2e). While oral administration of 300 mg/kg aspirin caused severe gastric damage 24 h later (Fig. 2f), oral administration of 1000 mg/kg AAD-2004 did not damage gastric mucosal membrane. Thus, AAD-2004, an mPGES-1 inhibitor and spin-trapping molecule, is expected to show better safety and efficacy in inhibiting PGE_2-mediated inflammation than aspirin.

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

Administration of AAD-2004 (i.p., b.i.d.) from 8 weeks of age alleviated motor function deficit and increased survival in $SOD1^{G93A}$. Maximal effects were observed from doses of 2.5 mg/kg (Fig. S1). In mice, the oral administration of 2.5 mg/kg AAD-2004 showed an area under the curve of 7.65 μg h/mL, which was approximately twofold higher than the intraperitoneal administration of 2.5 mg/kg AAD-2004 did (Fig. S2). Thus, the pharmacological effects of AAD-2004 were examined by the oral administration of 2.5 mg/kg (b.i.d.) in $SOD1^{G93A}$. As previously reported (Shin *et al.* 2007), oxidative stress was produced in the lumbar motor

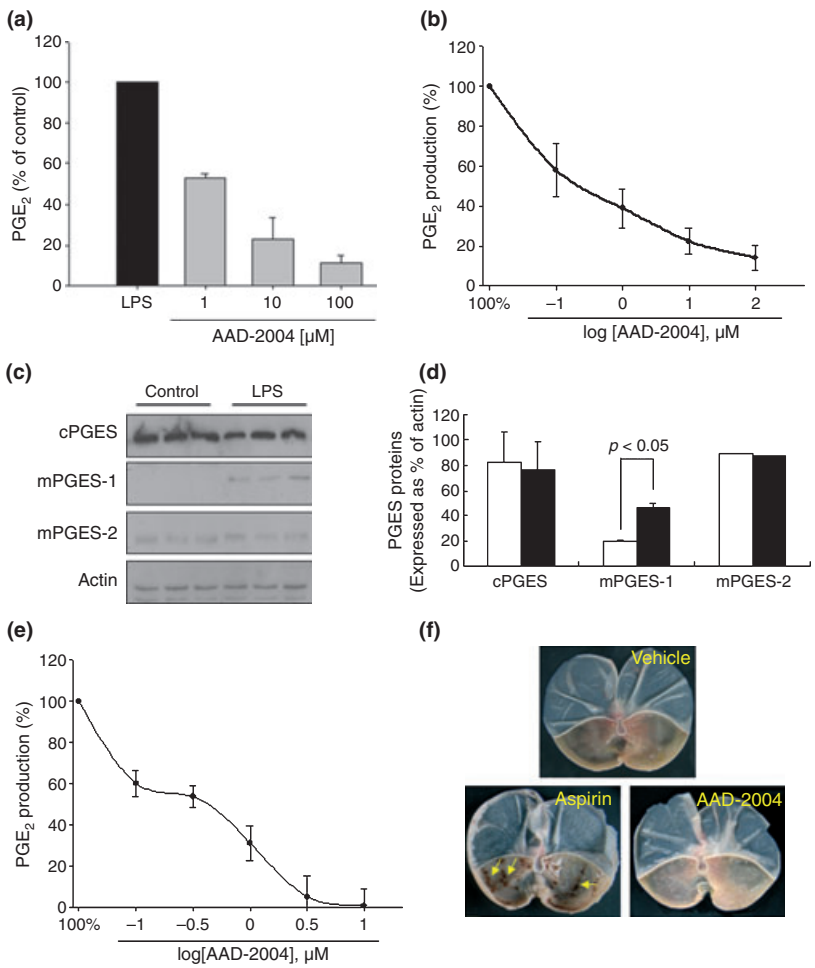

Fig. 2 AAD-2004 inhibits mPGES-1 activity without producing gastric damage. (a) Levels of PGE_2 24 h after exposure of BV2 cells to 1 μg/mL LPS, alone (= 100%) or with AAD-2004 ($n = 8$ cultures for each condition). (b) PGES-mediated PGE_2 production in BV2 cell lysates added with vehicle (= 100%) or AAD-2004 ($n = 4$ for each condition). (c, d) Western blot analysis of three isoforms of PGES 24 h following exposure of BV2 cells to sham control or 1 μg/mL LPS. Levels of each isoform were analyzed and scaled to actin ($n = 3$ cultures for each condition). (e) (rh) mPGES-1-induced PGE_2 production in the absence or presence of AAD-2004 ($n = 4$ for each condition). (f) Photomicrographs of rat stomach 24 h after the oral administration of vehicle, 300 mg/kg aspirin, or 1000 mg/kg AAD-2004.

neurons of 10-week-old SOD1^{G93A} as evidenced by increased immunoreactivity to nitrotyrosine and 8-OHdG (Fig. 3a). The administration of AAD-2004 from 8 weeks of age significantly blocked levels of nitrotyrosine and 8-OHdG elevated in SOD1^{G93A}. The number of microglia immunoreactive to Iba-1 (ionized calcium-binding adaptor molecule-1), a marker of microglia/macrophage, and levels of Iba-1 expression were increased in the ventral horn of the lumber spinal cord of 16-week-old SOD1^{G93A}, which were prevented by AAD-2004 (Fig. 4a–c). The expression of mPGES-1 was increased throughout the lumbar ventral horn of SOD1^{G93A} at 16 weeks of age (Fig. 4d–f). PGE_2 production was significantly increased in the lumbar spinal cord and also in plasma of SOD1^{G93A} (Fig. 4g). AAD-2004 blocked PGE_2 production induced in the spinal cord as well as plasma. As the maximum plasma concentration of AAD-2004 is approximately 5 μM following the oral administration of 2.5 mg/kg (Fig. S2), AAD-2004 is expected to prevent inflammation in the lumbar spinal cord of SOD1^{G93A} possibly through blockade of mPGES-1.

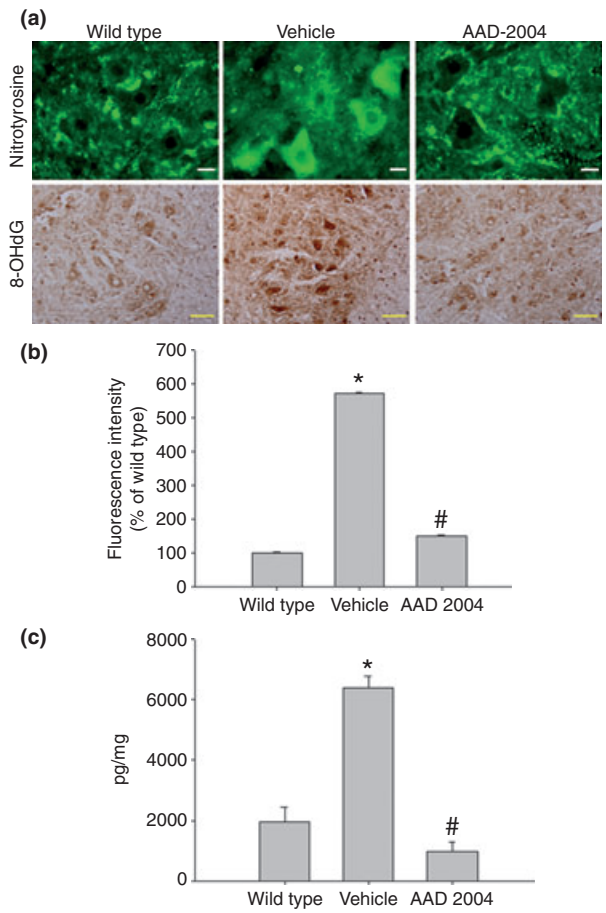

Fig. 3 AAD-2004 blocks oxidative stress in SOD1^{G93A}. (a) Fluorescent and bright-field photomicrographs of lumbar ventral sections immunolabeled with an antibody against nitrotyrosine (top panel; scale bar, 20 μm) or 8-OHdG (bottom panel; scale bar, 50 μm) in wild type or SOD1^{G93A} treated with saline (vehicle) or 2.5 mg/kg AAD-2004 (p.o., b.i.d.) for 2 weeks starting from 8 weeks of age. Levels of nitrotyrosine (b) and 8-OHdG (c) were analyzed by measuring immunofluorescence intensity of nitrotyrosine in the lumbar motor neurons (n = 20 sections from five 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.

AAD-2004 prevents motor neuron degeneration, axonal damage, and autophagosome formation in the lumbar spinal cord of SOD1^{G93A}

In the 16-week-old SOD1^{G93A}, neurodegeneration was extensive in the ventral horn of the lumbar spinal cord. The administration of 2.5 mg/kg AAD-2004 beginning at 8 weeks of age significantly prevented the loss of spinal motor neurons in the SOD1^{G93A} (Fig. 5a and 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 ameliorated by the administration of AAD-2004 (Fig. 5c and 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} as previously reported (Li *et al.* 2008). The conversion to LC3-II was sensitive to AAD-2004 (Fig. 5e and f). AAD-2004 also blocked the abnormal aggregation of mutant SOD1 observed in the lumbar spinal cord of SOD1^{G93A} (Fig. 5g).

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

Finally, we carried out a study comparing the functional efficacy of AAD-2004 with that of ibuprofen, a non-selective COX inhibitor, as well as riluzole. Mice received maximally effective doses of ibuprofen and riluzole as previously reported (Eriksen *et al.* 2003). As reported (Gurney *et al.* 1998), SOD1^{G93A} that received riluzole revealed significant improvement in motor function and survival (Fig. 6). Intraperitoneal administration of 25 mg/kg ibuprofen improved motor function and extended life span in SOD1^{G93A} comparable to riluzole. SOD1^{G93A} treated with 2.5 mg/kg AAD-2004 showed significantly better motor function and survival relative to riluzole or ibuprofen (Fig. 6a–d). The onset of Rotarod deficit was significantly delayed by 12% and 15.6% in the riluzole- and ibuprofen-treated groups, respectively, as compared with the vehicle group. The disease onset was further delayed by 36% in SOD1^{G93A} treated with AAD-2004 (Fig. 6e). Survival was extended by 8.2%, 9.4%, and 21% in the riluzole-, ibuprofen-, and AAD-2004-treated groups, respectively. AAD-2004 significantly

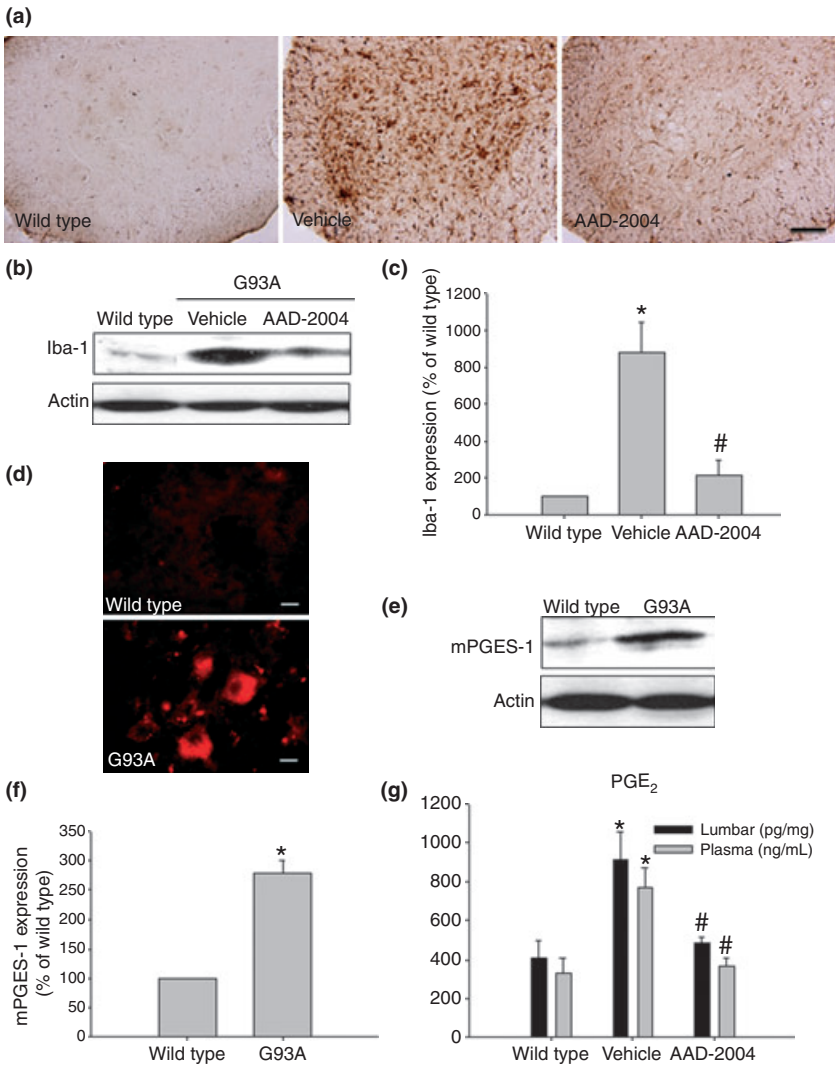

Fig. 4 AAD-2004 prevents microglia activation and PGE_2 production in $SOD1^{G93A}$. (a) Bright-field photomicrographs of lumbar ventral sections stained with an Iba-1 antibody, a marker of microglia/macrophage, in wild type or $SOD1^{G93A}$ treated with vehicle or 2.5 mg/kg AAD-2004 for 8 weeks starting from 8 weeks of age (bar, 100 μm). (b, c) Western blot analysis of Iba-1 in lumbar spinal cord of wild type and $SOD1^{G93A}$. Levels of Iba-1 were analyzed and scaled to actin ($n = 5$ mice for each condition). (d) Fluorescent photomicrographs of lumbar ventral sections immunolabeled with an mPGES-1 antibody from wild type and $SOD1^{G93A}$ (bar, 20 μm). (e, f) Western blot analysis of mPGES-1 in lumbar spinal cord from wild type and $SOD1^{G93A}$ ($n = 5$ mice for each condition). (g) Levels of PGE_2 were analyzed in lumbar spinal cord and plasma from wild type and $SOD1^{G93A}$ treated with vehicle or AAD-2004 ($n = 5$ mice for each condition). *Significant difference from wild type; #significant difference from vehicle.

improved motor performance and survival compared with riluzole or ibuprofen.

Discussion

AAD-2004, a dual-function drug derived from aspirin and sulfasalazine, is a potent spin-trapping molecule and mPGES-1 inhibitor effective at nanomolar concentrations. AAD-2004 improves motor function and survival in $SOD1^{G93A}$ with a maximally effective dose of 2.5 mg/kg, while no gastric damage is observed following oral administration of doses as high as 1000 mg/kg. AAD-2004 blocks oxidative stress and inflammation in $SOD1^{G93A}$, which results in blockade of neuronal death, axonopathy, and autophagosome formation. As a consequence, AAD-2004 significantly extends disease onset and survival compared with riluzole or ibuprofen.

Salicylate (2-hydroxybenzoate) can react with hydroxyl radical to produce catecol, 2, 3-dihydroxybenzoate, and 2, 5-dihydroxybenzoate (Udassin *et al.* 1991). However, salicylate weakly reacts with DPPH and does not reduce Fe^{2+}-induced free radical injury up to 1 mM, suggesting that salicylate is a poor antioxidant. Interestingly, sulfasalazine and 5-aminosalicylate prevented Fe^{2+}-induced free radical neurotoxicity at 30 μM (Ryu *et al.* 2003). The antioxidant effects of sulfasalazine and 5-aminosalicylate appear to be related to p-amine relative to the hydroxyl group of salicylate that increases stability of the peroxyl radical (Dinis *et al.* 1994). Furthermore, the antioxidant 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.

AAD-2004 reduced LPS-induced PGE_2 production with IC_{50} of 1.4 μM in BV2 cells. Unlike NSAIDs causing gastric damage at therapeutic doses, oral administration of 1000 mg/kg AAD-2004, which was 400-fold higher than maximal efficacy dose in $SOD1^{G93A}$, did not produce gastric bleeding. This led us to examine PGES, the terminal enzyme for PGE_2 biosynthesis, as a potential target of AAD-2004. AAD-2004

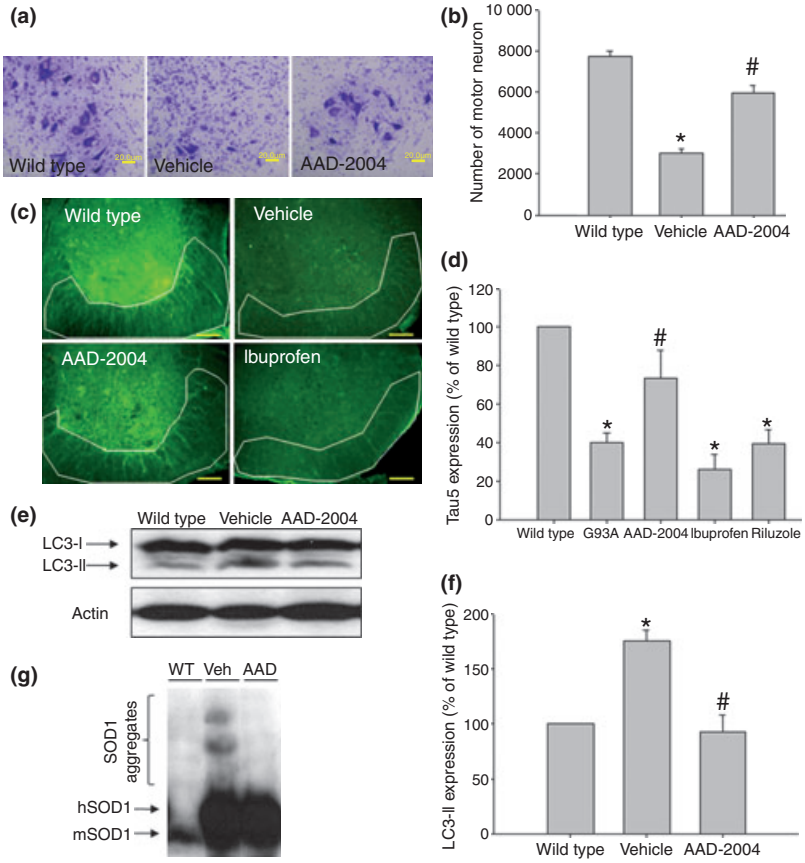

Fig. 5 AAD-2004 prevents motor neuron degeneration, axonopathy, and autophagosome formation in SOD1^{G93A}. (a) Bright-field photomicrographs of the lumbar ventral horn stained with cresyl violet in wild type and SOD1^{G93A} following 8-week administration of vehicle or 2.5 mg/kg AAD-2004 from 8 weeks of age (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} 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 (bar, 100 μm). (d) Western blot analysis of tau-5 in lumbar spinal cord from wild type and SOD1^{G93A} treated with vehicle, AAD-2004, ibuprofen, or riluzole (50 mg/kg/d in diet). Levels of tau-5 were analyzed and scaled to 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} treated with vehicle or AAD-2004 (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} treated with vehicle (veh) or AAD-2004 (AAD). *Significant difference from wild type; #significant difference from vehicle.

reduced conversion of PGH_2 to PGE_2 with IC_{50} of 0.23 μM in extracts of LPS-treated BV2 cells. As mPGES-1, an inducible form of PGES coupled to COX-2, mediates LPS-induced PGE_2 production and inflammation (Ikeda-matsuo *et al.* 2005), AAD-2004 is expected to act as an mPGES-1 inhibitor at submicromolar concentrations. In support of this, AAD-2004 reduced activity of (rh) mPGES-1 in a cell-free assay with IC_{50} of 0.249 μM, suggesting that AAD-2004 is a direct inhibitor of mPGES-1.

Recently, mPGES-1 was shown to play a proinflammatory role in fever, pain, and neurological diseases (Samuelsson *et al.* 2007). 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 (Ek *et al.* 2001; Yamagata *et al.* 2001; Engblom *et al.* 2002; Ozaki-okayama *et al.* 2004). Genetic deletion of *mPGES-1* reduced levels of PGE_2 in the CSF and fever following peripheral LPS injection (Engblom *et al.* 2003). Increased expression of mPGES-1 was also observed in neurons, astrocytes, and microglia as well as endothelial cells in postmortem brain of AD (Chaudhry *et al.* 2008). We found that levels of PGE_2 and mPGES-1 were significantly increased in the lumbar spinal cord of SOD1^{G93A}. The latter was observed in neurons, astrocytes, microglia, and endothelial cells in the ventral horn undergoing widespread neuronal death and inflammation in SOD1^{G93A}. Oral administration of AAD-2004 that reached a maximal plasma concentration of ~ 5 μM prevented PGE_2 formation and microglial activation in the spinal cord of SOD1^{G93A}, suggesting that AAD-2004 prevents inflammation in the CNS as an mPGES-1 inhibitor.

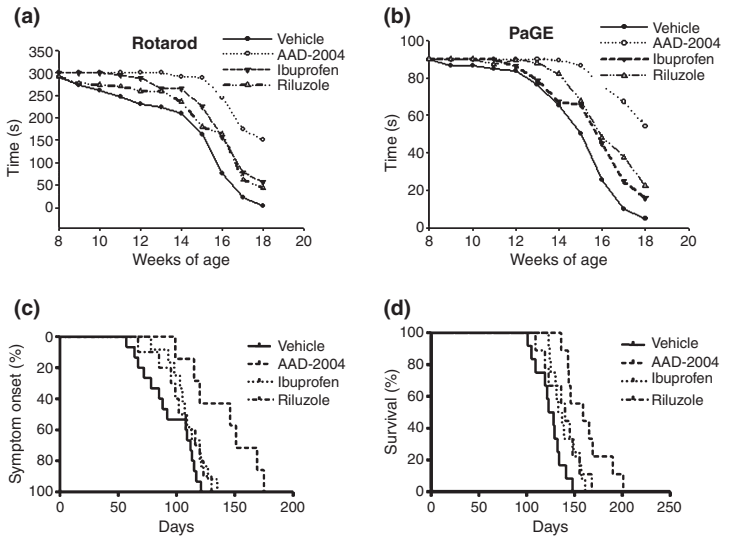

Fig. 6 AAD-2004 shows better motor function and survival than riluzole or ibuprofen in SOD1^{G93A}. SOD1^{G93A} received vehicle or 50 mg/kg riluzole in diet, 25 mg/kg ibuprofen (i.p., b.i.d.), or 2.5 mg/kg AAD-2004 (p.o., b.i.d.) from 8 weeks of age. (a, b) Motor function was analyzed using Rotarod test (a) and paw grip endurance test (b) at indicated points of age. (c, d) Cumulative probability of onset of Rotarod deficit (c) and survival (d). (e) Onset of Rotarod deficit and mortality of SOD1^{G93A}. [a]Significant difference from vehicle-treated group; [b]significant difference between AAD-2004-treated group and the group treated with ibuprofen or riluzole.

(e)

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

Levels of PGI_2 as well as PGE_2 were significantly increased in the lumbar segment of SOD1^{G93A} compared with wild type (Fig. S3). Administration of AAD-2004 that blocked PGE_2 production did not reduce PGI_2 production in the SOD1^{G93A}. 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 (Martinez-gonzalez and Badimon 2007). In addition, the pharmacological property of AAD-2004 as a spin-trapping molecule provides an additional safety profile. This is supported by studies demonstrating that antioxidants reduce NSAID-induced gastric injuries and cardiovascular risk (Rimm and Stampfer 1997; Fesharaki *et al.* 2006).

Although either antioxidants or NSAIDs improve motor function and prolong life span in SOD1^{G93A}, none of them have shown significant benefits in the translational studies for ALS patients (Benatar 2007; Orrell *et al.* 2008). 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 antioxidants through the BBB and adverse effects of NSAIDs that limit pharmacological action of antioxidants or NSAIDs in the CNS (De Boer and Gaillard 2007; Orrell 2010). 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} (Gordon *et al.* 2008), 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}. Compared with beneficial effects of riluzole or ibuprofen in SOD1^{G93A}, concurrent blockade of free radicals and PGE_2 with AAD-2004 significantly improved survival and onset of motor function deficit approximately up to two to threefold.

AAD-2004 or ibuprofen attenuates motor neuron death in the ventral horn of the lumbar spinal cord as riluzole or other COX-2 inhibitors do in SOD1^{G93A} (Drachman *et al.* 2002). The loss of ventral root axons correlates well with motor function deficit and appears before motor neuron death in SOD1^{G93A} and ALS patients (Fischer *et al.* 2004). While neither riluzole nor ibuprofen attenuated degeneration of ventral root axons, AAD-2004 significantly protected the axons in SOD1^{G93A}. Impaired autophagy has been proposed as a cause of progressive dystrophy and degeneration of axons (Komatsu *et al.* 2007; Morimoto *et al.* 2007; Li *et al.* 2008). 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}. This connotes that free radicals and PGE_2-mediated inflammation cause abnormal protein aggregates and autophagosome formation. In support of this, iron or mitochondrial reactive oxygen species induced autophagy and autophagic cell death (Chen and Gibson 2008; He *et al.* 2008). By inhibiting abnormal protein aggregation and axonopathy, AAD-2004 produces better motor function and survival in SOD1^{G93A} than riluzole or ibuprofen.

Undoubtedly, free radicals and 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 PGE_2-dependant inflammation *in vitro* and in $SOD1^{G93A}$. With the dual pharmacological actions, AAD-2004 did not cause gastric damage at a dose 400-fold higher than efficacy doses in $SOD1^{G93A}$, prevented protein aggregation and axonopathy, and improved neurological function and survival better than NSAIDs as well as riluzole. The protective mechanisms of AAD-2004 involve preventing mutant SOD1-induced superoxide production, mitochondrial dysfunction, axonal disorganization, and inflammation. It will be of interest to investigate if AAD-2004 prevents the other proposed mechanisms of pathology such as excitotoxicity, endoplasmic reticulum stress, and non-neuronal cell damage in $SOD1^{G93A}$ (Ilieva *et al.* 2009).

Conclusions

The present findings support the need for a novel medication that exhibits concurrent blockade of free radical and mPGES-1-mediated PGE_2 production as a means to combat devastating neurodegeneration in ALS and also has implications for the treatment of other neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.

Acknowledgements

This work was supported by grants from the 21C Frontier R&D Program in Neuroscience from Ministry of Education, Science, and Technology, a National Drug Discovery Program from Ministry of Health and Welfare, the Brain Korea 21 project at Ajou University School of Medicine, and GNT Pharma. The authors have no conflicts of interest.

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Materials and methods.

Figure S1. AAD-2004 improves motor function and increases survival in the SOD1G93A mice in a dose-dependent manner.

Figure S2. Pharmacokinetic profiles following intraperitoneal or oral administration of 2.5 mg/kg AAD-2004 in C57BL/6 mice.

Figure S3. AAD-2004 did not reduce PGI2 formation in the lumbar segment of SOD1 G93A mice.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

References

Almer G., Guegan C., Teismann P., Naini A., Rosoklija G., Hays A. P., Chen C. and Przedborski S. (2001) Increased expression of the pro-inflammatory enzyme cyclooxygenase-2 in amyotrophic lateral sclerosis. *Ann. Neurol.* **49**, 176–185.

Andreassen O. A., Dedeoglu A., Klivenyi P., Beal M. F. and Bush A. I. (2000) N-acetyl-L-cysteine improves survival and preserves motor performance in an animal model of familial amyotrophic lateral sclerosis. *NeuroReport* **11**, 2491–2493.

Babu G. N., Kumar A., Chandra R., Puri S. K., Kalita J. and Misra U. K. (2008) Elevated inflammatory markers in a group of amyotrophic lateral sclerosis patients from northern India. *Neurochem. Res.* **33**, 1145–1149.

Beal M. F., Ferrante R. J., Browne S. E., Matthews R. T., Kowall N. W. and Brown R. H. Jr (1997) Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann. Neurol.* **42**, 644–654.

Benatar M. (2007) Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol. Dis.* **26**, 1–13.

Bowling A. C., Schulz J. B., Brown R. H. Jr, and Beal M. F. (1993) Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *J. Neurochem.* **61**, 2322–2325.

Chaudhry U. A., Zhuang H., Crain B. J. and Dore S. (2008) Elevated microsomal prostaglandin-E synthase-1 in Alzheimer's disease. *Alzheimers Dement.* **4**, 6–13.

Chen Y. and Gibson S. B. (2008) Is mitochondrial generation of reactive oxygen species a trigger for autophagy? *Autophagy* **4**, 246–248.

Collier H. O. (1971) Prostaglandins and aspirin. *Nature* **232**, 17–19.

Crow J. P., Calingasan N. Y., Chen J., Hill J. L. and Beal M. F. (2005) Manganese porphyrin given at symptom onset markedly extends survival of ALS mice. *Ann. Neurol.* **58**, 258–265.

Cudkowicz M. E., Shefner J. M., Schoenfeld D. A., Zhang H., Andreasson K. I., Rothstein J. D. and Drachman D. B. (2006) Trial of celecoxib in amyotrophic lateral sclerosis. *Ann. Neurol.* **60**, 22–31.

De Boer A. G. and Gaillard P. J. (2007) Drug targeting to the brain. *Annu. Rev. Pharmacol. Toxicol.* **47**, 323–355.

Desnuelle C., Dib M., Garrel C. and Favier A. (2001) A double-blind, placebo-controlled randomized clinical trial of alpha-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis. ALS riluzole-tocopherol study group. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* **2**, 9–18.

Dinis T. C., Maderia V. M. and Almeida L. M. (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **315**, 161–169.

Drachman D. B., Frank K., Dykes-hoberg M., Teismann P., Almer G., Przedborski S. and Rothstein J. D. (2002) Cyclooxygenase 2 inhibition protects motor neurons and prolongs survival in a transgenic mouse model of ALS. *Ann. Neurol.* **52**, 771–778.

Ek M., Engblom D., Saha S., Blomqvist A., Jakobsson P. J. and Ericsson-dahlstrand A. (2001) Inflammatory response: pathway across the blood-brain barrier. *Nature* **410**, 430–431.

Engblom D., Saha S., Ericsson-dahlstrand A., Jakobsson P. J. and Blomqvist A. (2002) Prostaglandins as inflammatory messengers across the blood-brain barrier. *J. Mol. Med.* **80**, 5–15.

Engblom D., Saha S., Engstorm L., Westman M., Audoly L. P., Jakobsson P. J. and Blomqvist A. (2003) Microsomal prostaglandin E synthase-1 is the central switch during immune-induced pyresis. *Nat. Neurosci.* **6**, 1137–1138.

Eriksen J. L., Sagi S. A., Smith T. E. *et al.* (2003) NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo. *J. Clin. Invest.* **112**, 440–449.

Evans G., Packham M. A., Nishzawa E. E., Mustard J. F. and Murphy E. A. (1968) The effect of acetylsalicyclic acid on platelet function. *J. Exp. Med.* **128**, 877–894.

Ferrante R. J., Browne S. E., Shinobu L. A. *et al.* (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J. Neurochem.* **69**, 2064–2074.

Fesharaki M., Nasimi A., Mokhtari S., Mokhtari R., Moradian R. and Amirpoor N. (2006) Reactive oxygen metabolites and anti-oxidative defenses in aspirin-induced gastric damage in rats: gastroprotection by vitamin E. *Pathophysiology* **13**, 237–243.

Fischer L. R., Culver D. G., Tennant P., Davis A. A., Wang M., Castellano-sanchez A., Khan J., Polak M. A. and Glass J. D. (2004) Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp. Neurol.* **185**, 232–240.

Gordon P. H., Cheung Y. K., Levin B. *et al.* (2008) A novel, efficient, randomized selection trial comparing combinations of drug therapy for ALS. *Amyotroph. Lateral Scler.* **9**, 212–222.

Graf M., Ecker D., Horowski R. *et al.* (2005) High dose vitamin E therapy in amyotrophic lateral sclerosis as add-on therapy to riluzole: results of a placebo-controlled double-blind study. *J. Neural Transm.* **112**, 649–660.

Gurney M. E., Pu H., Chiu A. Y. *et al.* (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**, 1772–1775.

Gurney M. E., Fleck T. J., Himes C. S. and Hall E. D. (1998) Riluzole preserves motor function in a transgenic model of familial amyotrophic lateral sclerosis. *Neurology* **50**, 62–66.

He Y., Hua Y., Song S., Liu W., Keep R. F. and Xi G. (2008) Induction of autophagy in rat hippocampus and cultured neurons by iron. *Acta Neurochir. Suppl.* **105**, 29–32.

Ihara Y., Nobukuni K., Takata H. and Hayabara T. (2005) Oxidative stress and metal content in blood and cerebrospinal fluid of amyotrophic lateral sclerosis patients with and without a Cu, Zn-superoxide dismutase mutation. *Neurol. Res.* **27**, 105–108.

Ikeda-matsuo Y., Ikegaya Y., Matsuki N., Uematsu S., Akira S. and Sasaki Y. (2005) Microglia-specific expression of microsomal prostaglandin E2 synthase-1 contributes to lipopolysaccharide-induced prostaglandin E2 production. *J. Neurochem.* **94**, 1546–1558.

Ilieva H., Polymenidou M. and Cleveland D. W. (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *J. Cell Biol.* **187**, 761–772.

Ilzecka J. (2003) Prostaglandin E2 is increased in amyotrophic lateral sclerosis patients. *Acta Neurol. Scand.* **108**, 125–129.

Keizman D., Rogowski O., Berliner S., Ish-shalom M., Maimon N., Nefussy B., Artamonov I. and Drory V. E. (2009) Low-grade systemic inflammation in patients with amyotrophic lateral sclerosis. *Acta Neurol. Scand.* **119**, 383–389.

Komatsu M., Wang Q. J., Holstein G. R. *et al.* (2007) Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc. Natl Acad. Sci. USA* **104**, 14489–14494.

Li L., Zhang X. and Le W. (2008) Altered macroautophagy in the spinal cord of SOD1 mutant mice. *Autophagy* **4**, 290–293.

Liu R., Althaus J. S., Ellerbrock B. R., Becker D. A. and Gurney M. E. (1998) Enhanced oxygen radical production in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Ann. Neurol.* **44**, 763–770.

Maihofner C., Probst-cousin S., Bergmann M., Neuhuber W., Neundorfer B. and Heuss D. (2003) Expression and localization of cyclooxygenase-1 and -2 in human sporadic amyotrophic lateral sclerosis. *Eur. J. Neurosci.* **18**, 1527–1534.

Martinez-gonzalez J. and Badimon L. (2007) Mechanisms underlying the cardiovascular effects of COX-inhibition: benefits and risks. *Curr. Pharm. Des.* **13**, 2215–2227.

Morimoto N., Nagai M., Ohta Y. *et al.* (2007) Increased autophagy in transgenic mice with a G93A mutant SOD1 gene. *Brain Res.* **1167**, 112–117.

Orrell R. W. (2010) Motor neuron disease: systematic reviews of treatment for ALS and SMA. *Br. Med. Bull.* **93**, 145–159.

Orrell R. W., Lane R. J. and Ross M. (2008) A systematic review of antioxidant treatment for amyotrophic lateral sclerosis/motor neuron disease. *Amyotroph. Lateral Scler.* **9**, 195–211.

Ozaki-okayama Y., Matsumura K., Ibuki T., Ueda M., Yamazaki Y., Tanaka Y. and Kobayashi S. (2004) Burn injury enhances brain prostaglandin E2 production through induction of cyclooxygenase-2 and microsomal prostaglandin E synthase in cerebral vascular endothelial cells in rats. *Crit. Care Med.* **32**, 795–800.

Pedersen W. A., Fu W., Keller J. N., Markesbery W. R., Appel S., Smith R. G., Kasarskis E. and Mattson M. P. (1998) Protein modification by the lipid peroxidation product 4-hydroxynonenal in the spinal cords of amyotrophic lateral sclerosis patients. *Ann. Neurol.* **44**, 819–824.

Reilly I. A. and Fitzgerald G. A. (1988) Aspirin in cardiovascular disease. *Drugs* **35**, 154–176.

Rimm E. B. and Stampfer M. J. (1997) The role of antioxidants in preventive cardiology. *Curr. Opin. Cardiol.* **12**, 188–194.

Ryu B. R., Lee Y. A., Won S. J. *et al.* (2003) The novel neuroprotective action of sulfasalazine through blockade of NMDA receptors. *J. Pharmacol. Exp. Ther.* **305**, 48–56.

Samuelsson B., Morgenstern R. and Jakobsson P. J. (2007) Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacol. Rev.* **59**, 207–224.

Shadick N. A., Karlson E. W., Cook N. R., Maher N. E., Buring J. E. and Lee I. M. (2010) Low-dose aspirin in the primary prevention of rheumatoid arthritis: the women's health study. *Arthritis Care Res. (Hoboken)* **62**, 545–550.

Shin J. H., Cho S. I., Lim H. R. *et al.* (2007) Concurrent administration of Neu2000 and lithium produces marked improvement of motor neuron survival, motor function, and mortality in a mouse model of amyotrophic lateral sclerosis. *Mol. Pharmacol.* **71**, 965–975.

Udassin R., Ariel I., Haskel Y., Kitrossky N. and Chevion M. (1991) Salicylate as an *in vivo* free radical trap: studies on ischemic insult to the rat intestine. *Free Radic. Biol. Med.* **10**, 1–6.

Yamagata K., Matsumura K., Inoue W. *et al.* (2001) Coexpression of microsomal-type prostaglandin E synthase with cyclooxygenase-2 in brain endothelial cells of rats during endotoxin-induced fever. *J. Neurosci.* **21**, 2669–2677.

Yasojima K., Tourtellotte W. W., Mcgeer E. G. and Mcgeer P. L. (2001) Marked increase in cyclooxygenase-2 in ALS spinal cord: implications for therapy. *Neurology* **57**, 952–956.