EMBO Molecular Medicine

Novel telomerase-increasing compound in mouse brain delays the onset of amyotrophic lateral sclerosis

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Keywords: activator; ALS; brain; neuroprotector; telomerase

DOI 10.1002/emmm.201200212

Received May 01, 2011
Revised December 22, 2011
Accepted January 03, 2012

Telomerase is expressed in the neonatal brain, in distinct regions of adult brain, and was shown to protect developing neurons from apoptosis. Telomerase reactivation by gene manipulation reverses neurodegeneration in aged telomerase-deficient mice. Hence, we and others hypothesized that increasing telomerase expression by pharmaceutical compounds may protect brain cells from death caused by damaging agents. In this study, we demonstrate for the first time that the novel compound AGS-499 increases telomerase activity and expression in the mouse brain and spinal cord (SC). It exerts neuroprotective effects in NMDA-injected CD-1 mice, delays the onset and progression of the amyotrophic lateral sclerosis (ALS) disease in SOD1 transgenic mice, and, after the onset of ALS, it increases the survival of motor neurons in the SC by 60%. The survival of telomerase-expressing cells (*i.e.* motor neurons), but not telomerase-deficient cells, exposed to oxidative stress was increased by AGS-499 treatment, suggesting that the AGS-499 effects are telomerase-mediated. Therefore, a controlled and transient increase in telomerase expression and activity in the brain by AGS-499 may exert neuroprotective effects.

INTRODUCTION

Telomerase, a ribonucleoprotein complex, is responsible for the maintenance of telomere length and promotes genomic integrity and cell proliferation. The active enzyme consists of telomerase reverse transcriptase (TERT), an RNA subunit (TERC) and the dyskerin protein (Cohen et al, 2007; Park et al, 2009). While TERC is expressed in most cells, TERT is tightly regulated and consequently determines if telomerase is active (Kyo et al, 2003). It is mainly expressed in proliferating normal cells and the majority of cancers (Greenberg et al, 1998; Martin-Rivera et al, 1998). However, telomerase activation by itself is not oncogenic (Hackett and Greider, 2002; Zimmermann and Martens, 2008). Novel roles for telomerase beyond the telomeres, such as mediating resistance to apoptotic processes (Bermudez et al, 2006; Lee et al, 2008; Passos et al, 2007; Sung et al, 2005), have been suggested (Cong and Shay, 2008).

Developing neurons exhibit telomerase activity, which has been suggested to participate in neuronal development and protects them from apoptosis induced by DNA damage (Klapper et al, 2001). In the rodent brain, telomerase activity becomes undetectable by postnatal day 10, while TERT mRNA is maintained at lower levels into adulthood (Klapper et al, 2001). Other studies demonstrated telomerase activity within the adult subventricular zone, the olfactory bulb, the hippocampus and in the adult cerebellum and cortex (Caporaso et al, 2003; Flanary and Streit, 2003; Lee et al, 2009). TERT-deficient mice exhibit altered anxiety-like behaviours and abnormal olfaction (Lee et al, 2009), while TERT over-expressing transgenic mice show significant resistance to ischemic brain injury and NMDA-mediated neurotoxicity (Kang et al, 2004; Lee et al, 2008). In addition, telomerase activity in the brain increases after seizures, axotomy and ischemia (Baek et al,

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2004; Flanary and Streit, 2005; Fu et al, 2002). Recently, it was shown that multi-system degeneration in adult telomerase-deficient mice can be halted and reversed by the reactivation of the endogenous telomerase activity (Jaskelioff et al, 2011). In these mice, telomerase reactivation reversed neurodegeneration and restored the proliferation capacity of neural progenitors in the subventricular zone region of the brain (Jaskelioff et al, 2011). These accumulating data has led to the suggestion that increasing telomerase in a controlled manner may have beneficial therapeutic effects for neurodegenerative diseases (Fossel and Flanary, 2009; Harley, 2005; Klapper et al, 2001).

We synthesized novel triaryl compounds (designated AGS) and demonstrated that they increase the activity and expression of telomerase in various human cell lines (unpublished data, patent Priel et al WO2008/149353, WO2008/149345). Here, we show that AGS-499 increases telomerase expression in the forebrain (FB), brain stem (BS), and spinal cord (SC) of CD-1 mice in a time- and dose-dependent manner. AGS treatment provides resistance to NMDA-induced neurotoxicity in mice, as was previously demonstrated for telomerase-overexpressing transgenic mice (Kang et al, 2004). The notion that increasing telomerase expression may have some beneficial effects on neurodegenerative disease onset and progression was here examined using an amyotrophic lateral sclerosis (ALS) mouse model.

Amyotrophic lateral sclerosis is a devastating and rapidly fatal degenerative disease characterized by the death and dysfunction of motor neurons in the cerebral cortex, BS and SC (Boillee et al, 2006a; Nayak et al, 2006). Mutations in the Cu/Zn Superoxide Dismutase 1 gene (SOD1) account for ~20% of familial ALS (fALS) cases, corresponding to 2–3% of all ALS cases (Rosen, 1993).Transgenic mutant SOD1 mice exhibit the histopathological hallmarks observed clinically in sporadic and fALS. In these mice, massive death of motor neurons in the ventral horn of the SC and loss of myelinated axons in ventral motor roots leads to paralysis and muscle atrophy (Gurney et al, 1994; Jonsson et al, 2004, 2006a,b; Wong et al, 1995). ALS is believed to be multifactorial in origin and there is no known cause for ALS and no cure. Activation of an enzyme that may enhance the survival of motor neurons or may protect the neurons from the degenerative processes may delay the onset and the progression of ALS. Therefore, we examined the notion that increasing telomerase expression in the brain and SC by an AGS compound may increase the survival of motor neurons and delay the onset and progression of ALS.

RESULTS

The AGS-499 compound increases the expression and activity of telomerase in the forebrain of treated mice in a time- and dose-dependent manner

Previous studies demonstrated telomerase activity and expression in various adult (8–10 weeks) mouse central nervous system (CNS) regions (Caporaso et al, 2003; Flanary and Streit, 2003; Fu et al, 2000; Lee et al, 2009). We have synthesized novel triaryl compounds that increased telomerase expression

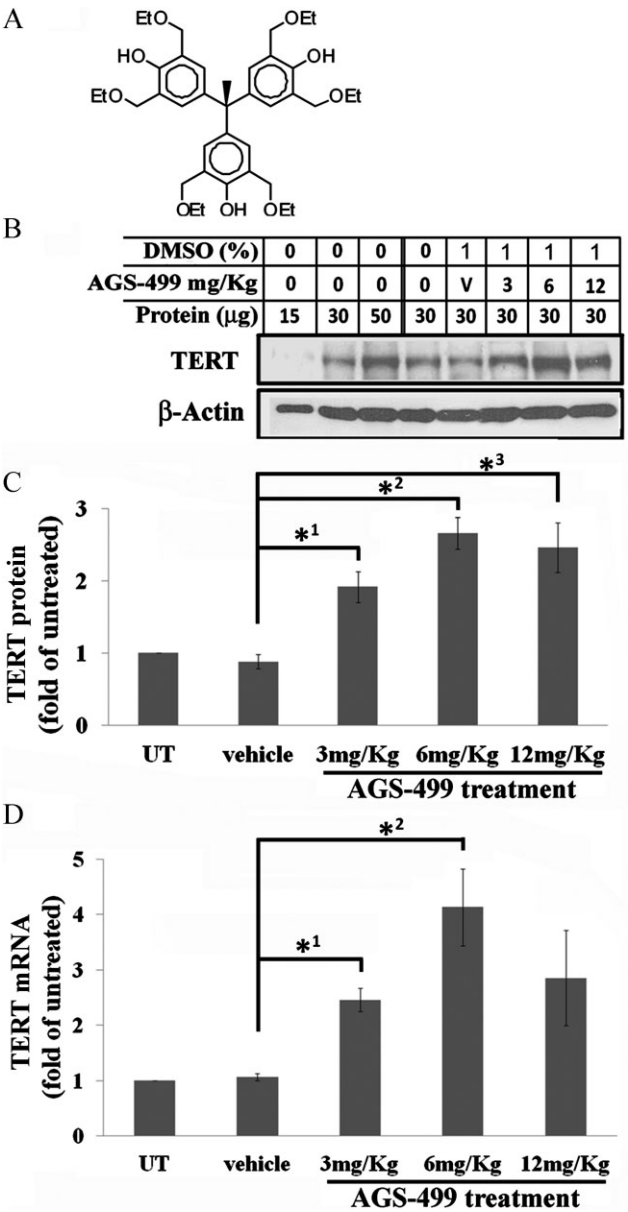

Figure 1. AGS-499 increases TERT protein in the FB of adult mice in a dose-dependent manner.

A. The AGS-499 molecule.

B. Mice were injected s.c. with different doses of AGS-499 or its vehicle DMSO. Twelve hours later, whole cell protein extract was prepared from the FB and 30 μg proteins were analysed by Western blot assay using three different anti-TERT antibodies. The results with anti-TERT monoclonal antibody (epitomic) and anti-β-actin antibody are shown.

C. Quantification of the results was performed using densitometric analysis by the EZquant software (mean ± s.e.m.; $n = 10$), Student's t-test $p = {}^{*1}0.019$, ${}^{*2}0.005$, ${}^{*3}0.021$.

D. Total RNA derived from mouse FB was examined for TERT mRNA by Real Time PCR, normalized to β-actin mRNA and the increase was calculated as fold of the vehicle (mean ± s.e.m.; $n = 3$, Student's *t*-test $p = {}^{*1}0.016$, ${}^{*2}0.046$). UT, untreated mice.

in various human cell lines and *in vivo* in animal models (patents WO 2008/149353, WO 2008/149345, Priel et al) One of these compounds, designated AGS-499 (chemical formula in Fig 1A), was examined for its ability to increase telomerase expression in the mouse brain.

Dose-dependent activation

Adult CD-1 mice (9–11 weeks old) were injected s.c. in the neck with AGS-499 at 3, 6 and 12 mg/kg. Twelve hours later, the mice were sacrificed and whole cell protein extracts or total RNA were prepared from the FB region (containing the cerebrum, thalamus, hypothalamus and limbic system). Equivalent amounts of the cell protein extracts were analysed by polyacrylamide gel electrophoresis and by Western blot using anti-TERT and anti β-actin antibodies. The results depicted in Fig 1B ($n = 5$ independent experiments) show an increase in TERT protein in the FB following AGS-499 treatments, while no effect on β-actin protein was observed. Quantification analysis of the results revealed a significant increase (1.9-, 2.7- and 2-fold, $p < 0.01$) in the level of telomerase protein in mice treated with 3, 6 and 12 mg/kg of AGS-499, respectively (Fig 1C). The increase in mTERT protein following AGS treatment was demonstrated by three different anti-TERT antibodies (Supporting information Fig 1A). Their specificity was previously shown (Tichon et al, 2009) and confirmed here by their ability to specifically inhibit telomerase activity in whole cell extracts derived from embryonic mouse brain (Supporting information Fig S1B). The antibodies recognized both the human and mouse TERT (Supporting information Fig S1C).

The examination of the effect of AGS-499 treatment on the expression of mTERT RNA transcripts in the mouse FB revealed a dose-dependent increase (up to 4 ± 1.05-fold compared to vehicle treatment, $p < 0.05$), peaking at 6 mg/Kg (Fig 1D), which is compatible with the expression pattern of TERT protein.

Telomerase activity in the aforementioned protein extracts was assayed by TRAP. As can be seen in Fig 2A ($n = 5$ independent experiments), telomerase activity in the FB of untreated or vehicle-treated mice was very low, while significant telomerase activity was detected 12 h post AGS-499 treatment. Quantification of telomerase activity from the TRAP assay data of five independent experiments revealed an increase of 3- ($p < 0.05$), 3.3- ($p < 0.01$) and 2.2- ($p < 0.05$) fold in telomerase activity in mice treated with 3, 6 and 12 mg/kg, respectively (Fig 2B). To confirm the increase in telomerase activity in the mouse FB following AGS treatment, a real time PCR-based TRAP assay was used. The results revealed that treatment of mice with AGS-499 increased telomerase activity in a dose-dependent manner. An increase of 2.4-, 3-, and 2-fold was observed when 3, 6 and 12 mg/kg of AGS 499 were injected, respectively (Fig 2C). Among the examined AGS doses, 6 mg/kg exhibited the most potent effect and therefore was used henceforth.

Time-dependent activation

To examine the time-dependent activation of telomerase in the brain following AGS treatment, AGS-499-treated mice were sacrificed at 3, 6, 12, 24 and 48 h after treatment. Telomerase protein level was examined in the cytoplasmic and nuclear fractions derived from the mouse FB. As can be seen in Fig 3A and C, telomerase protein level gradually increased with time in both the nucleus and cytoplasm (up to three- and twofold, respectively; $p < 0.01$) following AGS treatment, peaking at 12 h, decreasing to 1.5- and 2-fold ($p < 0.05$) activation at 24 h, and reaching the basal level at 48 h post treatment. Examination

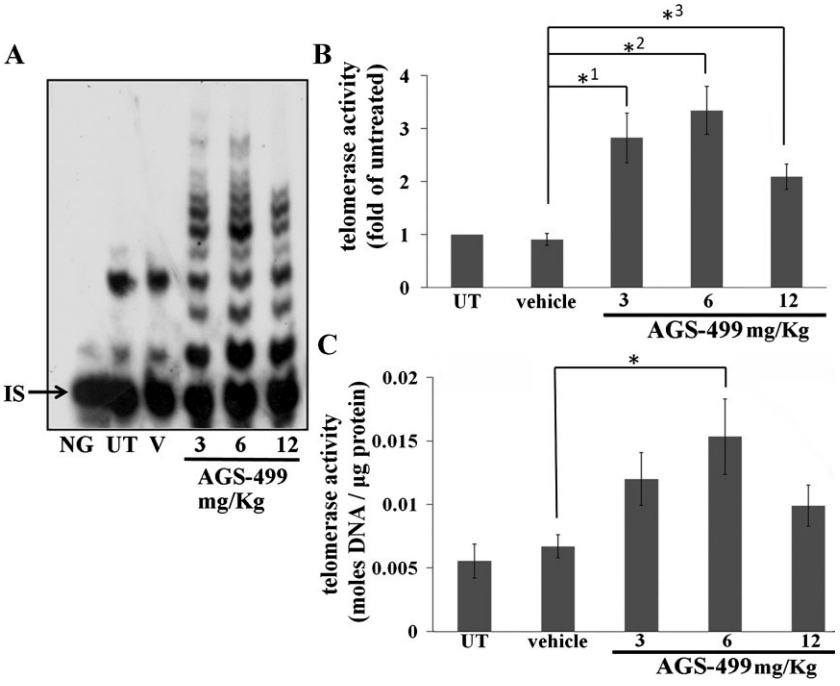

Figure 2. AGS-499 increases telomerase activity in the FB of adult mice in a dose-dependent manner.

A. Mice were injected s.c. with AGS-499 (3, 6 and 12 mg/kg of body weight) or its vehicle DMSO. Twelve hours later whole cell protein extract was prepared from the FB. Telomerase activity was determined by TRAP assay. Two micrograms of whole cell protein extract was added to the TRAP specific reaction mixture. Negative control (NG) contained CHAPS buffer instead of protein extract. IS, internal standard.

B. Quantification of the TRAP results by densitometric analysis using the EZquant software (mean ± s.e.m.; $n = 10$) and Student's *t*-test, $p = {}^{*1}0.019$, ${}^{*2}0.011$, ${}^{*3}0.030$.

C. Telomerase activity by the real-time PCR-based TRAP assay kit. Whole cell protein extract was prepared from FB of mice treated with AGS-499 (3, 6 and 12 mg/kg of body weight). One microgram of protein was added to the reaction according to the manufacturer's instructions and telomerase activity products were calculated as moles DNA/μg proteins (mean ± s.e.m.; $n = 6$). Student's *t*-test $p = {}^{*}0.08$. Symbols: UT, untreated mice; V, vehicle; NG, negative control.

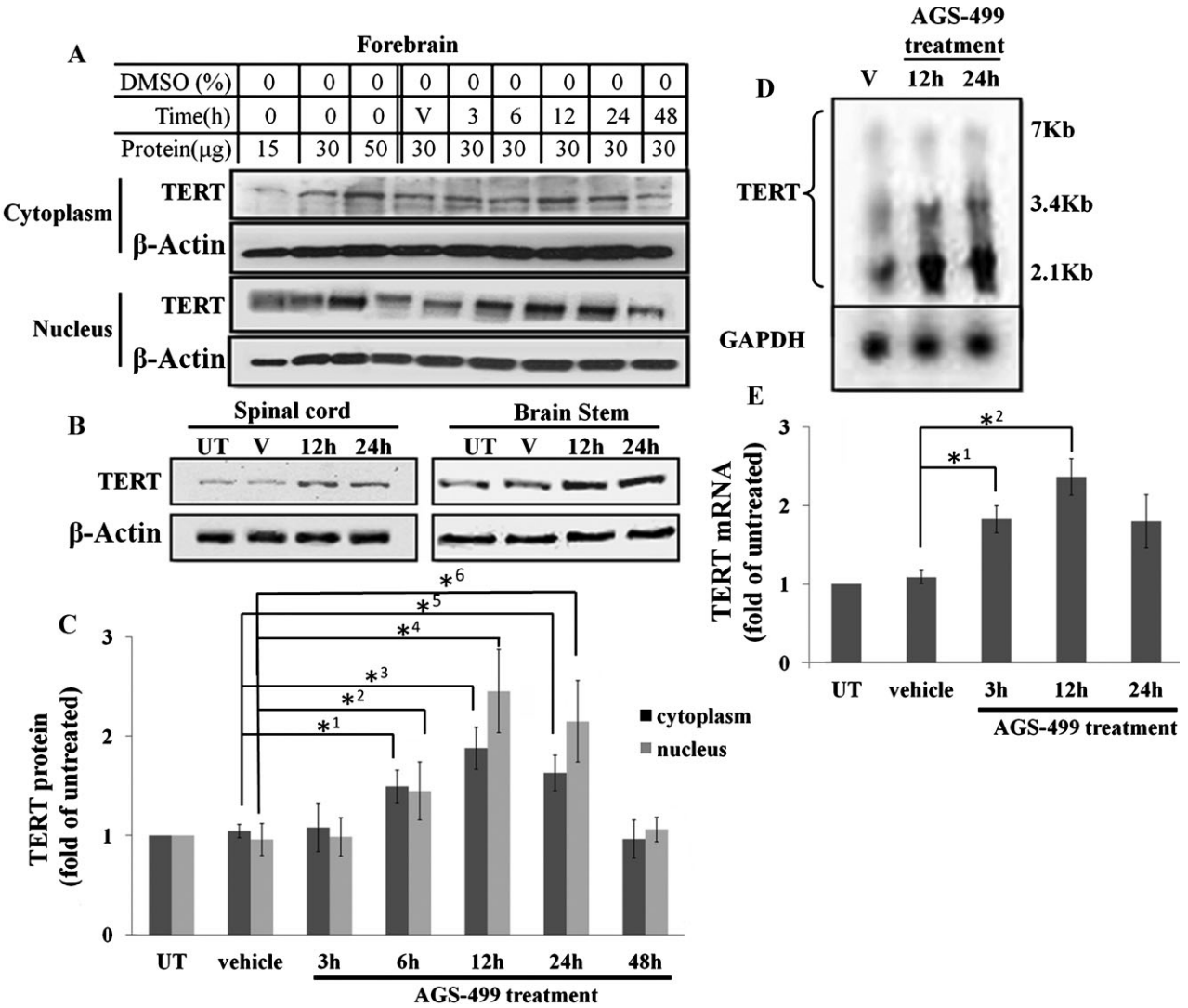

Figure 3. AGS-499 increases TERT protein level and TERT mRNA expression in the FB, BS and the SC in a time-dependent manner.

A-C. Mice were injected s.c. with AGS-499 (6 mg/kg) or its vehicle DMSO and cytoplasmic and nuclear protein extract was prepared from the FB (**A**,**C**) or whole cell protein extract from the BS and SC (**B**), at various time after the compound injection. Protein extracts (30 μg) were analysed by Western Blot assay using anti-TERT antibody (Rockland) or anti-β-actin antibody (**A**,**B**). Quantification of the Western blot results (depicted in A) by densitometric analysis using the EZquant software are shown in (**C**). (mean ± s.e.m.; $n = 10$) Student's t-test $p = {}^{*1}0.033$, ${}^{*2}0.035$, ${}^{*3}0.014$,${}^{*4}0.013$, ${}^{*5}0.024$, ${}^{*6}0.004$.

D-E. Total RNA extract was prepared from mouse FB that were injected with 6 mg/kg AGS-499, 12 and 24 h after injection. (**D**) Twenty micrograms RNA was subjected to Northern blot analysis using a specific [P^{32}] labelled TERT probe. (**E**) Real-time PCR was performed for quantification of TERT mRNA in mouse FB, normalized to β-actin mRNA and the increase in TERT transcripts was calculated as fold of vehicle (mean ± s.e.m.; $n = 3$, Student's t-test $p =: {}^{*1}0.033$, ${}^{*2}0.021$). Symbols: UT, untreated mice; V, vehicle.

of the effect of AGS treatment on telomerase protein in the BS and in the lumbar region of the SC demonstrates a significant increase in TERT protein 12 and 24 h after AGS injection (Fig 3B). In addition, the effect of AGS-499 injection on the TERT mRNA levels was determined by Northern blot analysis of total mRNA derived from the FB of AGS-treated and untreated mice. The results depicted in Fig 3D demonstrate the identification of the full-length mTERT mRNA (3.4 kb) and two additional unknown transcripts of 2.1 and 7 kb. In AGS-499-treated mice, a significant increase in the full-length TERT mRNA and the 2.1 kb transcript was observed. No change in the GAPDH transcript was detected, suggesting that AGS-499 specifically increases mTERT RNA expression. Quantification of the increase in mTERT RNA transcripts by real-time PCR analysis revealed a time-dependent increase (up to 3.87 ± 0.74, $p < 0.05$ compared to vehicle) peaking at 12 h after AGS injection (Fig 3E), which is compatible with the mTERT protein results (Fig 3A and C).

Telomerase activity was determined by TRAP assay and by real time PCR TRAP using the same assay conditions as above. Telomerase activity in the FB, the BS and the SC increased with time following AGS treatment and exhibited a similar time-dependent activation to that found with TERT protein (Fig 4). As

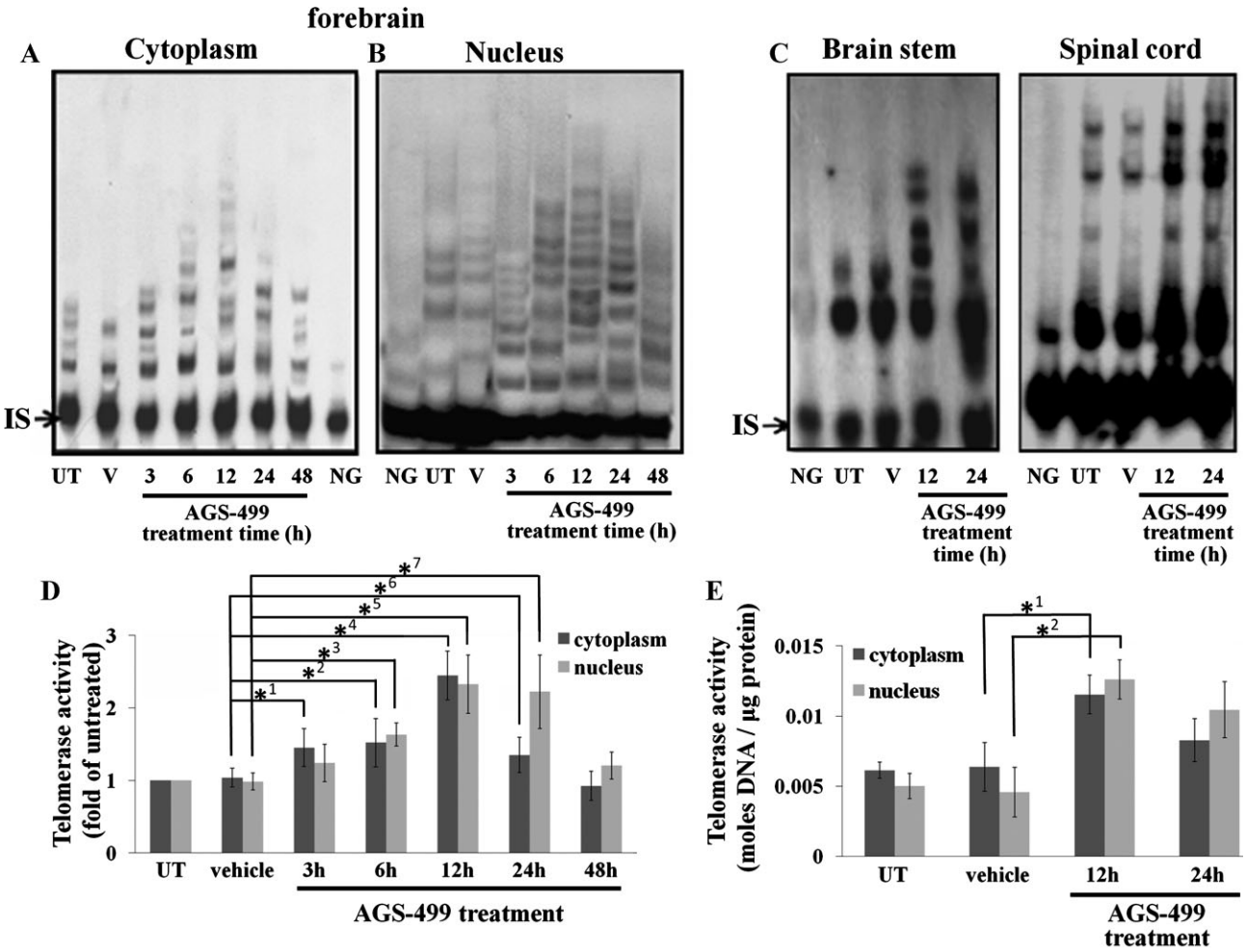

Figure 4. AGS-499 increases telomerase activity in the forebrain, BS and SC in a time-dependent manner. Mice were injected s.c. with AGS-499 (6 mg/kg) or its vehicle DMSO. Cytoplasmic (**A**) and nuclear (**B**) protein extracts from the FB, and whole cell protein extract from the BS or SC (**C**) at various intervals after compound injection were prepared.

A-C. Telomerase activity by TRAP assay. Protein extracts (2 μg) were added to the TRAP-specific reaction mixture. Negative control (NG) contained CHAPS buffer instead of the protein extract. IS, internal standard.

D. Quantification of the TRAP results by densitometric analysis using the EZquant software (mean ± s.e.m.; $n = 10$), Student's *t*-test $p = {}^{*1}0.092$, $^{*2}0.007$, $^{*3}0.028$, $^{*4}0.005$, $^{*5}0.0002$, $^{*6}0.004$, $^{*7}0.045$.

E. Quantification of telomerase activity by the real-time PCR-based TRAP assay kit. Cytoplasmic and nuclear protein extracts (1 μg) derived from the FB of AGS-treated or untreated mice for various intervals, were added to the reaction according to the manufacturer's instructions and telomerase activity products were calculated as moles DNA/μg protein (mean ± s.e.m.; $n = 6$), Student's *t*-test $p = {}^{*1}0.043$, $^{*2}0.026$. Symbols: UT, untreated mice; V, vehicle.

can be seen in Fig 4A–D for TRAP assay and Fig. 4E for real time PCR-based TRAP assay, telomerase activity in the cytoplasmic and nuclear extracts derived from FB and in the whole cell protein extract derived from the BS and SC exhibited a gradual increase with time, with a peak at 12 h (nucleus 2.54-fold, $p < 0.01$; cytoplasm 2.63-fold, $p < 0.01$), a decrease at 24 h (nucleus 2.44-fold, $p < 0.01$; cytoplasm 1.54-fold, $p < 0.01$) and reverting to the basal level 48 h after AGS treatment.

Examination of toxic effects

To examine the toxicity of this compound, CD-1 mice ($n = 5$ per group) were s.c. injected with a daily dose of 6, 30, or 60 mg/kg of AGS 499 for 3 weeks and no toxic effects were observed. The mice were kept for 12 month and exhibited good health and normal behaviour.

In summary, these results suggest that injection of a single dose of AGS-499 transiently increased telomerase expression and activity in the FB of adult CD-1 mice in a dose- and time-dependent manner and telomerase level returned to its basal state 48 h post treatment.

AGS protects mice from NMDA excitotoxicity

Transgenic mice that overexpress TERT were shown to be more resistant to NMDA-induced excitotoxicity (Lee et al, 2008). Since the aforementioned results suggest that the AGS-499 compound increased telomerase expression and activity in the

brain, we examined whether AGS-499 treatment may provide protection against NMDA-induced excitotoxicity. AGS-499 was administered s.c. 24 and 12 h prior to i.p. injection of NMDA, and the effect of dual doses of AGS-499 on telomerase in the FB was measured by TRAP assay. The dual dose of AGS-499 significantly increased telomerase activity as demonstrated in Fig 5A. Peripheral injection of NMDA causes epileptic-like seizures that can lead to mortality (Feigenbaum et al, 1989). Pretreatment of mice with the AGS-499 compound increased the survival rate of the NMDA-injected mice from 64 to 85% ($p < 0.05$) (Fig 5B). Surprisingly, pretreatment of mice with the vehicle (DMSO), increased the mortality in NMDA-injected mice by 25%. Thus, the AGS compound increased the survival rate by twofold ($p < 0.01$) as compared to vehicle. In addition, the rate of seizures and behavioural freezing in the surviving mice was blindly monitored by nine independent observers using a neurological score (0–5: 0,1, no seizures and freezing episodes and 5, many seizures or freezing episodes). The results depicted in Fig 5C show that AGS-499 reduced seizures and increased mouse movement in NMDA-injected mice.

AGS injection increases the lifespan and delays the onset and progression of ALS in SOD1 transgenic mice

The potential of telomerase activation for the treatment of degenerative and neurodegenerative diseases was previously suggested (Fossel and Flanary, 2009; Fu et al, 2000; Harley, 2005). ALS, a neurodegenerative disease with unknown cause and no cure, is characterized by the death and dysfunction of motor neurons in the cerebral cortex, BS and SC (Boillee et al, 2006a; Nayak et al, 2006). We hypothesized that increasing telomerase expression in the BS and in the SC by AGS treatment may have some neuroprotective effects on the motor neurons. As can be seen in Figs 3C and 4C, AGS treatment indeed increased the expression of TERT protein (Fig 3B) and telomerase activity (Fig 4C) in the BS and SC. Since AGS-499 increased telomerase expression in the relevant regions of the mouse brain and in the SC and decreased the excitotoxicity of NMDA, we hypothesized that AGS administration to SOD1 Tg mice that develop ALS may affect the onset and the progression of the disease. Fifty-six B6SJL-Tg ($SOD1^{G93A}$) 1Gur/J male mice were divided into five treatment groups, which were age- and weight-matched as described in the 'Materials and Methods' section. Injection of AGS-499 to the SOD1 Tg mice significantly increased their lifespan, reduced their weight loss, increased their motor performance, and delayed the onset and progression of the disease in a dose- and time-dependent manner (Fig 6). Treatment of mice with AGS-499 at 6 mg/kg injected every 24 h (Fig 6A) demonstrated the most efficient effect among the different treatment groups (Fig 6B) and led to a significant increase of survival from 123 ± 6.3 days (control and vehicle) to 141 ± 6.7 days [6 mg/kg every 24 h, an increase of 16.4% ($p < 0.01$) in the lifespan]. Injection of AGS-499 (6 mg/kg) every 48 h or at lower doses (1.5 mg/kg) every 24 h also increased the lifespan of the SOD1 Tg mice to 138 ± 5.1 and 133 ± 6.8 days, respectively, an extension of 12.2% ($p < 0.01$) and 8.1% ($p < 0.09$). These results suggest that the AGS compound increased the survival of ALS mice in a dose- and time-dependent manner.

AGS treatments significantly ameliorated body weight loss of the ALS mice (Fig 6C) and improved the motor performance as assessed by Rotarod test. Between days 115–120, the average time on the rotating rod in the AGS-499-treated group increased at least twofold in comparison to the vehicle- or control-treated mice (Fig 6D). A significant delay (15 days) in the appearance and progression of neurological symptoms was observed in

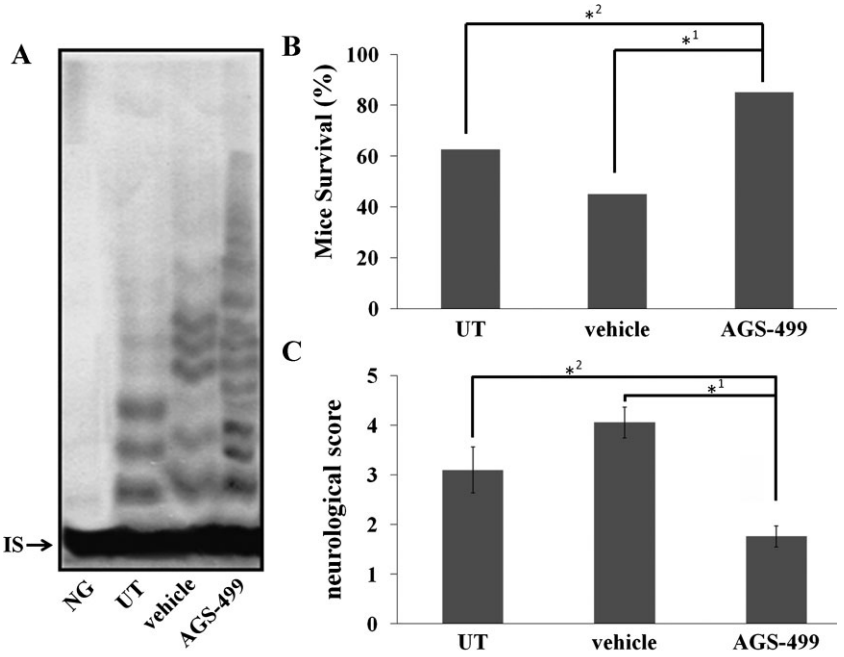

Figure 5. AGS-499 protects mice from NMDA-induced excitotoxicity.

A. Mice were twice injected with AGS-499 (6 mg/kg), or its vehicle DMSO, 24 and 12 h before inter-peritoneal injection of NMDA (110 mg/kg). Telomerase activity was determined by TRAP assay, in which 2 μg protein from the protein extracts were added to the TRAP specific reaction mixture. Negative control (NG) contained CHAPS buffer instead of the protein extract. IS, internal standard.

B. Mouse survival rate after inter-peritoneal NMDA injection (untreated $n = 16$, vehicle $n = 20$, AGS $n = 20$ $^{*1}p = 0.011$, $^{*2}p = 0.0003$ determined by chi test χ^2)

C. Seizures and 'freezing' in the surviving mice were blind-monitored by nine independent observers, using a neurological score range of 0–5: 0–1, no seizures and freezing episodes and 5, many seizures or freezing episodes (mean ± s.d.; untreated $n = 10$, vehicle $n = 9$, AGS $n = 17$, Student's t-test, $^{*1}p = 0.013$, $^{*2}p = 0.000$). Symbols: UT, untreated mice.

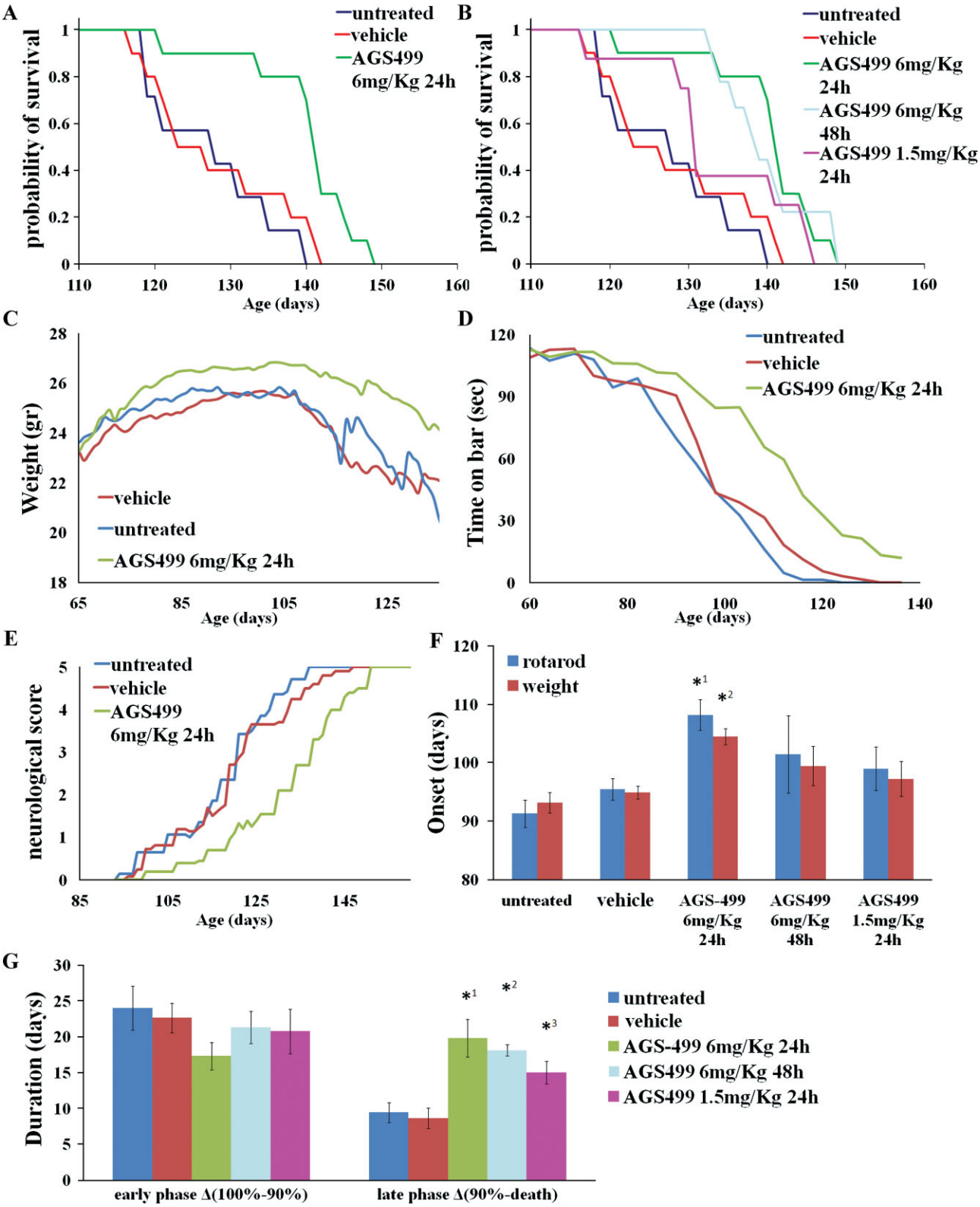

Figure 6.

the AGS-499-treated mice as determined by neurological score assessment (Fig 6E). Among the examined treatments, AGS administered at 6 mg/kg every 24 h was again the most efficient. To determine the time of disease onset in each mouse group, we analysed the data received from the measurement of body weight, neurological score and Rotarod performance. For each mouse in a treatment group, the age in which the peak in body weight and Rotarod performance was observed was recorded, and the average mouse age for each treatment group was calculated and is presented in Fig 6F. The disease onset in the untreated or vehicle-treated groups was at the age of 91 ± 5.4 days and at 106 ± 6.8 days for the AGS-499 (6 mg/kg, every 24 h) treatment group, a delay of 14.6% ($p < 0.01$) in the age of onset of the disease. The neurological score data are compatible with these results.

To determine the effect of AGS-499 treatment on ALS progression, the stages of the disease were defined as previously described (Boillee et al, 2006b). The duration of the early disease phase for each group was calculated from onset to 10% weight loss and the late disease phase from 10% weight loss to the end of stage. The results depicted in Fig 6G show that AGS-499 treatment did not affect the early stage of the disease but significantly delayed the progression of the late phase of the disease in a dose-dependent manner. At 6 mg/kg administered daily, AGS-499 doubled the duration of survival of the mice in the late phase of the disease.

Survival of motor neurons

Motor neuron survival was assessed morphologically by Nissl staining of lumbar SC sections (L5-T10; Fig 7A) derived from SOD1 Tg mice at the age of 100 days, treated with vehicle or AGS-499 (6 mg/kg) starting at 70 days of age. Similar sections derived from untreated SOD1 Tg mouse at 70 days of age were also examined for assessing the number of motor neurons prior the beginning of the treatment. The representative micrographs in Fig 7B show that a significant loss in motor neurons in the lumbar SC is observed at the age of 100 days as compared to the pre-symptomatic stage of 70 days. At the age of 100 days, a substantial attenuation in motor neuron loss in the AGS-499-treated mice was observed as compared to the vehicle-treated mice. Counting the number of motor neurons in 10 sections from each animal revealed that the mean number of motor neurons per section in vehicle-treated animals was 26.7 ± 1.96 and 43.6 ± 4.26 in AGS-499-treated mice, an increase of 60% in motor neuron survival (Fig 7C).

TERT expression during ALS progression

Examination of the expression of mTERT transcripts in the BS region and of mTERT protein in the FB and the BS regions of SOD1 Tg mice was performed at the following disease stages: pre-symptomatic (40–70 days), after the onset of disease (100 days), and at the terminal stage (neurological score 5). Untreated SOD1 Tg mice exhibited a significant decrease in mTERT protein (Fig 8A and B) and a three- to fourfold decrease in the expression of mTERT RNA transcripts (Fig 8C) during the progression of the disease. In contrast, AGS-treated SOD1 mice preserved the expression of mTERT protein in the FB and BS regions at the age of 100 days, after the onset of disease (Fig 8D).

Our results therefore suggest that AGS-499 treatment significantly delay the onset of ALS and slows its progression in a dose- and time-dependent manner. The results suggest that the enhancement in the survival of SOD1 Tg mice by AGS-499 is mediated by an increase in the survival of motor neurons.

Telomerase mediates the AGS-499-induced enhancement of the survival of NSC-34 motor neuron-like cells exposed to oxidative stress

To directly examine the effect of the AGS compound on motor neuron survival after insult, we used the NSC-34 cell line, a common *in vitro* model for motor neuron-like cells (Matusica et al, 2008). Consistent with our previous results, the AGS compound increased telomerase expression and activity in these cells (Fig 9A, lanes 3 and 4, and Supporting information Fig 2A and B). NSC-34 cells were transfected with hTERT-GFP vector (NSC-$34^{\text{hTERT-GFP}}$; Fig 9A, lane 1) and two TERT shRNA vectors (NSC-34^{shRNA69}, NSC-34^{shRNA72}), which reduced TERT expression and activity. NSC-34^{shRNA69} exhibited a 73% decrease in TERT expression, while NSC-34^{shRNA72} exhibited a 54% decrease (Fig 9A, lanes 5 and 7, and Supporting information Fig 2C). AGS treatment did not increase TERT expression in the shRNA-transfected cells but increased the expression of the endogenous TERT in NSC-$34^{\text{hTERT-GFP}}$ cells (Fig 9A).

Figure 6. AGS treatment increases the survival of SOD1 Tg mice and delays disease onset and progression. SOD1 B6SJL-Tg ($SOD1^{G93A}$) 1 Gur/J male mice (56) were divided into five control and treatment groups. The groups were age and weight matched.

A. Probability of survival with AGS-499 (6 mg/kg, 24 h) treatment ($n = 10$) started at symptoms onset as compared to untreated or vehicle treated mice ($n = 7$–10 per group).

B. Probability of survival with different AGS-499 doses administered every 24 or 48 h ($n = 9$–10 per group) started at disease onset as compared to untreated or vehicle treated mice.

C. Decline in body weight during disease progression.

D. Motor performance was evaluated by the Rotarod test. Analysis was performed by repeated measures (mean ± s.e.m.).

E. Neurological score: 0, no symptoms, 1, trembling of hind legs during tail suspension, 2, part of foot is dragging along cage, 3, one limb is paralysed, 4, two limbs are paralysed, 5, cannot right itself within 30 s after being placed on either side.

F. Disease onset: the age in which the peaks of the body weight and Rotarod performance were recorded, and the average mouse age for each treatment group was calculated and is presented as means ± s.e.m. (Student's *t*-test $p = {}^{*1}0.0004$, ${}^{*2}0.00002$ compared to vehicle).

G. Duration of the early disease phase for each group was calculated from onset to 10% weight loss and the late disease phase from 10% weight loss to the end of stage. The results are means ± s.e.m. (Student's *t*-test $p = {}^{*1}0.002$, ${}^{*2}0.000$, ${}^{*3}0.007$).

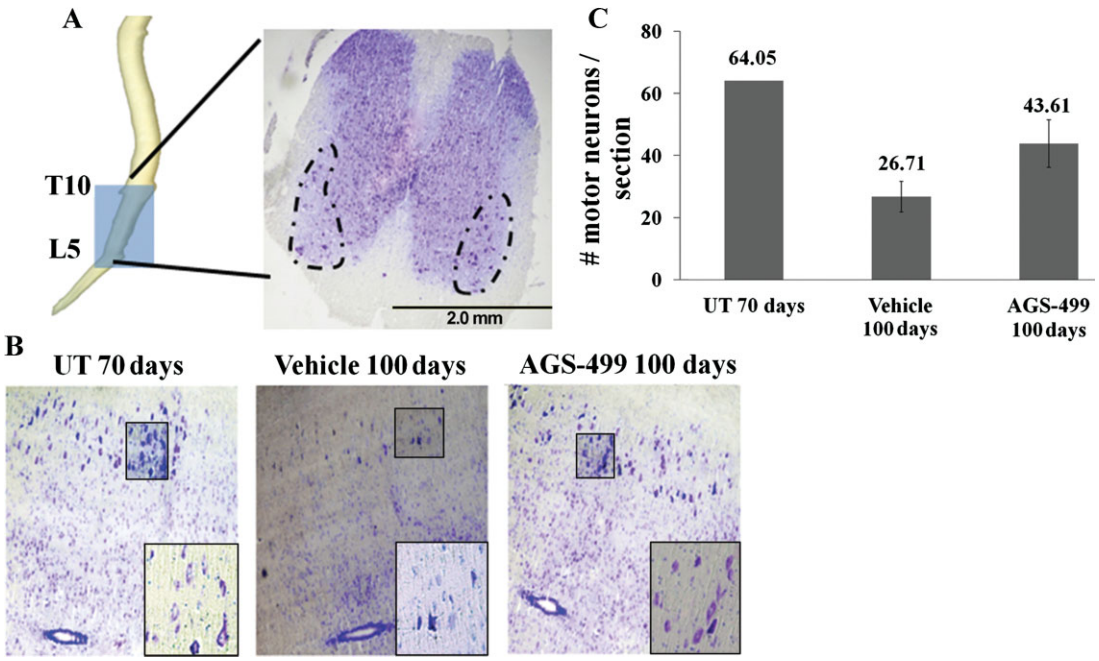

Figure 7. Attenuation of motor neuron loss by AGS-499 treatment in the lumbar SC of SOD1 Tg mice.

A,B. Photomicrographs of Nissl-stained sections derived from the ventral horn of the lumbar SC from untreated mice at the age of 70 days, vehicle-treated and AGS-499-treated mice at the age of 100 days.

C. Quantification of number of motor neurons per section was performed by counting motor neurons from serial micrographs ($n = 10$ from each animal) taken from the T10-L5. The results are shown as mean ± s.e.m.

Several studies have demonstrated the sensitivity of motor neurons to oxidative stress and have discussed the implications of oxidative stress and mitochondrial dysfunction on the progression of ALS (Cozzolino and Carri, 2011). Since telomerase protects cells from oxidative stress, we first examined the effect of telomerase expression on the survival of NSC-34 cells challenged by H_2O_2. Figure 9B shows that the survival of NSC-34$^{hTERT\text{-}GFP}$ cells following H_2O_2 increased (by up to 2 ± 0.5-fold compared to NSC-34 cell, $p < 0.01$) and significantly decreased in both NSC-34^{shRNA69} and NSC-34^{shRNA72} cells (by 2.3 ± 0.4 and 1.7 ± 0.2, $p < 0.05$, respectively), suggesting that telomerase plays an anti-oxidative stress protective role in NSC-34 cells. AGS-499 treatment of NSC-34 cells that were challenged with H_2O_2 revealed a dose-dependent increase (up to $60\% \pm 11\%$ compared to vehicle treatment, $p < 0.01$) in cell survival (Fig 9C). AGS also decreased caspase 3 cleavage, a marker for apoptosis, in a dose-dependent manner (Supporting information Fig 2D). These results suggest that AGS-499 protects NSC-34 cells from oxidative stress. AGS-499 did not increase the survival of NSC-34^{shRNA69} and NSC-34^{shRNA72} cells that were challenged with H_2O_2 (Fig 9D) and did not further enhance the survival of NSC-34$^{hTERT\text{-}GFP}$ (Fig 9D). However, AGS-499 enhanced the survival of H_2O_2-challenged differentiated NSC-34 cells that had attained the morphology of motor neurons (Matusica et al, 2008) by 60% (Supporting information Fig 3A and B). Taken together, these results support the idea that the increase in the survival of motor neuron-like cells by AGS-499 is mediated by telomerase.

AGS-499 does not increase cell survival of H_2O_2 treated embryonic stem cells derived from TERT-knockout mice

To substantiate the notion that AGS-499 protects cells from oxidative stress by increasing telomerase expression we used TERT-deficient embryonic stem cells (mES *Tert*$^{-/-}$) derived from a TERT KO mouse (Liu et al, 2000, 2002). mES *Tert*$^{-/-}$ cells and their wild type counterparts (*Tert*$^{+/+}$) were exposed to various concentrations of H_2O_2 (unpublished observations) or to 0.2 mM H_2O_2 for various intervals, and cell survival was examined by the XTT assay. TERT-deficient *mES* cells (*Tert*$^{-/-}$) exhibited a 1.8-fold increased sensitivity to oxidative stress compared to their WT counterparts (Fig 10A). To induce similar oxidative damage in both cell types, *Tert*$^{-/-}$ cells were treated with 0.2 mM H_2O_2 for 1 h and *Tert*$^{+/+}$ cells were treated for 2 h. After removal of the H_2O_2-containing medium, fresh medium containing AGS 499 (100 nM) was added for an additional 2 h. H_2O_2-induced DNA damage (DNA double-strand breaks) in the treated cells was determined by anti-γH2AX (phosphorylated histone) immunolabelling (Burma et al, 2001). Figure 10B depicts the γH2AX foci observed in *Tert*$^{-/-}$ and *Tert*$^{+/+}$ cells treated with vehicle or with AGS-499. The ratio between the number of cells with γH2AX foci and the total number of cells in the slide was calculated for each sample and is shown in Fig 10C. AGS treatment significantly reduced (by twofold) DNA damage induced by oxidative stress in *Tert*$^{+/+}$ cells, but had no significant effect on *Tert*$^{-/-}$ cells, suggesting that the protective activity of AGS-499 depends on TERT.

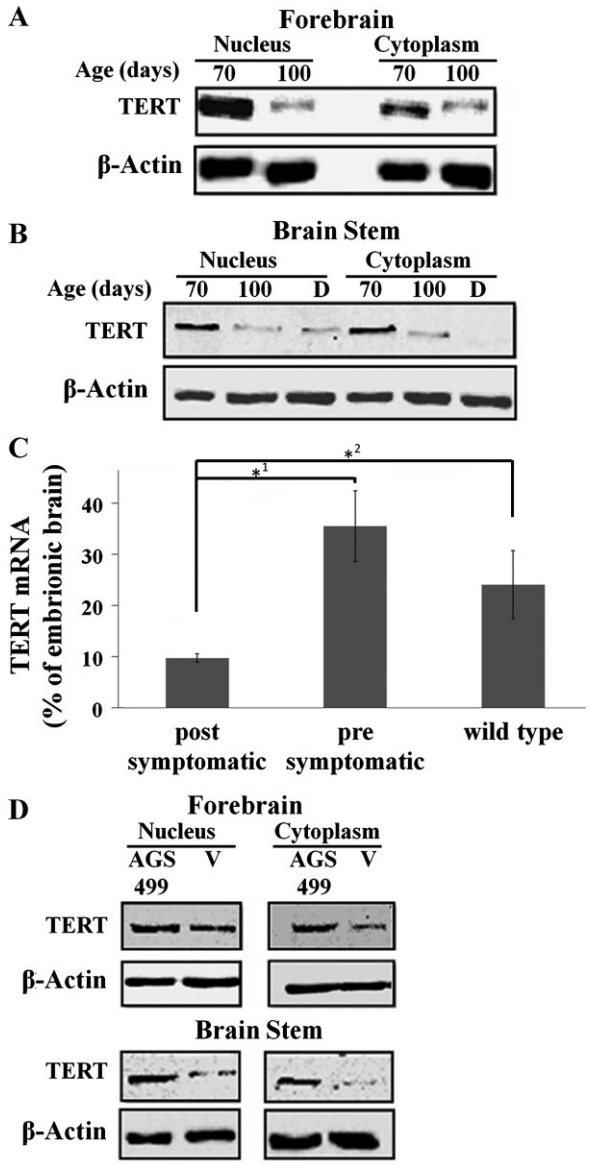

Figure 8. Expression of TERT protein in the FB and BS of SOD1 Tg mice before and after disease onset and the effect of AGS-499 treatment on its expression. Nuclear and cytoplasmic proteins extracts were prepared from the FB and BS regions (**A**,**B**,**D**) and total RNA was extracted from the BS regions (**C**).

A-B. Extracts derived from SOD1 Tg mice at the pre-symptomatic stage (40–70 days), after disease onset (100 days), before death (neurological score 5) were analysed (25 μg) by Western blotting using anti TERT or anti-β actin antibodies.

C. TERT RNA transcripts were detected by Real time PCR, normalized to β-actin and the quantification of TERT expression was calculated relatively to the value obtained for mouse embryonic brain (E17). The results are mean ± s.e.m., $n = 3$–9 per group, Student's t-test $^{*1}p = 0.063$, $^{*2}p = 0.006$.

D. Extracts derived from AGS-499 treated or vehicle treated SOD1 mice at the age of 100 days were analysed (25 μg) by Western blotting using anti TERT or anti-β actin antibodies.

AGS-499 increases telomerase levels in human cells and enhances the survival of cells exposed to oxidative stress in a telomerase-dependent manner

To confirm our results using human cells, experiments were performed using HEK 293T cells and adult human dermal fibroblasts. HEK 293T cells were transfected either with a vector expressing hTERT shRNA, a vector expressing scrambled shRNA or with a hTERT-GFP expression vector. The expression of TERT protein and activity in the various transfectants is shown in Supporting information Fig 4A and B. Our measurements indicate that TERT expression and activity were significantly abolished in HEK^{shRNA} cells and were not affected in $HEK^{scrambled\ shRNA}$ cells. The expression of TERT-GFP is also demonstrated. AGS treatment increased telomerase activity in HEK cells and did not show a synergistic effect in TERT-GFP-overexpressing cells (Supporting information Fig 4A and B). The transfectants were then exposed to increasing concentrations of H_2O_2 in the presence or absence of AGS-499. A significant increase in cell viability was observed in HEK cells or $HEK^{TERT\text{-}GFP}$ cells treated with AGS-499 (Supporting information Fig 4C and D). AGS-499 did not increase the viability of HEK^{shRNA} cells and did not further enhance the survival of $HEK^{TERT\text{-}GFP}$ cells (Supporting information Fig 4C and D).

Since HEK 293T are transformed human cells, we examined the ability of AGS-499 to increase telomerase levels in normal adult human primary cells. AGS-499 (50 and 200 nM) significantly increases the expression and the activity of telomerase in the adult human dermal fibroblasts (HDF-a) treated with the compound for 6 h (Supporting information Fig 5).

Together, these results indicate that AGS-499 increases telomerase expression and activity in human cells and that the survival and viability of human cells exposed to oxidative stress is mediated by telomerase.

DISCUSSION

TERT has been reported to inhibit cell death and to promote survival in many cell types including neurons (Cong and Shay, 2008; Klapper et al, 2001; Saretzki, 2009). The presence of telomerase expression in neurons, which are non-dividing cells, suggests that it possesses roles beyond its function on telomeres. Several studies (Cong and Shay, 2008; Parkinson et al, 2008; Saretzki, 2009) demonstrate that telomerase has a role in the regulation of apoptosis independently of its classical function in telomere maintenance. In fibroblasts and endothelial cells, TERT migrates to the mitochondria during oxidative stress (Ahmed et al, 2008; Santos et al, 2006) and regulates the mitochondrial complex 1, improving the efficiency of the respiratory chain (Haendeler et al, 2009). TERT is also involved in the repair of double-stranded DNA breaks in several cell types, including embryonic neurons (Klapper et al, 2001; Masutomi et al, 2005; Nergadze et al, 2007). Altogether, these studies suggest the importance of TERT as a protector from cellular damage through a mechanism that is as yet

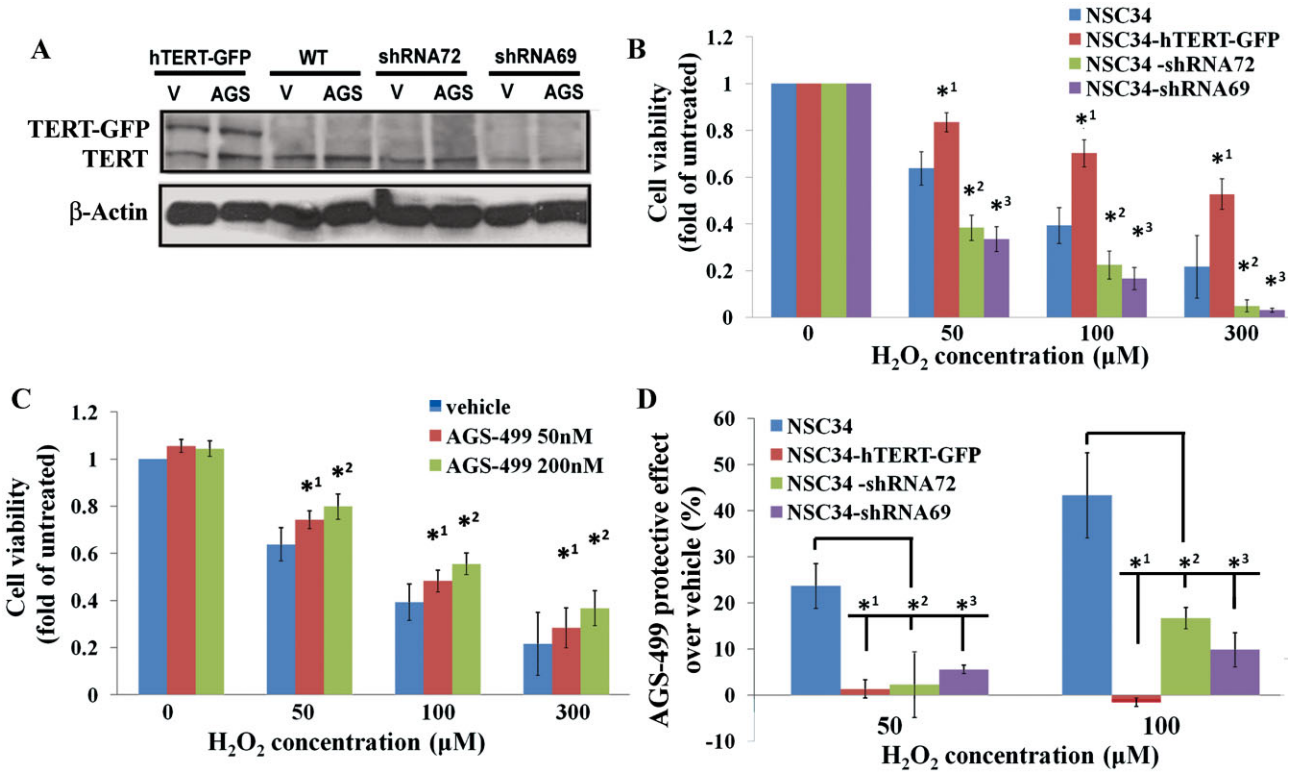

Figure 9. AGS-499 increases the survival of NSC-34 cells exposed to oxidative stress in a telomerase-dependent manner.

A. TERT protein expression was examined in vehicle-treated and AGS-499 treated (200 nM) NSC-34, NSC-34$^{TERT\text{-}GFP}$, NSC-34^{shRNA68} and NSC-34^{shRNA72} cells for 4 h.

Cells were exposed to various H_2O_2 concentrations for 4 hrs (**B**) and AGS-499(50 nM or 200 nM) was added (**C,D**). The medium was replaced by fresh serum-containing medium for 24 hrs. Cell viability was examined by XTT assay (**B–D**).

B. Represent the effect of H_2O_2 without AGS treatment. The results are mean ± s.e.m., Two Way ANOVA test $p = {}^{*1}0.0017$, $^{*2}0.002$, $^{*3}0.000$ compared to none-transfected cells.

C. Treatment of NSC-34 cells only with AGS-499. The results are fold of vehicle (0 μM-H_2O_2), mean ± s.e.m., Two Way ANOVA test $p = {}^{*1}0.045$, $^{*2}0.003$ relative to vehicle.

D. The increase in cell survival by AGS-499 (200 nM) in NSC-34, NSC-34$^{TERT\text{-}GFP}$, NSC-34^{shRNA68} and NSC-34^{shRNA72} cells calculated as follow: (% of cells treated with AGS/% of vehicle treated cells) − 1. Shown are mean ± s.e.m., $n = 5$ independent experiments. Two Way ANOVA $p = {}^{*1}0.0018$, $^{*2}0.02$, $^{*3}0.006$.

unclear. Since neurons are susceptible to damage caused by free oxygen radicals, due to a high oxidative load in the brain (Barzilai, 2007; McKinnon, 2009), and they are long lived cells with a low capacity of regeneration, one may assume that the presence of TERT in neurons is important for the ability of these cells to overcome various insults (*i.e.* oxidative stress) and for the maintenance of their genome stability. Accumulating data based on telomerase gene transfection led many to suggest that increasing telomerase expression in a controlled manner may have beneficial therapeutic effects on neurodegenerative diseases (Fossel and Flanary, 2009; Fu et al, 2000; Harley, 2005). Indeed, a recent study demonstrates that reactivation of the endogenous telomerase gene in adult mice that do not express telomerase not only halted degenerative processes, including neurodegeneration, but reversed them (Jaskelioff et al, 2011). Currently, gene therapy strategies have severe limitations therefore possible activation of the cellular telomerase gene by specific compounds and the ability to control this activation is a preferable therapeutic strategy.

Here, we demonstrate, for the first time, the ability of the novel compound AGS-499 to significantly increase telomerase expression (mRNA and protein) and activity in various regions of the brain and SC of adult mice in a dose- and time-dependent manner. The increase in TERT expression and activity is transient and reaches its maximal value 12 h after s.c. injection of 6 mg/kg of the AGS-499 compound, suggesting the ability to control the increase in telomerase by using this compound. The mechanism by which AGS-499 increases the expression of TERT gene in the brain is not known and is currently under investigation.

The biological significance of increasing telomerase expression in the brain by AGS-499 is shown in this study by its effect on NMDA-induced excitotoxicity in CD-1 mice and by its influence on the onset and progression of ALS in SOD1 Tg mice. Pretreatment of mice with AGS-499 12 and 24 h prior to NMDA

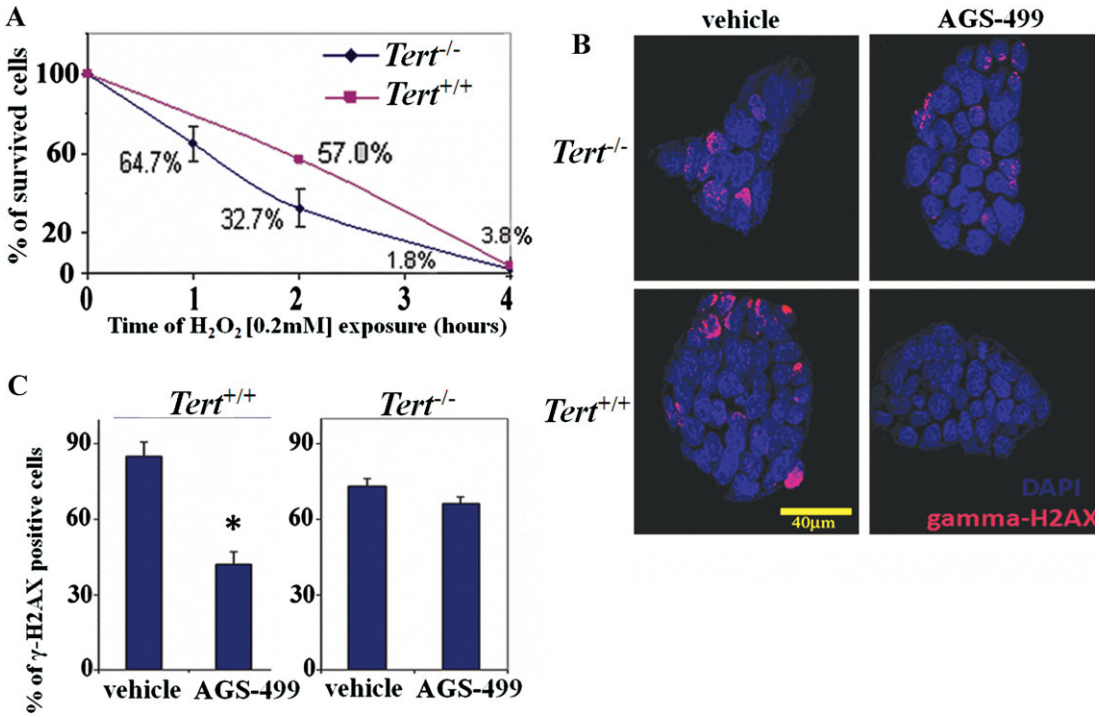

Figure 10. AGS compounds decreases the number of cells containing γH2AX foci induced by H_2O_2 in TERT-expressing mES cells but not in TERT-deficient ones.

A. TERT deficient mES cells (*Tert*$^{-/-}$) and their TERT expressing littermates (*Tert*$^{+/+}$) were treated with various concentrations of H_2O_2 in a serum free medium for 2 h and replaced with fresh medium for 24 h. Cell viability was determined by XTT assay. *mES Tert*$^{-/-}$ cells were treated with 0.2 mM H_2O_2 for 1 h and *Tert*$^{+/+}$ (WT) cells for 2 h. The medium was removed and a fresh medium containing AGS-499 (100 nM) was added for an additional 2 h.

B. H_2O_2 induced-DNA damage (DNA double-strand breaks) in the treated cells was determined by anti-γH2AX (phosphorylated histone) immunolabelling (red). Nuclei were stained using DAPI (blue) representative pictures. Bar, 40 μm.

C. The ratio between the number of cells containing γH2AX foci and the total number of cells in the slide ($n = 138$) was calculated for each sample. The results are mean ± s.e.m. of $n = 3$ experiments, Student's *t*-test, $p = 0.005$.

injection increases the survival rate and improves neurological symptoms in the surviving mice. These results indicate that AGS-499 protects neurons from the excitotoxicity effect of NMDA. Is it a consequence of telomerase activation in the brain? Several studies showed that increasing telomerase in the brain protected neurons from various insults. Specifically, an increased surviving rate was observed following NMDA injection (Kang et al, 2004; Lee et al, 2008) and ischemia in Tert-transgenic mice (Kang et al, 2004). In Tert-knockout mice, an increase in the susceptibility to stroke (Zhang et al, in press) and impaired memory (Lee et al, 2009) was observed. We showed here that increasing telomerase expression by AGS in motor neuron-like cells protected them from oxidative stress suggesting that AGS mimicked the results obtained in TERT transgenic mice challenged with NMDA.

The SOD1 Tg mice develop a rapid and progressive neurodegenerative disease, ALS, with a massive death of motor neurons and therefore they are a suitable animal model for testing the idea that telomerase increasing compounds, such as AGS-499, may have beneficial therapeutic effects on neurodegenerative diseases (Fossel and Flanary, 2009; Fu et al, 2000; Harley, 2005). Common parameters for examining the onset and progression of ALS were employed (Leitner et al, 2009), and the mouse groups were matched by gender, weight and age to avoid biological variables that may influence the interpretation of the results (Scott et al, 2008). AGS-499 delayed the onset (by 15%) and the progression of ALS (by doubling the survival duration in the late phase of the disease) and increased the lifespan (by 16%) of the SOD1 Tg mice. The influence of AGS-499 on ALS onset and progression exhibited a dose and time dependent pattern in which 6 mg/kg, daily, demonstrated the most efficient effects as revealed by all the tested parameters. The mode of action of AGS-499 on ALS development in the SOD1 Tg mice is not known. Several mechanisms to explain motor degeneration in SOD1 Tg mice have been proposed including mitochondrial dysfunction and oxidative damage (Bruijn et al, 2004). Since telomerase protects mitochondria and cells from oxidative stress (Haendeler et al, 2009), it is possible that AGS-499 that increases telomerase in neurons may protects the motor neurons (and/or other cells) in the mouse brain from degeneration caused by oxidative damage. Indeed, we observed a significant increase in the survival of motor neurons in the SOD1 lumbar region of the SC of AGS treated mice.

To substantiate this notion, we performed *in vitro* studies using a motor neuron-like cell line and demonstrated that AGS treatment significantly increased the survival of motor neurons exposed to oxidative stress. Our results indicated that the AGS effect is mediated by telomerase, since depletion of TERT expression by TERT shRNA prevented the effect of AGS-499. Additional support for this claim is provided by our finding that, while overexpression of TERT in these cells significantly increased their survival after oxidative stress, AGS-499 treatment did not enhance it further. To strengthen our claim that AGS-499 protected cells from oxidative stress by increasing telomerase expression and activity, we examined the effect of AGS-499 on TERT-deficient embryonic stem cells and their WT counterparts. We demonstrated that AGS decreased the number of cells containing DNA damage induced by oxidative stress in TERT-expressing cells but not in the TERT-deficient ones. Moreover, this effect is not specific to mouse cells but was also observed in human embryonic kidney cells. Our results therefore implicate telomerase in mediating the neuroprotective effects of AGS-499 in motor neurons and other cell types, which were exposed to oxidative stress.

In the present study, we demonstrate a decrease in telomerase protein and mTERT RNA transcripts in the FB and BS of SOD1 mice during disease progression, and this decrease in telomerase expression is prevented or restored by AGS treatment. Several studies compared the gene expression pattern in the SC or other tissues of SOD1 mutant Tg mice to their normal counterpart using microarray analysis (Ferraiuolo et al, 2007; Kudo et al, 2010; Ticozzi et al, 2011). The complete data can be found in the NCBI GEO dataset. We downloaded the relevant data and normalized them by Affymetrix software MAS-5 and the expression values of TERT in the SC for SOD1 mutant and the WT mice were collected and analysed. A decrease in TERT expression in motor neurons derived from SOD1 mutant mice compared to their littermate non-transgenic control is observed after ALS onset and during ALS progression, while TERT expression in other tissues was not affected (Supporting information Table 1). This data analysis is compatible with our result suggesting that telomerase expression is decreased during ALS progression.

Here, we suggest that increasing telomerase expression in the brain and SC of ALS mice by the AGS compound may be responsible for the enhancement of survival of motor neurons. However, we cannot rule out the possibility that AGS-499 activates telomerase-independent processes in the brain and SC that may also affect the onset or progression of ALS. However, our *in vitro* results, which demonstrate that AGS-499-induced enhancement of cell survival after oxidative stress is mediated by telomerase, strengthen the notion that AGS-499 delays ALS onset and progression by increasing the expression and function of telomerase in motor neurons.

In conclusion, our results suggest that increasing TERT expression and telomerase activity in the brain in a controlled and transient manner by the AGS-499 compound can be used as a potential novel therapy for neurodegenerative disorders.

MATERIALS AND METHODS

In vivo experiments

Animals

CD-1 male mice (5–8 weeks) were used for the investigation of telomerase in the mouse brain and for NMDA excitotoxicity. B6SJL-Tg ($SOD1^{G93A}$) 1 Gur/J male mice (60 mice) were purchased from the Jackson laboratory (Maine). B6SJL-Tg ($SOD1^{G93A}$) 1 Gur/J mice (7–16 weeks) for the mTERT analysis were kindly received from Dr. Daniel Offen of Tel Aviv University, Israel. All animal procedures were approved by the animal experimentation ethics committee at Ben-Gurion University.

AGS-499 injection

AGS solution in DMSO was freshly prepared for each experiment and 1.5, 3, 6 and 12 mg/kg of AGS (1 μl AGS/DMSO mixture in 100 μl PBS) or an equivalent amount of the vehicle (1 μl DMSO in 100 μl PBS) were administrated by subcutaneous injection into the mouse neck.

Preparation of nuclear and cytoplasmic proteins extracts

Brains were quickly removed from the skull and washed with ringer solution at 4°C. The FB, BS and the lumbar region of the SC were isolated. The tissues were homogenized (pestle B) and centrifuged at 500 *g* at 4°C for 7 min. The pellets were subjected to nuclear and cytoplasmic extractions. The cytoplasmic extract was prepared as previously described (Auer et al, 1982; Bendetz-Nezer et al, 2004; Zehorai et al, 2008).

Nuclear extract

The pellet from the cytoplasmic preparation was re-suspended in CHAPS buffer (10 mM TRIS HCl, pH-7, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 0.5% CHAPS (3[(3 Cholamidopropyl)dimethylammonio]-propanesulfonic acid), 10% Glycerol) at 4°C for 30 min following by for 30 min centrifugation at 9300 *g* at 4°C, and the supernatant was collected. Total protein concentration was determined using the BIO-Rad protein assay kit (Bio-Rad Laboratories).

Whole cell extract

Cell pellets, obtained following brain or SC homogenization, were re-suspended in CHAPS buffer for 30 min, centrifuged (9300 *g* at 4°C), and the supernatant was kept as the whole cell protein extract.

Western blot analysis

Antibodies

Anti-hTERT polyclonal antibodies: SC-7212 (Santa Cruz Biotechnology, CA) and Rockland 600-401-252 (Rockland Immunochemicals, Inc., PA). Anti-hTERT monoclonal antibody (1531-1, Epitomics, CA). Anti -β actin antibody (691001) were purchased from ICN (Irvine, CA).

Separation of proteins by gel electrophoresis and detection

Protein extract (as indicated in the various experiments) derived from nuclear and cytoplasmic fractions were analysed by poly acrylamide gel electrophoresis and Western blotting as previously described (Kaufmann and Svingen, 1999; Maniatis et al, 1980), using either

anti-hTERT antibody (1:1000) or anti-β-actin antibody (1:10,000). The immunocomplexes were detected by enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology).

Northern blot assay

RNA extract was prepared with 'Total RNA Extraction Reagent' (BioFlux) according to the manufacturer instructions. Total RNA (20 μg) derived from mice brains was analysed by Northern blot as previously described (Tichon et al, 2009) cDNA was prepared using the ReverAid First strand CDNA synthesis kit (Fermentas). The probe used for the identification of telomerase mRNA was described by Klapper et al. (Klapper et al, 2001). mTERT-primer Fw 5′-CGGAAGAGTGTCTG-GAGCAA-3′ and mTERT primer Rv 5′-GGATGAAGCGGAGTCTGGA-3′. GAPDH probe was used as a control. GAPDH-primer Fw 5′-TTCACCACCATGGAGAAGGC-3′ and GAPDH primer Rv 5′-GGCATG-GACTGTGGTCATGA-3′. Both probes were labelled by dCTP [P^{32}] using 'Ready-to-go' kit (Amersham). The membrane was blotted with the probes overnight at 65°C, washed with buffer containing SSCx2 and SDS, and finally the radioactive labelling was detected with autoradiography or phosphoimager (Bio-Rad Laboratories, USA).

m*Tert* transcripts detection

Total RNA and cDNA from the mouse FB and BS was prepared as described above. Real time PCR was performed using the following primers: FW: 5′-GAAAGTAGAGGATTGCCACTGGC-3′, REV: 5′-CGTATGTGTCCATCAGCCAGAAC-3′ using the Green JumpStart Taq ready mix for quantitative PCR (Sigma, Israel) with the 7500 Real time PCR System (Applied BioSystems).

TRAP assay

Telomerase activity was performed as described (Harley et al, 1994). In brief, protein extract (at the indicated amount) was incubated with TS primer (5′-AATCCGTCGAGCAGAGTT-3′) for 45 min at 30°C followed by PCR assay with [α-P^{32}] dCTP using CX primer (5′-CCCTTACCCT-TACCCTTACCCTTA-3′) or ACX primer (5′-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3′). Internal standard primers used as a control: IS primer (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′) and ISR primer (5′-ATCGCTTCTCGGCCTTTT-3′). For detection of telomerase products in the brain extracts, 32 PCR cycles were used, and the internal standard primers were diluted to concentration of 5×10^{-15} M. The PCR products were separated on 12.5% polyacrylamide gel, and the radioactive products were detected with phosphoimager (Bio-Rad Laboratories, USA) or by autoradiography using X ray films.

Quantification of telomerase activity

Using the Telomerase detection kit, Real Time-PCR based TRAP assay was performed according to the manufacturer protocol (Allied Biotech, Inc.), with an RT-PCR machine (Bio-Rad Laboratories, USA).

NMDA treatment

Mice were injected with AGS-499, 24 and 12 h before the NMDA (110 mg/kg) intraperitoneal (i.p.) injection. The seizures where started 5 min after NMDA treatment and re-occurring seizures were monitored during 40 min after injection (Feigenbaum et al, 1989). Dead mice were immediately removed from the cage and the remained surviving mice were subjected to a blinded neurological score estimation by nine independent observers.

ALS mouse model

Animals

Adult male B6SJL Tg ($SOD1^{G93A}$) mice with a high copy number of the mutant allele and a rapid disease onset were used. Mice were housed 4–5 per cage with free access to food and water, under standard conditions. Fifty-six mice were separated into treatment, vehicle and untreated groups (5 groups, $n = 7$–10 per group). The mice in the groups were matched by weight and by age to ensure that the initial body weights and the average age in each group were as similar as possible (Scott et al, 2008).

Treatment

Mice received subcutaneous injections (into the neck) of AGS-499 (6 or 1.5 mg/kg, volume 100 μl in PBS) or vehicle (1% DMSO in PBS) every 24 or every 48 h, starting at 70 days of age.

Survival

Mice were observed daily and were considered clinically dead and sacrificed when found unable to roll over within 20 s after being pushed on their side (Esposito et al, 2007). Date and cause of death were recorded for each mouse.

Behavioural and weight assessment

Motor performance was assessed using an Economex Rotarod apparatus (Columbus Instruments, Columbus, OH). Mice were given two Rotarod training sessions to acclimate them to the apparatus and then tested twice a week starting from 70 days of age. Each mouse undertook three 120-s trails per test session and the best result was recorded. Mice were weighted twice a week.

Neurological score

Neurological score of four limbs was blindly performed by an independent physiotherapist using the scale of 0–5, with 0 being normal and 5 being completely paralysed as suggested in the guidelines for preclinical testing with ALS mice (Leitner et al, 2009).

Motor neurons survival

Untreated mice at the age of 70 days and vehicle or AGS (6 mg/kg, every 24 h) treated mice at the age of 100 days were sacrificed. The lumbar region of the SC (T10-L5) was removed, and Para-formaldehyde-fixed, paraffin embedded, 10 μm transverse sections were cut and subjected to Nissl staining. Quantification of the number of motor neurons per section was performed, by counting serial micrographs ($n = 10$) from each animal ($n = 2$), as previously described (Ilieva et al, 2008).

Cell culture experiments

Mouse NSC-34 cells

Mouse NSC-34 cells, a motor neuron-like cell line (Matusica et al, 2008), was kindly received from Dr. Daniel Offen, Tel Aviv University, Israel. The cells were cultured in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% penicillin–streptomycin and 1% non-essential amino acids.

The paper explained

PROBLEM:

Neurodegenerative diseases are characterized by the loss and death of neurons. Amyotrophic lateral sclerosis (ALS) is a devastating and rapidly fatal degenerative disease characterized by the death and dysfunction of motor neurons in the cerebral cortex, BS and SC. No cure for this disease is available and the cause for the death of the motor neurons is not known. However, oxidative stress is involved in the pathogenesis of ALS. Therefore, one may suggest that protecting of motor neurons from oxidative stress may provide a possible therapeutic strategy. Increasing telomerase expression in neurons by gene manipulation protected them from various insults such as oxidative stress and reactivation of the endogenous telomerase gene in engineered adult mice reversed neurodegeneration and restored the proliferation capacity of neural progenitors in the subventricular zone region of the brain (Jaskelioff et al, 2011). Thus, increasing telomerase activity in a controlled manner may have beneficial therapeutic effects for neurodegenerative diseases.

RESULTS:

In this study, we show that the novel compound AGS-499 transiently increased the expression and activity of telomerase in mouse FB, BS and SC in a dose- and time-dependent manner. This compound protected mice from NMDA-induced excitotoxicity. Most importantly, AGS-499 delayed the onset and progression of ALS in the SOD1 Tg mouse model and increased their survival. In these mice, a significant increase in the survival of SC motor neurons was detected after AGS-499 treatment. The neuroprotective effect of AGS-499 from oxidative stress was confirmed *in vitro* using motor-neuron-like cell lines and is mediated by telomerase. These results suggest that the AGS-499 compound exerts its neuroprotective effect by increasing telomerase expression and activity.

IMPACT:

The controlled increase of telomerase in motor neurons by compounds such as AGS-499 and their neuroprotective effect against oxidative stress may provide a possible new therapeutic strategy for the treatment of neurodegenerative diseases like ALS.

Mouse embryonic stem cells

Mouse embryonic stem cells (mESc) derived from TERT deficient mice (m*Tert*$^{-/-}$) and mESc derived from their wild type littermates (*mTert*$^{+/+}$) were kindly obtained from Dr. L. Harrington, (Edinburgh Univ. Scotland) (Liu et al, 2000, 2002). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, 1% penicillin–streptomycin, leukaemia inhibitory factor (LIF) 1.08×10^3 U/ml, 0.05 mM β-mercaptoethanol and 1 mM pyruvate.

Human epithelial kidney cells

Human epithelial kidney cells (HEK) were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% penicillin–streptomycin.

Plasmids

pMKO.1 puro hTERT shRNA (Masutomi et al, 2005), and pBABEhygroHigheGFPhTERT (Wong et al, 2002) were purchased from Addgene (Cambridge, MA). TRC1.5 Vector: pLKO.1-puro containing mTERT shRNA and Scrambled shRNA were purchased from Sigma, Israel.

Retrovirus production

Packaging of the plasmids (pMKO.1 puro hTERT shRNA and pBABEhygroHigheGFPhTERT) for retroviral infection was performed as previously described (Braiman et al, 2006) using pVSV-G (Clontech) and GP2-293 packaging cells (Clontech).

Lentivirus production

Packaging of the TRC1.5 Vector: pLKO.1-puro plasmids was performed as previously described (Regev et al, 2010) using pCMV-VSV-G, pMDL.gp.RRE and pRSV-Rev plasmids kindly provided by Dr. A. Chen (Weizmann Institute of Science, Rehovot, Israel).

Treatment of cells with H_2O_2

Cells were exposed to various concentrations of H_2O_2 for 4 h, in serum-free medium and AGS-499 was added at the indicated doses. The medium was replaced with fresh serum-containing medium for 24 h, followed by the XTT assay.

Statistical analysis

Results are the mean of at least three independent experiments ± standard deviation or standard error. Statistical significance of the results was obtained using the student's T or χ^2 test or the Two Way ANOVA using the OriginPro7 software.

Author contributions

EP and EE designed research; EE and AT performed research; EP, EE and DG analysed data; AG, SS and EP designed compounds; AG synthesized compounds; EP and EE wrote the paper; all authors edited the manuscript.

Acknowledgements

We thank Dr. L. Harrington (Scotland) for providing the TERT-deficient mESC, Dr. Daniel Offen for the NSC-34 cells, Dr. Chen A for the lentivirus production system, Dr. Alex Braiman for the retroviral production system, Rafael Peleg for graphical assistance, Noam Eitan for performing the neurological scores

in SOD1 mice and Sylvia Tsori for technical assistance. This study was supported by the IsrALS association fund and by the Seed Research fund of the Ben-Gurion University.

Supporting information is available at EMBO Molecular Medicine online.

Conflict of interest statement: EP, AG and SS filed a patent on AGS-499 and other derivatives as telomerase increasing compounds.

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