Table 1. Summary of regional immunohistochemical findings

	*Lower motor neurones**	*Motor cortex*	*Non-motor cortex†*	*Dentate gyrus*	*Hippocampal CA4*	*Cerebellar granule cells*
Neuronal loss	++	+	–	–	–	–
TDP-43	+	+	–	–	–	–
pTDP-43	+	+	–	–	–	–
RBM45	+	+	–	–	–	–
p62	+	++	++	++	++	++
Ubiquitin	+	+	+	+	+	++
Ubiquilin	–	+	+	++	+	++
DPR‡	–	++	++	+++	++	+++

Severity of neuronal loss are represented as: – = not noted, + = mild, ++ = moderate-severe.
Neurones containing each antibody-positive inclusions were counted per 100 neurones in high-power fields, and ratio is represented as: – = none, + = ~10%, ++ = 10~30%, +++ = >30%.
*Cervical anterior horn cells and hypoglossal nucleus.
†Frontal and temporal cortex were examined.
‡poly Gly-Ala.

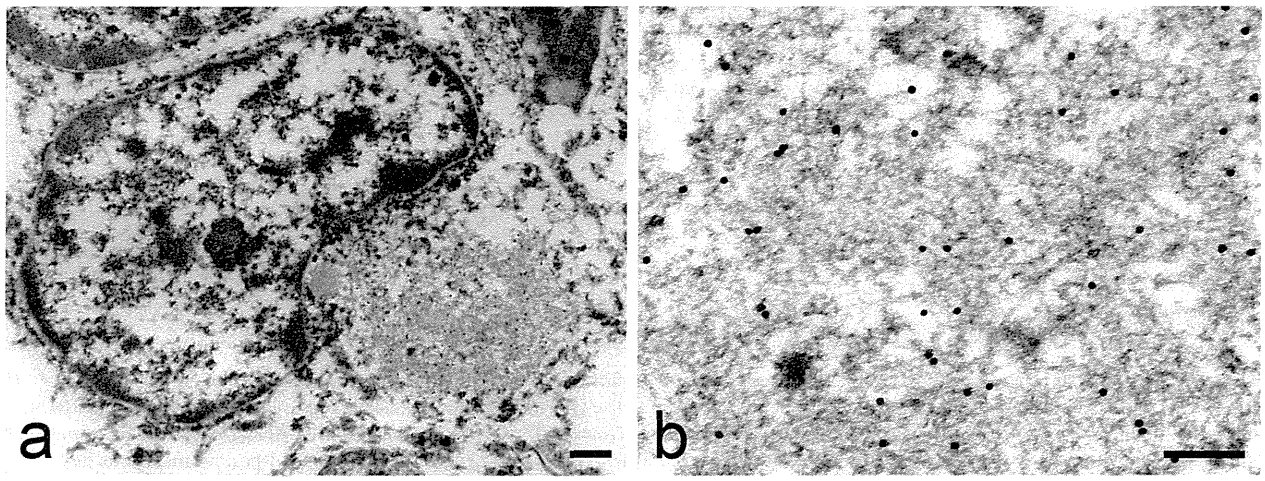

Figure 2. Immunoelectron microscopy for the presence of ubiquilin. (**a**) A cerebellar granule cell is seen to have a round filamentous cytoplasmic aggregate. (**b**) Higher-magnification view of the aggregate, showing immunogold particles on the randomly arranged, approximately 10-nm-wide filamentous structures. Bars: (**a**) 500 nm, (**b**) 200 nm.

(Figure 1**e–h,j**) that generally differed in morphology from TDP-43- and RBM45-positive NCIs (Figure 1**a–d**). Such inclusions were frequently labelled with antibodies against ubiquilin, ubiquitin (polyclonal, Dako, Glostrup, Denmark; 1:800) and p62 (monoclonal, BD biosciences, San Jose, CA, USA; 1:500) (Figure 1**j–m**). The immunohistochemical findings are summarized in Table 1.

Under immunogold-labelling electron microscopy for ubiquilin in the cerebellar granular layer tissue, the labelled NCIs were shown to be composed of randomly arranged filamentous structures (Figure 2**a,b**); their morphological features were very similar to those of ubiquitin-positive and TDP-43-negative NCIs in the entorhinal cortex in a case of c9FTD/ALS [12]. Double-labelling immunofluorescence revealed that co-localization of TDP-43 (monoclonal, clone 2E2-D3, Abnova, Taipei, Taiwan; 1:250) and RBM45 (Figure 3**a–c**), but not TDP-43 (2E2-D3) and DPR proteins (Figure 3**d–f**), was a feature in the cytoplasmic inclusions. The majority of p62-positive NCIs were positive for DPR proteins, whereas p62-positive GCIs were negative for DPR proteins (Figure 3**g–i**).

In the present Japanese case of c9ALS, the histological and molecular pathology of sporadic ALS was observed: especially, the occurrence of Bunina bodies and TDP-43-

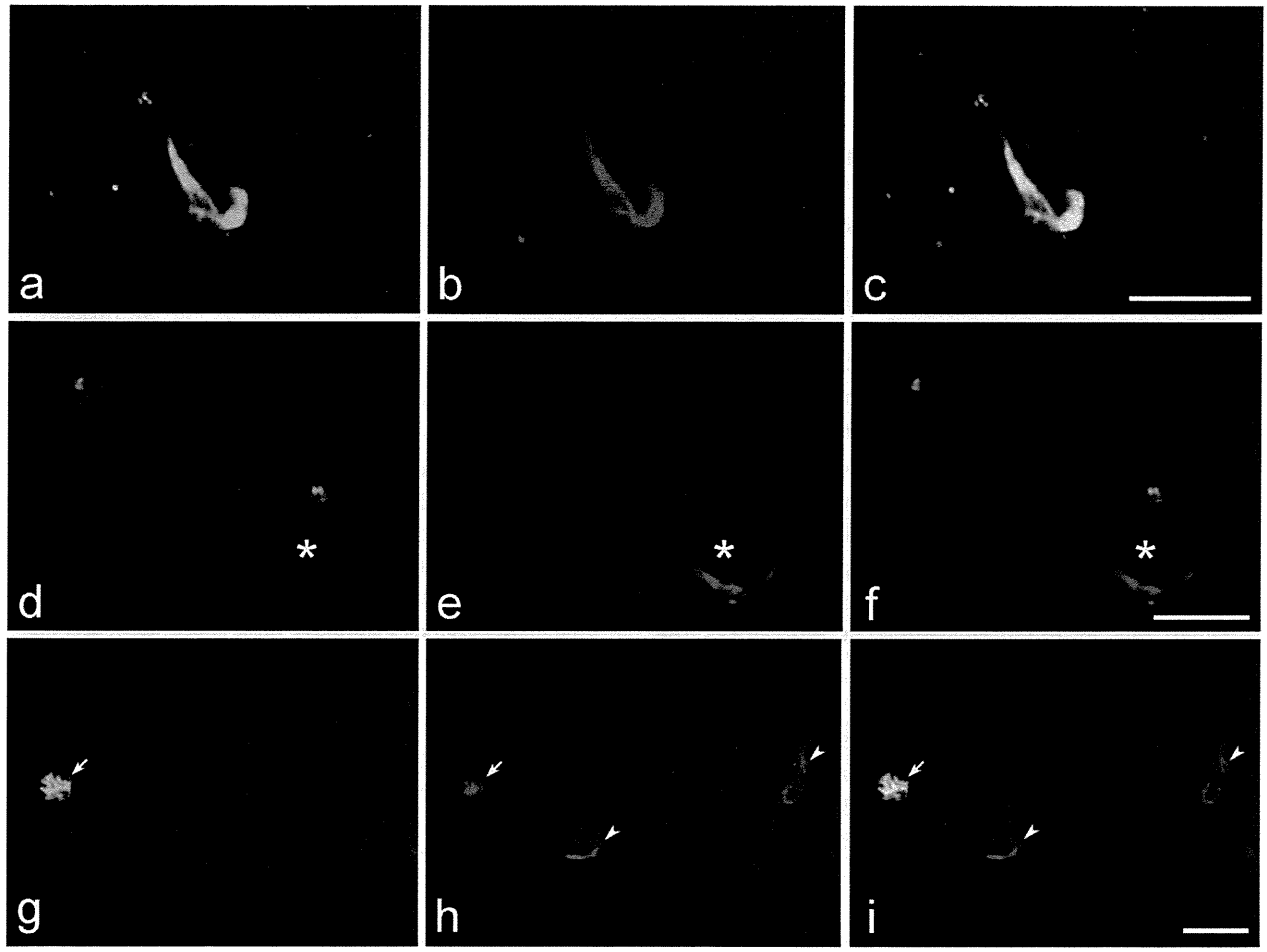

Figure 3. Double-labelling immunofluorescence for the presence of TDP-43, RBM45, DPR proteins and p62. (**a–i**) Inclusions observed in the motor cortex cells. (**a–c**) Colocalization of RBM45 (**a**, green) and TDP-43 (**b**, red) is evident in the cytoplasmic inclusions in an oligodendrocytic cell (**c**, merge). (**d–f**) Poly Gly-Ala (GA) (**d**, green) and TDP-43 (**e**, red) never co-localize in the cytoplasmic inclusions (**f**, merge), although co-existence of poly GA- and TDP-43-positive inclusions are rarely observed in the same cells, mostly in the neuronal cells (asterisks) (**d–f**). (**g–i**) Co-localization of poly GA (**g**, green, arrow) and p62 (**h**, red, arrow) is evident in cytoplasmic inclusions in a neuronal cell (**i**, merge, arrow). Poly GA-negative (**g**, green) and p62-positive inclusions (**h**, red, arrowheads) are also evident in two oligodendrocytic cells (**i**, merge, arrowheads). Bars: 10 μm (**a–i**).

positive NCIs was confirmed. It is important to note that the TDP-43 pathology and neuronal loss were correlated with each other; this was also the case for another RNA-binding protein, RBM45, which has been reported to be increased in the cerebrospinal fluid of ALS patients, and also colocalized with TDP-43 in inclusions of patients with ALS and FTLD-TDP [10]. In addition, the occurrence of p62-positive, and TDP-43-negative granular or star-shaped NCIs in the cerebellar cortex and hippocampus was a feature. Importantly, such p62-positive and TDP-43-negative NCIs were also labelled with antibodies against DPR proteins we generated. The DPR protein pathology was distributed independently of TDP-43 pathology: co-localization of DPR proteins and TDP-43 was extremely rare, if present, in the same inclusion, indicating that unlike TDP-43 and RBM45, there was no correlation between DPR protein pathology and neuronal loss (Table 1), or between DPR protein pathology and clinical symptoms, as pointed out previously in Caucasian cases of c9FTD/ALS [13].

It is noteworthy that most recently, the DPR proteins have been reported to be translated from not only a sense direction of the repeats but also an antisense direction (CCCCGG); in fact, two additional DPR proteins (poly

Ala-Pro and poly Pro-Arg) have been detected by immunohistochemistry [8,11]. Although the present study did not investigate antisense strand-derived DPR proteins, it appears almost certain that the overall neuropathological picture, including the immunohistochemical features, was indistinguishable from that described previously in Caucasian cases of c9FTD/ALS [8,11,13], leading us to conclude that the neuropathology of c9FTD/ALS is specifically and strongly tied to the abnormal *C9ORF72* repeat expansions, irrespective of any difference in genetic background or ethnicity.

At present, several pathomechanisms, by which *C9ORF72* repeat expansions cause c9FTD/ALS, can be considered [14]: loss of C9ORF72 function (decrease in *C9ORF72* mRNA), gain of RNA toxic function (formation of RNA foci and sequestration of protein into them), and aberrant dipeptide aggregation resulting from RAN translation. It remains unknown which mechanism might play a major role in the pathogenesis of c9FTD/ALS, or whether these mechanisms may mutually affect each other and generate synergistic toxicity. Considering the possible pathomechanisms, it appears significant that there was no apparent correlation between DPR protein pathology and neuronal loss [13], which was also evident in the present Japanese case of c9ALS (Table 1). It is also of great interest that the co-occurrence of sense/antisense RNA foci and DPR protein-positive inclusions is rare in the same cells [8]. Taken together, it may be possible to consider that the production of, and subsequent aggregate formation by DPR proteins is either neuroprotective or not a major factor in the pathogenesis of c9FTD/ALS.

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Author contributions

T.K., M.T. and H.T. managed the study and were principally responsible for writing. A.T. and H.E. examined the patient, T.K., M.T., A.S., A.K. and H.T. performed the neuropathological observation and evaluation, T.K, M.N. and O.O. performed the *C9ORF72* repeat expansion analysis, and M.M.-S. and M.H. generated anti-polypeptide antibodies used in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Confirmatory double-blind, parallel-group, placebo-controlled study of efficacy and safety of edaravone (MCI-186) in amyotrophic lateral sclerosis patients

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Abstract

Our objective was to confirm the efficacy and safety of edaravone in amyotrophic lateral sclerosis (ALS) patients. We conducted a 36-week confirmatory study, consisting of 12-week pre-observation period followed by 24-week treatment period. Patients received placebo or edaravone i.v. infusion over 60 min for the first 14 days in cycle 1, and for 10 of the first 14 days during cycles 2 to 6. The efficacy primary endpoint was changed in the revised ALS functional rating scale (ALSFRS-R) scores during the 24-week treatment. Patients were treated with placebo ($n = 104$) and edaravone ($n = 102$). Changes in ALSFRS-R during the 24-week treatment were -6.35 ± 0.84 in the placebo group ($n = 99$) and -5.70 ± 0.85 in the edaravone group ($n = 100$), with a difference of 0.65 ± 0.78 ($p = 0.411$). Adverse events amounted to 88.5% (92/104) in the placebo group and 89.2% (91/102) in the edaravone group. In conclusion, the reduction of ALSFRS-R was smaller in the edaravone group than in the placebo group, but efficacy of edaravone for treatment of ALS was not demonstrated. Levels and frequencies of reported adverse events were similar in the two groups.

Key words: *Amyotrophic lateral sclerosis, ALSFRS-R, edaravone, placebo, randomized trial*

Introduction

Amyotrophic lateral sclerosis (ALS) is a refractory and progressive disease that causes selective degeneration of upper and lower motor neurons (1). Median survival from onset to death in ALS is reported to vary from 20 to 48 months (2).

Oxidative stress has been considered to be involved in the onset and progression of ALS (3). An established marker of oxidative stress is 3-nitrotyrosine (3NT), formed by reaction of the free radical peroxynitrite with tyrosine residues. A significant increase of 3NT was reported in spinal cord of transgenic mice expressing mutated SOD1 (4) and in autopsied spinal cord of FALS patients with genetic mutation of SOD1 and sporadic ALS (SALS) patients (5). 3NT was found in motor neurons (6,7) and was elevated in cerebrospinal fluid of SALS patients (8). Moreover, oxidative stress induces nuclear translocation and activation of Nrf-2, a transcription factor that generates an anti-oxidative response (9). Nrf-2 translocation also occurs in mutant TDP-43 transfected cultured motor neuron cell lines (10–12). Consequently, drugs that eliminate free radicals might protect motor neurons from oxidative stress and free radical damage in ALS patients.

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Edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one, Mitsubishi Tanabe Pharma Corporation, Tokyo, Japan) is a free radical scavenger approved for treatment of acute cerebral infarction in Japan in 2001 (13). Edaravone eliminates lipid peroxides and hydroxyl radicals during cerebral ischemia and protects nerve cells within or around the ischemic region from free radical damage (14–16). Beneficial effects of edaravone have been reported in wobbler mice with ALS-like symptoms (17) and in ALS-model animals (18,19).

A phase II trial was conducted to investigate the safety and efficacy of edaravone in ALS patients, and found that progression of motor dysfunction was slowed and no clinically significant adverse drug reactions occurred. The level of 3NT was low in cerebrospinal fluid of almost all patients in the phase II trial, suggesting that edaravone could protect neuronal cells from oxidative stress (20). Therefore we designed a clinical trial to confirm the efficacy and safety of edaravone in ALS patients.

Methods

Standard protocol approvals

Twenty-nine sites in Japan participated in the study between May 2006 and September 2008. An institutional review board at each site approved the study protocol. The study was conducted in compliance with Good Clinical Practice (GCP). All participants provided written informed consent before the pre-observation stage. The study sponsor was Mitsubishi Tanabe Pharma Corporation. The study is registered in ClinicalTrials.gov with a registration number NCT00330681.

Patients

Inclusion criteria were: age 20–75 years; diagnosis of 'definite', 'probable' or 'probable laboratory-supported' ALS (21,22) according to the revised Airlie House diagnostic criteria; forced vital capacity (FVC) of at least 70%; duration of disease within three years; and change in revised ALS functional rating scale (ALSFRS-R) (23,24) score during the 12-week pre-observation period of –1 to –4 points. Patients also had a Japanese ALS severity classification (25) of 1 or 2. (The Japanese ALS severity classification score ranges from 1 to 5 according to the severity classification of the Specified Disease Treatment Research Program for ALS of the Ministry of Health, Labor and Welfare of Japan. Severity Classification: 1) able to work or perform housework; 2) independent living but unable to work; 3) requiring assistance for eating, excretion or ambulation; 4) presence of respiratory insufficiency, difficulty in coughing out sputum or dysphagia; and 5) using a tracheostomy tube, tube feeding or tracheostomy positive pressure ventilation.)

Exclusion criteria were: reduced respiratory function and complaints of dyspnea; complications that may substantially influence evaluation of drug efficacy, such as Parkinson's disease, schizophrenia and dementia; complications that require hospitalization, including liver, cardiac and renal diseases; infections that require antibiotic therapy; deteriorated general condition as judged by investigators; renal dysfunction with creatinine clearance of 50 ml/min or below within 28 days before treatment; and undergoing cancer treatment.

Patient eligibility was assessed with inclusion and exclusion criteria at the start and end of pre-observation.

Administration regimen of riluzole was required not to be changed during the study.

Study medication

Mitsubishi Tanabe Pharma Corporation provided the investigational drugs in ampoules. Only authorized personnel, independent of the sponsor and investigators, had access to the key code until unblinding. The dose of edaravone was 60 mg per day, which was indicated to show efficacy in the phase II trial (20), and placebo was chosen since no suitable comparator drug for ALS has been approved. Saline (placebo) or edaravone was administered once daily by i.v. infusion over 60 min.

Design

After the 12-week pre-observation period, eligible patients were randomized to placebo or edaravone group. Dynamic allocation was used to minimize the effects of the following three factors, which may substantially influence the evaluation of edaravone:

- Factor 1: change in ALSFRS-R score during pre-observation period: two categories: –4, –3 or –2, –1.
- Factor 2: initial symptom: two categories – bulbar or limb.
- Factor 3: use of riluzole: two categories – yes or no.

The study period was 36 weeks, consisting of a 12-week pre-observation period before the start of the first cycle, followed by a 24-week treatment period (Figure 1).

A single treatment cycle consisted of 14 days of study drug administration period followed by a 14-day observation period. Study drugs were administered every day for 14 days in the administration period of the first cycle, and for 10 out of 14 days in the administration periods of cycles 2 to 6. The end of the administration period in each cycle was followed by a 14-day observation period.

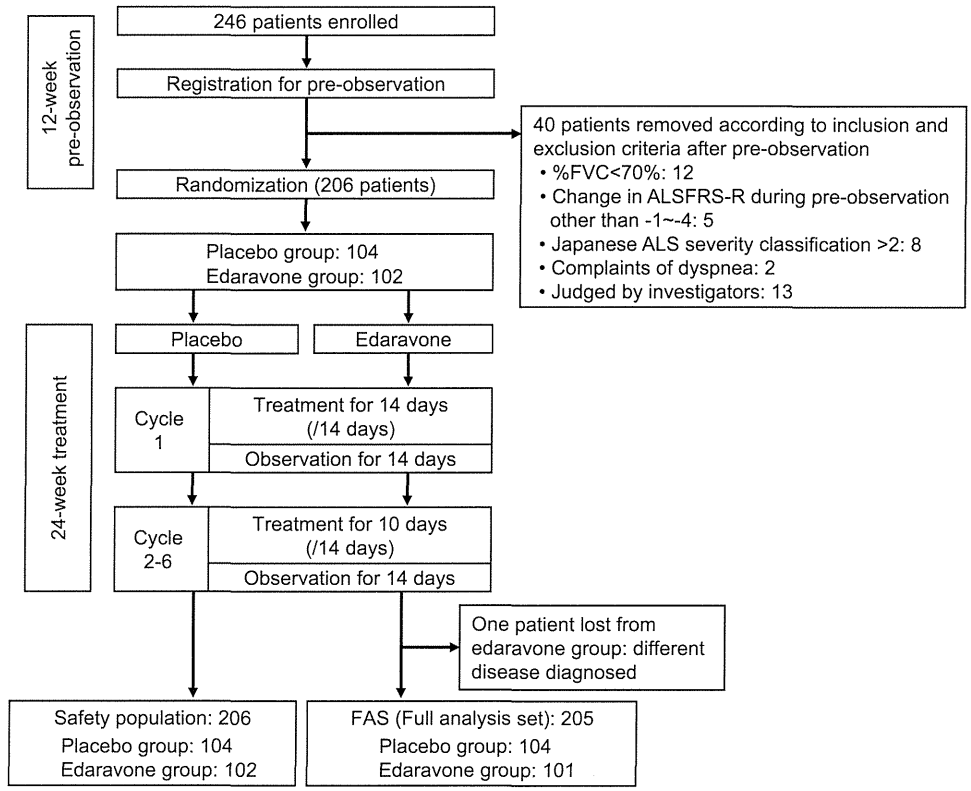

Figure 1. Trial profile.

Efficacy evaluation

Primary efficacy endpoint was the change in ALS-FRS-R score. Secondary endpoints were: changes of FVC, grip strength (left/right mean), pinch strength (left/right mean), Modified Norris Scale score (26,27), ALSAQ-40 (ALS Assessment Questionnaire) (28,29), and time to death or a specified state of disease progression (incapable of independent ambulation, loss of function in upper limbs, tracheotomy, artificial respirator with intubation, or tube feeding). The evaluations were carried out at the following times: before pre-observation, before the start of the first treatment cycle and at the end of each treatment cycle (after 14 days observation and before the first dosage of the next cycle).

Safety evaluation

Safety was assessed in terms of number and severity of adverse events (AE), adverse drug reactions and the results of clinical laboratory tests and sensory tests. Serious adverse events were identified from the adverse events according to the GCP guideline.

Statistical analysis

Based upon the experience of the phase II trial (20), we considered that it would be difficult to enroll more than 100 patients per group for the trial and the target number of patients for enrollment was set at 200. In the phase II trial, the difference of the change of ALSFRS-R between the edaravone and placebo groups was 2.2 for patients with matched severity and dose to those of the present study; and on the assumption of a standard deviation of 5.2, the statistical power of this study can be calculated as 85% when 100 patients per group were enrolled.

The primary population used for the efficacy analysis was the full analysis set (FAS). For ALS-FRS-R scores, analysis of covariance (ANCOVA) was performed on the change in score during treatment, defined as the difference between the score before the start of the first treatment cycle (before treatment) and the score at two weeks after the end of the sixth treatment cycle (after treatment). Three factors were used for dynamic allocation as covariates, after which the inter-group difference was assessed. Repeated measures analysis of variance was also performed using the treatment group, period, and interaction between treatment group and period (treatment group × period) as design factors, and baseline value and the three factors used for dynamic allocation as covariates, after which the inter-group difference was assessed. Compound symmetry was assumed as a covariance structure of repeated measurement. Edaravone efficacy would be verified if a significant inter-group difference were found in at least one of the above analyses. A two-sided level of significance of 5% and a two-sided 95% confidence interval were chosen for interpretation of main effect. A two-sided level of significance of 15% was chosen for

determining the existence of effect of interaction. A stratified analysis was also performed on the changes of ALSFRS-R score by diagnostic category. The level of significance for differences of patient characteristics was set at 15%.

ANCOVA and repeated measures analysis of variance were similarly performed on the secondary endpoints. Time to death or a specified state of disease progression was defined as an event and the other endpoints were followed until cut-off. A stratified, generalized Wilcoxon test and log-rank test were performed using the change in ALSFRS-R score during the pre-observation period as a stratification factor. For patients with more than one event, the onset date of the first event was defined as the survival time. In censored cases, the cut-off date was the end date of observations.

For patients with missing data at 24 weeks after starting treatment, the last observation carried forward (LOCF) method was applied to impute missing data. Patients who completed the third cycle were eligible for LOCF.

To evaluate safety, AE and adverse drug reactions were assessed in the safety population. Proportions of AE, adverse drug reactions, serious adverse events (SAE) and serious adverse drug reactions were calculated and compared between the groups using Fisher's exact test. A two-sided level of significance of 5% and a two-sided 95% confidence interval were chosen for interpretation. Statistical analysis was performed using SAS software (version 9.1, SAS Institute, Cary, NC).

Results

Subject background

Two hundred and forty-six patients were prospectively registered. After the 12-week pre-observation period, 40 patients were excluded according to the inclusion and exclusion criteria, and the remaining 206 patients were randomized (Figure 1).

The FAS included 205 patients after exclusion of one patient who was diagnosed with a different disease. For the safety evaluation, the number of patients in the safety population was 206, which included all patients treated with the study medication. The treatment was discontinued for 23 patients (edaravone group: patients' request 5, AE 3, tracheotomy 1; placebo group: patients' request 5, AE 6, tracheotomy 2, protocol violation 1). There was no imbalance between the groups in either analysis set on discontinuation (FAS: $p = 0.378$; safety population: $p = 0.377$).

All patients in the safety population received at least 80% of the assigned dosages of study drug.

Patient characteristics are summarized in Table I. Among the patient characteristics, those for which inter-group differences were found at a significance level below 15% were the duration of disease ($p = 0.104$, paired t-test), ALSFRS-R score before pre-observation ($p = 0.065$, paired t-test), and ALSFRS-R score at the start of the first cycle ($p = 0.146$, paired t-test).

Table I. Subject demographic characteristics.

Item	Placebo (104) *n* (%)	Edaravone (101) *n* (%)
Gender		
male	69 (66.3)	63 (62.4)
Initial symptom		
bulbar	20 (19.2)	18 (17.8)
limb	84 (80.8)	83 (82.2)
Diagnosis (El Escorial revisited)		
definite	21 (20.2)	29 (28.7)
probable	54 (51.9)	52 (51.5)
probable laboratory-supported	28 (26.9)	20 (19.8)
possible	1 (1.0)	0 (0.0)
The Japanese severity classification		
grade 1	40 (38.5)	36 (35.6)
grade 2	64 (61.5)	65 (64.4)
Use of riluzole		
yes	92 (88.5)	90 (89.1)
Change in ALSFRS-R score during pre-observation		
−4, −3	32 (30.8)	29 (28.7)
−2, −1	72 (69.2)	72 (71.3)
Item	Placebo (104) median (min-max)	Edaravone (101) median (min-max)
Age (years old)	58.5 (28–75)	58.0 (29–73)
Body weight (kg)	57.0 (37–109)	57.0 (35–77)
Duration of disease (years)	1.20 (0.3–3.0)	1.30 (0.4–2.9)
ALSFRS-R score before pre-observation	44.0 (35–48)	43.0 (31–48)
ALSFRS-R score before treatment period	42.0 (32–47)	41.0 (29–47)

ALSFRS-R: the revised amyotrophic lateral sclerosis functional rating scale.

Table II. Change in endpoints during treatment.

	Change in endpoints during treatment (ANCOVA)				Repeated-measures analysis			
	Adjusted mean change LS Mean ± S.E.		Inter-group difference in adjusted mean change LS Mean ± S.E. (95% C.I.)	p value	Adjusted mean LS Mean ± S.E.		Inter-group difference in adjusted mean LS Mean ± S.E. (95% C.I.)	p value
	Placebo	Edaravone			Placebo	Edaravone		
Primary endpoint								
ALSFRS-R	−6.35 ± 0.84 (99)	−5.70 ± 0.85 (100)	0.65 ± 0.78 (−0.90 – 2.19)	0.411	37.43 ± 0.46	38.08 ± 0.47	0.65 ± 0.44 (−0.22 – 1.52)	0.141
Secondary endpoint								
%FVC	−17.49 ± 2.39 (99)	−14.57 ± 2.41 (100)	2.92 ± 2.24 (−1.49, 7.33)	0.193	87.30 ± 1.56	88.56 ± 1.59	1.26 ± 1.46 (−1.63, 4.15)	0.390
Grip strength	−5.71 ± 0.69 (99)	−4.81 ± 0.69 (100)	0.89 ± 0.64 (−0.37, 2.16)	0.165	13.22 ± 0.42	13.83 ± 0.43	0.60 ± 0.40 (−0.18, 1.38)	0.130
Pinch strength	−1.03 ± 0.15 (99)	−0.83 ± 0.15 (100)	0.20 ± 0.14 −0.08, 0.48)	0.165	2.62 ± 0.11	2.83 ± 0.11	0.21 ± 0.10 (0.01, 0.41)	0.038
Modified Norris scale	−16.15 ± 2.00 (97)	−14.12 ± 2.05 (95)	2.03 ± 1.89 (−1.69, 5.75)	0.284	NA	NA	NA	NA
ALSAQ40	19.13 ± 3.79 (95)	19.60 ± 3.82 (95)	0.48 ± 3.50 (−6.44, 7.39)	0.892	NA	NA	NA	NA

ALSFRS-R: interaction between treatment group and period ($p = 0.915$). ALSFRS-R: the revised amyotrophic lateral sclerosis functional rating scale. NA: not applicable. For Modified Norris scale and ALSAQ40, repeated measures analysis was not conducted

Efficacy

The results of ANCOVA for the change of ALSFRS-R score during treatment and the results of repeated measures analysis of variance are shown in Table II. In both analyses, no significant inter-group difference was observed.

The changes in ALSFRS-R score during treatment according to diagnostic category, i.e. 'definite', 'probable' and 'probable laboratory-supported', are shown in Figure 2.

The results of secondary endpoints are presented in Table II. The pinch strength analyzed by repeated measures analysis of variance showed a statistically significant difference, as there was no interaction between the treatment group and period ($p = 0.292$). The other endpoints did not show a significant difference.

The proportion of events of death or a particular state of disease progression was documented in 27 patients in the placebo group (14 patients with –4, –3 change in ALSFRS-R score during pre-observation, and 13 patients with –2, –1 change) and 32 in the edaravone group (12 patients with –4, –3 change and 20 patients with –2, –1 change). There was no significant inter-group difference (stratified log-rank test: $p = 0.381$, stratified generalized Wilcoxon test: $p = 0.399$).

Safety

The proportion of AE reported in the safety population was 88.5% in the placebo group and 89.2% in the edaravone group. All AE and SAE with a proportion of at least 5% in either group are listed (Table III). The inter-group difference in proportion with 95% confidence interval is 0.8% (–7.8% to 9.4%). There were no significant inter-group differences in the proportion of AE ($p = 1.000$), and in adverse drug reactions ($p = 0.349$). The proportion of SAE was 23.1% in the placebo group and 17.6% in the edaravone group. Two cases of respiratory failure in the placebo group resulted in death; in the edaravone group, there were three deaths (two cases of respira-

Figure 2. Change of ALSFRS-R score during treatment by diagnostic category. ALSFRS-R: the revised amyotrophic lateral sclerosis functional rating scale.

Table III. Adverse events and serious adverse events.

	AE				SAE			
	Placebo (104)		Edaravone (102)		Placebo (104)		Edaravone (102)	
Treatment	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Total	92	(88.5)	91	(89.2)	24	(23.1)	18	(17.6)
Constipation	17	(16.3)	13	(12.7)				
Dysphagia	12	(11.5)	8	(7.8)	11	(10.6)	8	(7.8)
Nasopharyngitis	22	(21.2)	22	(21.6)				
Muscular weakness	9	(8.7)	7	(6.9)	1	(1.0)	1	(1.0)
Contusion	5	(4.8)	12	(11.8)				
Headache	3	(2.9)	8	(7.8)				
Insomnia	10	(9.6)	9	(8.8)				
Gait disturbance	16	(15.4)	20	(19.6)	2	(1.9)	3	(2.9)
Eczema	2	(1.9)	7	(6.9)				
Glucose urine present	3	(2.9)	6	(5.9)				

All AE with an incidence greater than 5% are tabulated by the primary term, MedDRA version 11.1.

tory disorder and one case of respiratory failure). The investigators determined that the deaths were due to the primary disease and were not related to the study drug. There was no significant inter-group difference in the proportion of SAE ($p = 0.389$). No serious adverse drug reactions occurred in either group.

Discussion

The results of prior clinical trials (20,30) indicated that edaravone may delay the progression of symptoms in some ALS patients. Because evaluation of edaravone would be difficult in patients in whom ALS progression was either acute or non-existent, only patients whose ALSFRS-R score changed by –1 to –4 points during the 12-week pre-observation period were eligible for the study. Since efficacy was found over the 24-week treatment period in the phase II trial, a treatment period of 24 weeks was also chosen for this study.

Based on the results of the previous phase II trial, the inter-group difference in the change of ALSFRS-R score at the end of treatment was expected to be 2 points in this trial. However, the actual inter-group difference was only 0.65 points by ANCOVA and this was not statistically significant. No significant inter-group difference was found by repeated measures analysis of variance either. The results of ANCOVA for pinch strength, a secondary endpoint, suggested a beneficial effect in the edaravone group compared to the placebo group.

Additionally, stratified analysis by diagnostic category (Figure 2) revealed that the change in ALSFRS-R score during treatment was greater in those patients fulfilling the criteria for clinically definite ALS using the Airlie House diagnostic classification. This trial enrolled patients with longer duration of disease and higher ALSFRS-R scores at the start of treatment compared to those of the patients in other trials (31–34). As shown in Table I, the mean duration of disease for the edaravone group and the placebo group was 1.3 years and 1.2 years, respectively, and the mean ALSFRS-R score at the start of treatment was 41 and 42, respectively. While the mean change in ALSFRS-R score during the treatment was –5.70 for the edaravone group and –6.35 for the placebo group, our internal analysis showed that 25% of patients in the edaravone group and 26% of patients in the placebo group showed the change of 0 or –1 point in ALSFRS-R score indicating a more slowly progressive form of the disease than had originally been anticipated when the trial was designed, and thus attenuating the power of the study. Future trials will aim to enroll patients with more rapidly progressive illness. AE occurred in nearly 90% of both groups, i.e. 88.5% of patients in the placebo group and 89.2% in the edaravone group, with no significant differences between the two groups.

In conclusion, although the elimination of free radicals to inhibit the degeneration of motor neurons appears to be a promising new strategy for the treatment of ALS, this study failed to demonstrate efficacy of edaravone to delay the progression of ALS. While the primary endpoint was not achieved, we consider that the results are helpful to identify the patient population in which edaravone could be expected to show efficacy. On the basis of this information, we have designed and are conducting a phase III study.

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Cystatin C protects neuronal cells against mutant copper-zinc superoxide dismutase-mediated toxicity

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective and progressive loss of motor neurons. Cystatin C (CysC), an endogenous cysteine protease inhibitor, is a major protein component of Bunina bodies observed in the spinal motor neurons of sporadic ALS and is decreased in the cerebrospinal fluid of ALS patients. Despite prominent deposition of CysC in ALS, the roles of CysC in the central nervous system remain unknown. Here, we identified the neuroprotective activity of CysC against ALS-linked mutant Cu/Zn-superoxide dismutase (SOD1)-mediated toxicity. We found that exogenously added CysC protected neuronal cells including primary cultured motor neurons. Moreover, the neuroprotective property of CysC was dependent on the coordinated activation of two distinct pathways: autophagy induction through AMPK-mTOR pathway and inhibition of cathepsin B. Furthermore, exogenously added CysC was transduced into the cells and aggregated in the cytosol under oxidative stress conditions, implying a relationship between the neuroprotective activity of CysC and Bunina body formation. These data suggest CysC is an endogenous neuroprotective agent and targeting CysC in motor neurons may provide a novel therapeutic strategy for ALS.
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Failure of protein quality control and degradation is deeply involved in the pathomechanisms of neurodegenerative diseases. Prominent deposition of disease-specific proteins is characteristic in neurodegenerative diseases, such as amyloid-β in Alzheimer's disease or huntingtin in Huntington's disease. Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease characterized by the selective loss of motor neurons. While 90% of ALS is sporadic, 10% is inherited. Among the inherited ALS cases, dominant mutations in Cu/Zn superoxide dismutase (SOD1) are the frequent cause of inherited ALS.[1] Transgenic mice and rats expressing a human gene for SOD1 with an ALS-linked mutation develop an ALS phenotype, whereas those with deletion of wild-type SOD1 do not, indicating that acquired toxicity mediated by mutant SOD1 is involved in neurodegeneration.[2,3] In SOD1-linked ALS, SOD1-containing inclusions or oligomerized protein complexes have been specifically found in the spinal motor neurons and astrocytes.[4] It has been proposed that mutant SOD1 proteins are misfolded and consequently aggregated, gaining toxic properties at some stage in their formation.[5] Furthermore, recent studies have suggested that the accumulation of misfolded SOD1 proteins is involved in the pathomechanisms of sporadic ALS.[6,7] Therefore, a reduction of misfolded SOD1 proteins might be one of the viable therapeutic approaches for ALS.

Cystatin C (CysC) is an endogenous cysteine protease inhibitor and expressed in various tissues.[8] In the central nervous system, CysC is mainly secreted from the choroid plexus into the cerebrospinal fluid. CysC is a member of the type-II Cystatin family and inhibits cathepsin B, S and F.[9] Although its precise function, especially in the central nervous system, is still uncertain, some studies have revealed that CysC has a neuroprotective role in neurodegenerative diseases.[10] In a mouse model for Alzheimer's disease, overexpression of human CysC in the mice reduced deposits of amyloid-β fibrils.[11] CysC has been shown to improve the survival of dopaminergic neurons in a rat model of Parkinson's disease.[12] In sporadic ALS, CysC is a major component of Bunina bodies, which are ALS-specific inclusion bodies, found in remaining motor neurons,[13] and the levels of CysC are decreased in the cerebrospinal fluid of ALS patients.[14,15] Intriguingly, it was also reported that the concentration of CysC in the cerebrospinal fluid is correlated with the survival time of ALS patients,[15] implying a potent neuroprotective property of CysC in ALS.

Previous reports showed that CysC induces autophagy to protect neuronal cells against various stresses including serum or growth-factor deprivation and oxidative stresses.[10,16]

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Abbreviations: AICAR, AICA-riboside; ALS, amyotrophic lateral sclerosis; AMPK, AMP-activated protein kinase; CatB, cathepsin B; CC, Compound C; CysC, cystatin C; dbcAMP, N,N-dibutyladenosine 3′,5′-phosphoric acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; LMP, lysosomal membrane permeabilization; 3-MA, 3-methyl adenine; mTOR, mammalian target of rapamycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; N2a, neuro2a; pAMPK, phosphorylated-AMPK; PBS, phosphate buffered saline; PKCδ, protein kinase C δ; pPKCδ, phosphorylated-PKCδ; SOD1, copper-zinc superoxide dismutase

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Autophagy is a major intracellular proteolytic pathway that targets misfolded or aggregated proteins as well as the ubiquitin-proteasome pathway. Because the ubiquitin-proteasome pathway is impaired in both SOD1-linked[17,18] and SOD1-unrelated[19,20] ALS models, autophagy activation may complementally degrade the abnormal proteins to rescue motor neurons. Indeed, involvement of autophagy is implicated in the experimental models of ALS.[21,22] Moreover, recent studies have shown that cathepsin B (CatB), a member of the cysteine protease family that is inhibited by CysC, is deeply involved in motor neuronal degeneration. Increased immunoreactivity of CatB was often found in the neurons of sporadic ALS patients[23] or ALS model mice[24] and CatB-knockout mice showed a lower rate of motor neuron death after nerve injury,[25] suggesting that inhibition of CatB is beneficial for motor neuronal survival. These previous data suggest the possibility that CysC is a promising therapeutic candidate for ALS. However, no evidence has been provided for the role of CysC in neuroprotection in ALS models.

Here, we performed direct tests of the neuroprotective property of CysC using neuroblastoma cell Neuro2a (N2a) and primary mix-cultured motor neurons derived from mutant SOD1 transgenic mice and identified that CysC is a novel neuroprotective agent against mutant SOD1-mediated neurotoxicity that acts through induction of autophagy and inhibition of CatB.

Results

CysC is enriched in the healthy motor neurons in mouse spinal cords. Although Bunina bodies are the specific structures in patients with sporadic ALS, they are not found in some sporadic ALS cases as well as cases of familial ALS caused by SOD1 mutations.[26,27] In a previous study, CysC was immunostained in remaining neurons as a condensed form and activated astrocytes in SOD1^{G93A} mouse spinal cord.[24] Therefore, we first confirmed the localization of endogenous murine CysC and its alternations in SOD1^{G93A} mouse spinal cord (Figure 1). The punctate staining of murine CysC was observed in SOD1 wild-type mouse spinal cord. Murine CysC immunoreactivity was strongly positive in the anterior horn neurons, whereas it was weak or negative in the posterior horn neurons and the glial cells (Figure 1a). Murine CysC was partially co-localized with lysosome-associated membrane protein 2 (LAMP-2), a marker protein of lysosomes (Figure 1b). In SOD1^{G93A} mouse spinal cord, murine

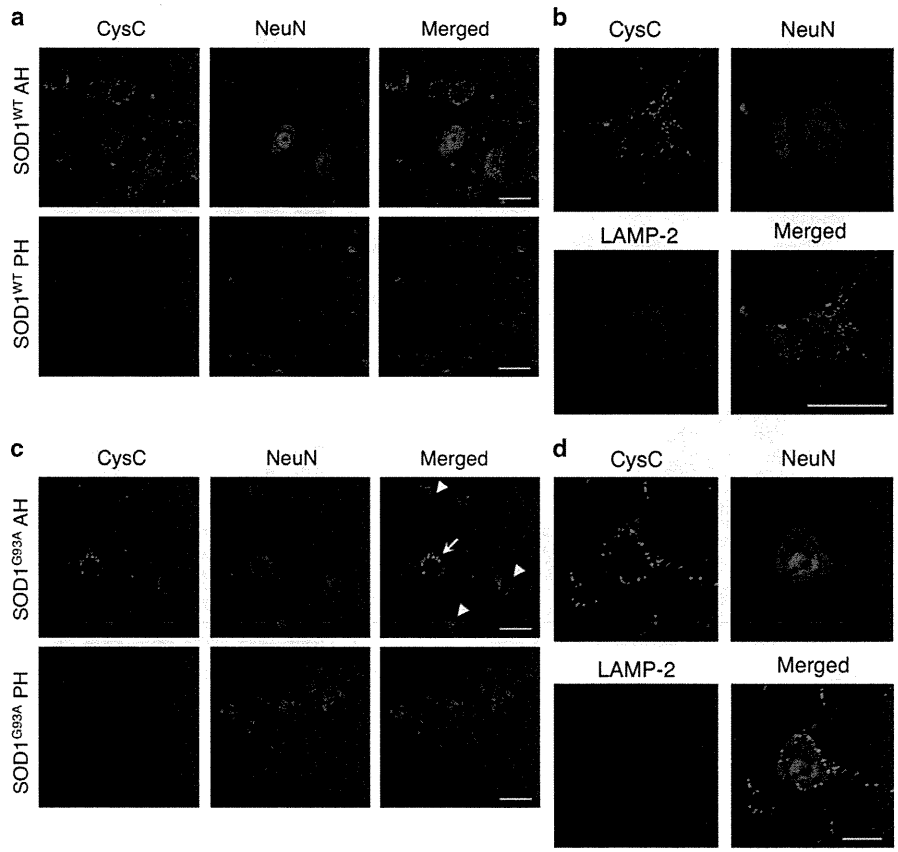

Figure 1 CysC expression in wild-type and mutant SOD1 mouse spinal cords. Double or triple immunostaining for neurons, lysosomes and Cystatin C was performed using antibodies for NeuN, LAMP-2 and CysC, respectively. Transverse sections in the anterior horn (AH) and the posterior horn (PH) of 5-month-old SOD1^{WT} (**a** and **b**) or SOD1^{G93A} (**c** and **d**) mouse spinal cords were analyzed by confocal microscopy. The arrow represents the remaining normal-shaped neuron, and the arrowheads represent the shrunken neurons, respectively. Scale bars: 50 μm in **a**–**c**, 25 μm in **d**

CysC immunoreactivity was generally reduced and condensed in the remaining neurons, and it was also found in the outside of neurons (Figures 1c and d). Interestingly, murine CysC was still immunostained in remaining, normal-appearing neurons (Figure 1c, arrows), whereas murine CysC immunoreactivity was almost undetectable in shrunken neurons (Figure 1c, arrowheads). These results are similar to that of human sporadic ALS cases,[13] suggesting that murine CysC immunoreactivity correlates with motor neuronal survival. Moreover, LAMP-2 immunoreactivity was also diminished in the SOD1^{G93A} anterior horn neurons (Figure 1d), implying the involvement of lysosomal dysfunction in motor neuronal degeneration.

CysC protects N2a cells against mutant SOD1-mediated neurotoxicity. In order to investigate whether CysC is involved in motor neuronal survival, we examined the neuroprotective activity of exogenously added recombinant human CysC against mutant SOD1-mediated toxicity. Phase microscopic images showed that both of G85R and G93A mutant SOD1 were toxic to differentiated N2a cells and the addition of CysC to the culture medium reduced their toxicity (Figure 2a). The neuroprotective effect of CysC against mutant SOD1-mediated toxicity was further confirmed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 2b) and by quantitation of the live cells excluding trypan blue dye (Figure 2c). To evaluate the concentration dependence of neuroprotection by CysC, we added CysC into the culture medium at dose of 0, 0.2, 0.6 and 1 μM. As shown in Figure 2d, CysC protected the N2a cells in a concentration-dependent manner. These results provide strong evidence that CysC is involved in neuroprotection against mutant SOD1-mediated toxicity.

CysC reduces toxic SOD1 species by induction of autophagy. A previous study demonstrated that CysC protected neuronal cells against various stresses by induction of autophagy.[16] To examine whether autophagy is involved in the neuroprotective activity of CysC against SOD1-mediated toxicity, we first evaluated the number of intracellular SOD1 aggregates with fluorescent microscopy.

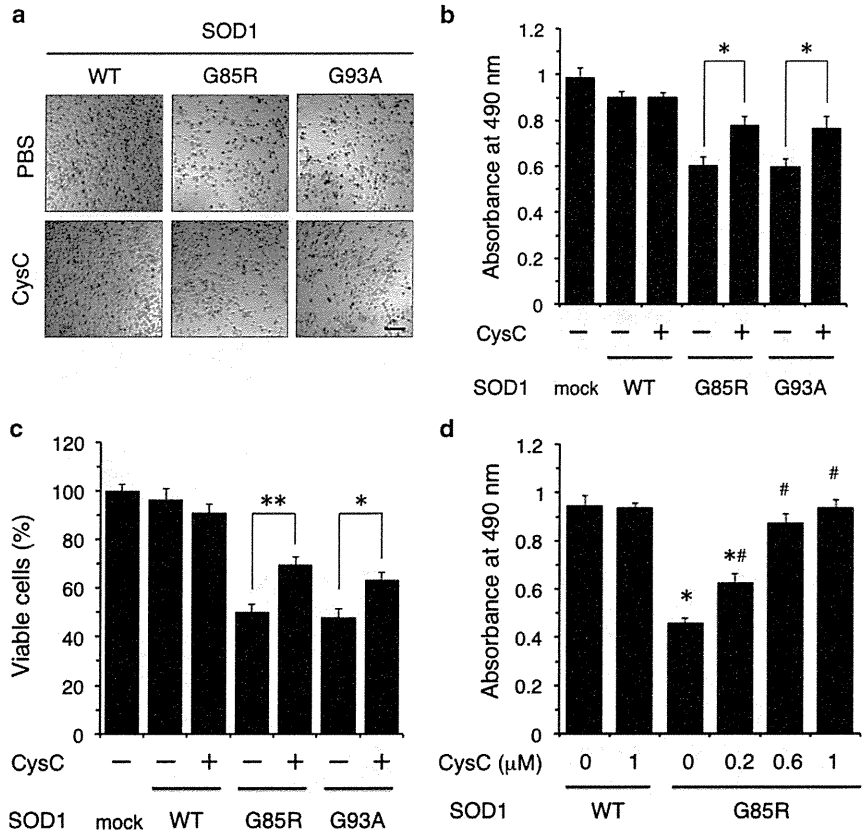

Figure 2 CysC protects N2a cells against mutant SOD1-mediated cytotoxicity. (**a**–**c**) N2a cells were transfected with SOD1^{WT} (WT), SOD1^{G85R} (G85R) or SOD1^{G93A} (G93A), and incubated for 48 h in the differentiation medium with or without 0.2 μM CysC. Light microscopy images (**a**), the cell viability measured by the MTS assay (**b**), or quantification of viable cells with trypan blue staining (**c**) were shown. (**d**) Dose-dependent cytoprotection of CysC. Transfected N2a cells were incubated with CysC at the indicated concentrations. Cytoprotective activity of CysC was measured by the MTS assay. All data are expressed as means ± standard error of the mean (S.E.M.) from three independent experiments, each performed in triplicate. *$P<0.05$, **$P<0.01$ in (**b**) and (**c**). *$P<0.05$ compared to SOD1^{WT} without CysC, #$P<0.05$ compared to SOD1^{G85R} without CysC in D. Scale bar: 200 μm

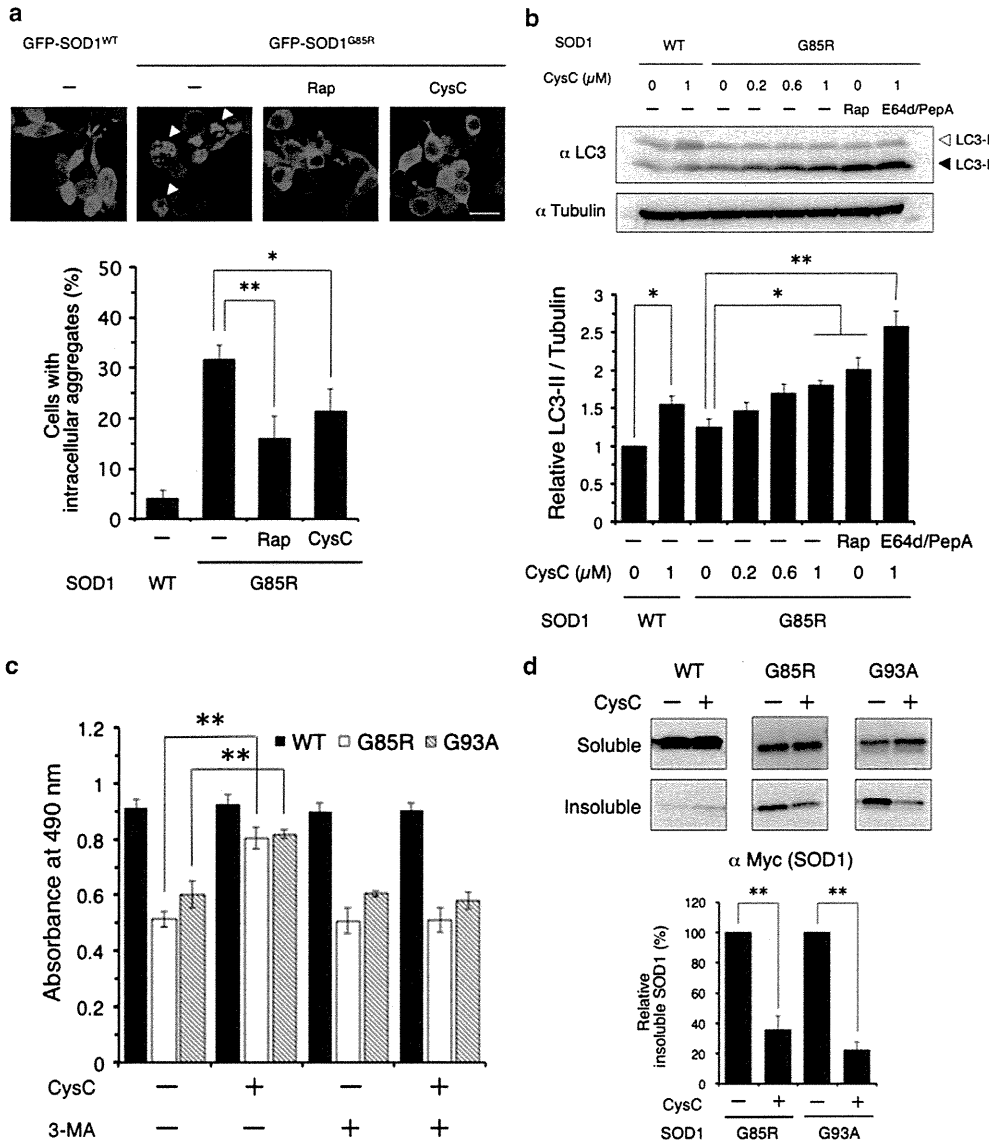

Figure 3 CysC induces autophagy and reduces detergent-insoluble mutant SOD1 proteins in cells. (**a**) Representative fluorescent microscopy images of N2a cells expressing GFP-SOD1 incubated for 24 h with rapamycin (Rap.; 300 nM) or CysC (0.2 μM). The arrowheads indicate the intracellular mutant SOD1 aggregates. Quantified data are expressed as means ± S.E.M. from three independent experiments. In each experiment, at least 100 cells were counted. (**b**) Induction of LC3-II, an autophagy marker, by CysC. N2a cells were transfected with $SOD1^{WT}$ or $SOD1^{G85R}$, and incubated with CysC at the indicated concentrations for 12 h. Lysates were analyzed by immunoblotting with anti-LC3 and α-tubulin antibodies. Relative levels of LC3-II normalized by the expression of α-tubulin were quantified. (**c**) The effect of autophagy inhibition on neuroprotection by CysC. N2a cells expressing wild-type or mutant SOD1 were treated with CysC (0.2 μM) and/or 3-MA (10 mM). Cell viability was measured by the MTS assay. Data are expressed as means ± S.E.M. from three independent experiments. Each experiment was performed in triplicate. (**d**) Reduction of Triton-insoluble mutant SOD1 species by CysC. After 24 h of CysC treatment, N2a cells expressing SOD1 constructs were lysed with 1% Triton X-100. Triton-insoluble fractions were resuspended with 2% sodium dodecyl sulfate and analyzed by immunoblotting. $^{**}P<0.01$. $^{*}P<0.05$. Scale bar: 50 μm

As shown in Figure 3a, the green fluorescent protein (GFP)-fused $SOD1^{G85R}$ mutant formed intracellular aggregates in about 30% of total transfected N2a cells. Rapamycin, a representative inducer of autophagy, remarkably reduced these intracellular aggregates of $SOD1^{G85R}$ to about 50% of untreated cells. CysC treatment also decreased the aggregates like rapamycin did to about 70% of untreated cells, although difference in the degree of reducing aggregates between CysC and rapamycin treatment was not statistically significant. These data imply that CysC protected N2a cells through induction of autophagy. LC3, a selective marker of autophagy, normally appears as a cytosolic form (LC3-I) and becomes cleaved, membrane-bound form (LC3-II) under autophagosome formation process. Western blot analyses showed that CysC enhanced the formation of LC3-II (Figure 3b). Lysosomal proteinase inhibitors, E64d and pepstatin A, further increased the level of LC3-II, indicating the upregulation of autophagy by CysC. Interestingly, the

induction of autophagy was independent of the transfected SOD1 species, suggesting that the regulation of autophagy by CysC is physiologically constitutive and independent of SOD1-mediated toxicity (Figure 3b). Next, in order to confirm the relationship between neuroprotective activity of CysC against mutant SOD1-mediated toxicity and induction of autophagy, the MTS assay was performed in the presence of 3-methyladenine (3-MA), an inhibitor of macroautophagy. As shown in Figure 3c, the protective effects of CysC were completely abolished by 3-MA treatment. Moreover, CysC treatment remarkably reduced Triton X-100-insoluble SOD1 mutants (Figure 3d), suggesting degradation of the misfolded toxic SOD1 species by autophagy. All of these data indicate that the induction of autophagy is essential for the neuroprotective activity of CysC.

CysC induces autophagy via AMPK-mTOR pathway. CysC has been shown to inhibit the mammalian target of rapamycin (mTOR) complex, which suppresses autophagy.[16] However, the mechanisms through which CysC inhibits mTOR are still uncertain. To identify the signal transduction pathway mediated by CysC, we investigated the following pathways that regulate mTOR activity. (i) AMP-activated kinase (AMPK) is a regulator of cellular metabolism and energy consumption. AMPK inactivates mTOR under malnutritic conditions, such as those involving a low glucose supply.[28] (ii) Akt is a major signal transducer that promotes protein synthesis, cellular proliferation and cell survival. Akt phosphorylates mTOR at Ser2448 and induces its activity.[29] (iii) Protein kinase C δ (PKCδ) is activated in the spinal cord of the wobbler mouse, an ALS model.[30] PKCδ activates mTOR via the transglutaminase-Akt pathway.[31] In order to examine the effects of CysC on these pathways, N2a cells expressing SOD1 were treated with 1 μM CysC for 8 h and analyzed by immunoblotting (Figure 4a). AMPK activation was remarkably inhibited when SOD1 mutants were expressed (Figures 4a and b) and the reduced activation of AMPK by SOD1 mutants was partially recovered by CysC treatment (Figures 4a and c). In addition to this, compound C (CC), an AMPK inhibitor, clearly inhibited the AMPK activation and the conversion to LC3-II by CysC treatment (Figure 4d). These data indicate that CysC induces autophagy through the AMPK-mTOR pathway, whereas the activities of the other mTOR-regulating factors, Akt and PKCδ, were not affected by CysC treatment. To examine the role of AMPK activation in the neuroprotection by CysC, we measured the cell viability with CysC, AMPK inhibitor or activator (Figure 4e). CC also inhibited the neuroprotective activity of CysC. However, AICA-riboside (AICAR), an AMPK activator, exacerbated the mutant SOD1-mediated toxicity. These data indicate that AMPK activation is essential but not sufficient for the neuroprotection by CysC. To confirm the changes in AMPK activity *in vivo*, we analyzed the spinal cord lysates of SOD1 transgenic mice at various ages (Figure 4f). The phosphorylation levels of AMPK were reduced at the end stages of the disease (Figure 4f). This is consistent with the results of the N2a cells and implies that a reduction of AMPK activity is involved in the disease.

CysC is transduced into neuronal cells via clathrin-dependent endocytotic pathway. CysC transduction into some human non-neuronal cell lines[32,33] has been previously reported. However, it has not been clarified whether CysC is also transduced into neuronal cells. Moreover, the transduction pathway by which CysC is transduced into neuronal cells is unknown. To address these questions, we added fluorescein isothiocyanate (FITC)-labeled CysC (FITC-CysC) into the culture medium. We found that CysC was transduced into neuronal cells and localized to lysosomal acidic components, which were labeled by Lysotracker-Red, as like in the case of other cells (Figure 5a). Immunoblot analyses of isolated intact lysosomes of the cells treated with biotinylated-CysC (Biotin-CysC) revealed intact full-length CysC delivery (Figure 5b). There are multiple endocytotic pathways including clathrin-dependent endocytosis, lipid raft-caveolae-dependent endocytosis and macropinocytosis. To determine the endocytotic pathway responsible for CysC transduction, we administered the pathway-specific inhibitors, chlorpromazine (clathrin-dependent endocytosis),[34] Filipin-III (lipid raft-caveolae-dependent endocytosis)[35] or 5-(N-Ethyl-N-isopropyl) amiloride (macropinocytosis)[36] into the cells. Although Filipin-III and 5-(N-Ethyl-N-isopropyl) amiloride did not inhibit CysC transduction at all, chlorpromazine clearly inhibited it (Figure 5c). These data showed that CysC is transduced into the cells via the clathrin-dependent pathway and localized to lysosomes.

Transduced CysC leaks from lysosome and forms aggregates under oxidative stress conditions. Intriguingly, transduced CysC was leaked from the lysosomes and aggregated in the cytosol when the G85R or G93A SOD1 mutant was expressed (Figure 6a), whereas wild-type SOD1 expression did not cause the CysC leakage. Previous studies have revealed that lysosomal membrane permeabilization (LMP) is induced by various stresses including oxidative stress.[37,38] In order to investigate the possible involvement of oxidative stress in CysC leakage, we treated N2a cells with hydrogen peroxide (H_2O_2) and found that H_2O_2 treatment caused CysC leakage, which was similar to the effects of the mutant SOD1 expression (Figure 6b). Moreover, N-acetyl-L-cysteine, a scavenger of reactive oxygen species, inhibited H_2O_2-induced CysC release from the lysosomes. Furthermore, N-acetyl-L-cysteine also inhibited the leakage of CysC from lysosomes when G85R SOD1 mutant was expressed (Figure 6c). These data suggest that LMP caused by oxidative stress is a major cause of CysC leakage from lysosomes.

CatB inhibitory activity of CysC is required for its neuroprotection. It is of interest whether the CatB inhibitory activity of CysC is also involved in its neuroprotective activity, because the previous reports have shown that CatB is activated in ALS pathology[23,24] and involved in motor neuronal degeneration.[25] First, we confirmed the activation of CatB in the mutant SOD1 model. The immunoreactivity of CatB and the amount of the CatB active form was substantially increased in SOD1^{G93A} mouse spinal cord, which was similar to the findings of previous studies (Figures 7a and b). Next, to examine the inhibitory activity of CysC treatment on CatB activation by mutant SOD1 expression, we

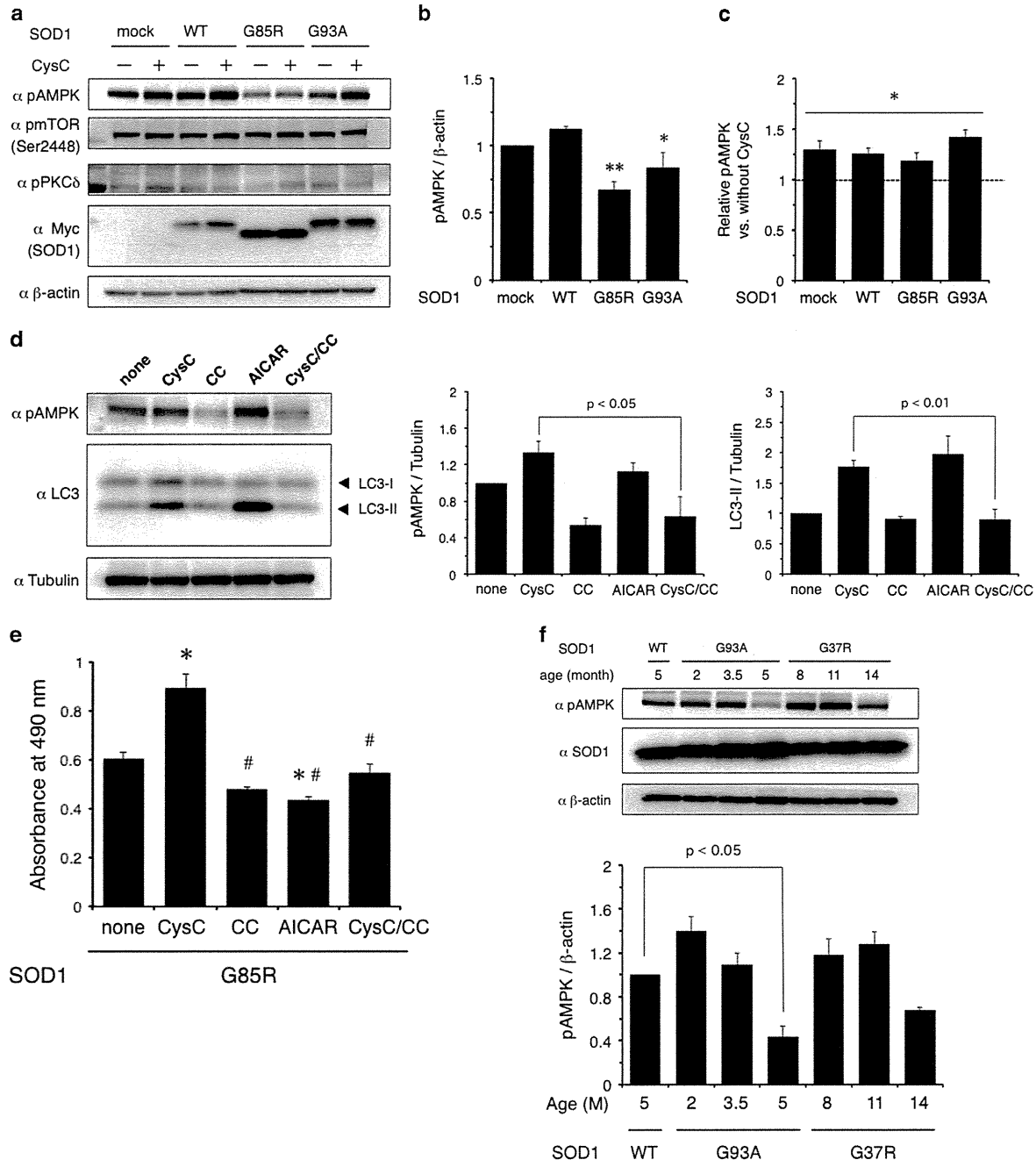

Figure 4 CysC regulates AMPK activity during the induction of autophagy. (**a**) Immunoblotting analysis of autophagy regulators. N2a cells expressing SOD1 were treated with or without CysC (1 μM) for 6 h. The lysates were analyzed by immunoblotting using antibodies for phosphorylated AMPK (pAMPK), phosphorylated mTOR (pmTOR), phosphorylated PKCδ (pPKCδ), Myc and β-actin. (**b**) Inactivation of AMPK by mutant SOD1 expression. Each relative pAMPK level normalized by β-actin in (**a**) is quantified. $^{*}P<0.05$, $^{**}P<0.01$ *versus* mock. (**c**) Activation of AMPK by CysC treatment. Relative levels of pAMPK for CysC-treated samples normalized by that of PBS-treated control, which is shown as the broken line, in (**a**) were quantified. $^{*}P<0.05$ *versus* PBS-treated controls. (**d**) CysC induced autophagy through the AMPK activation. N2a cells were treated with CysC (1 μM), CC (5 μM) or AICA-riboside (AICAR, 5 mM) for 12 h. The lysates were analyzed by immunoblotting using antibodies against pAMPK, LC3 and Tubulin (left panel). Quantification of immunoblots was plotted (right panel). (**e**) The effect of pAMPK activation on neuroprotection by CysC. N2a cells expressing G85R SOD1 mutant were treated with CysC (0.2 μM), CC (5 μM) or AICAR (5 mM). Cell viability was measured by the MTS assay. Data are expressed as means $\pm$ S.E.M. from three independent experiments. Each experiment was performed in triplicate. $^{*}P<0.01$ compared to non-treated control, $^{\#}P<0.01$ compared to CysC-treated one. (**f**) Immunoblotting analysis of pAMPK in SOD1 transgenic mouse spinal cords. The spinal cord lysates from the transgenic mice at indicated ages were analyzed for the levels of pAMPK, SOD1 and β-actin (upper panel). The expression levels of pAMPK were normalized by β-actin (lower panel). All data are expressed as means $\pm$ S.E.M. from three independent experiments

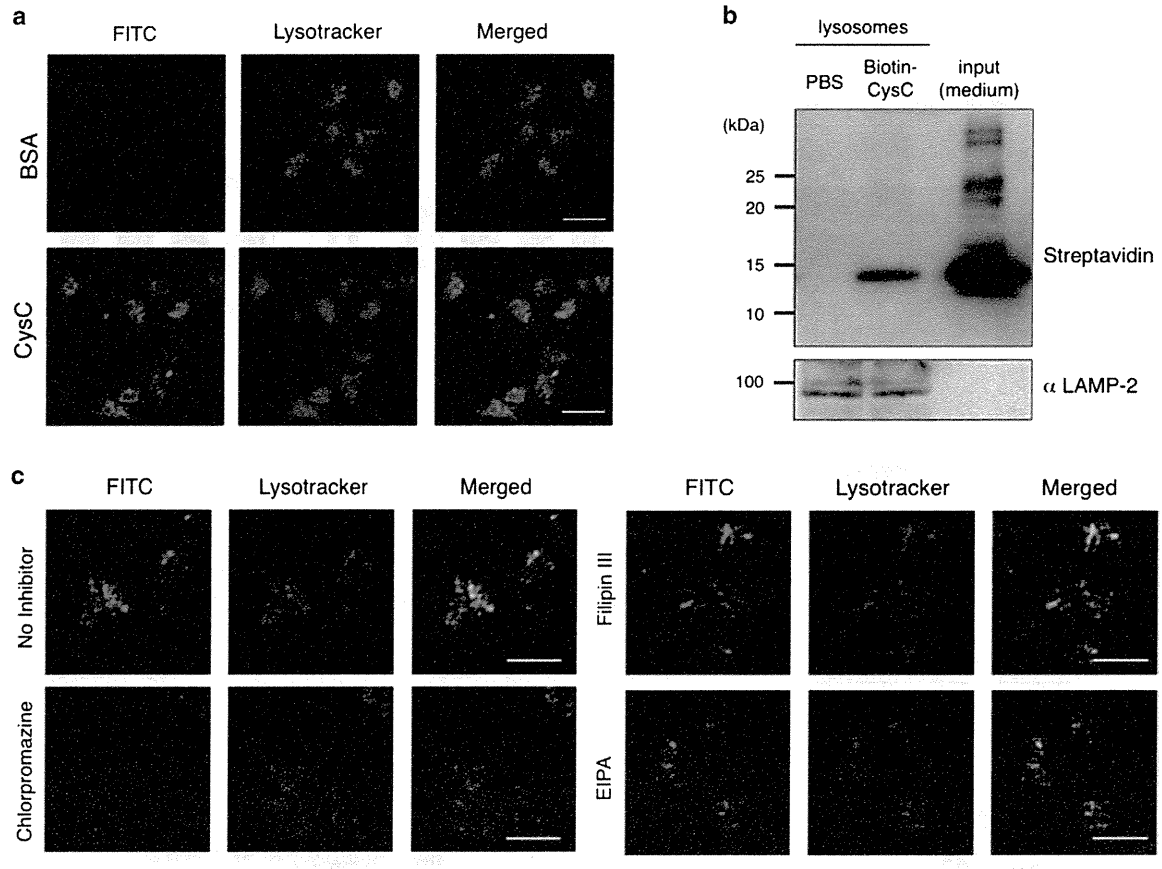

Figure 5 Transduction of exogenously added CysC into N2a cells. (**a**) Transduction and localization of CysC to lysosomes in N2a cells. FITC-labeled CysC or bovine serum albumin (1 μM) was added to N2a cells for 3 h, and the cells were then briefly treated by LysoTracker and analyzed by confocal microscopy. (**b**) Immunoblotting detection of transduced full-length CysC in purified lysosomal fractions. Biotin-conjugated transduced CysC was detected by horseradish peroxidase-streptavidin. Immunoblot using anti-LAMP-2 antibody indicates enrichment of lysosome. (**c**) Clathrin-dependent transduction of CysC. N2a cells were pre-treated with chlorpromazine (25 μM), filipin III (5 mg/ml) or 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) (25 μM) for 1 h. Then, the cells were incubated with FITC-labeled CysC for 1 h, and analyzed by confocal microscopy. Scale bars: 25 μm

created a W106G CysC mutant that specifically lacked the inhibitory activity against CatB.[39] Similar to the wild-type, the W106G mutant induced autophagy (Figure 7c) and transduced into N2a cells (Figure 7d). As shown in Figure 7e, the CatB activity induced by mutant SOD1 was completely inhibited by wild-type CysC treatment, whereas the W106G CysC mutant did not inhibit the activation of CatB, suggesting that transduced CysC functioned as the endogenous intracellular CatB inhibitor. Surprisingly, CysC treatment did not affect the CatB activity at all in the absence of mutant SOD1, implying that CysC was effective only to inhibit the aberrant activation of CatB. Moreover, to assess the contribution of CatB inhibition by CysC to its neuroprotective activity, we examined the neuroprotective activity of the wild-type or W106G CysC mutant to mutant SOD1-mediated toxicity by the MTS assay. As shown in Figure 7f, W106G CysC did not reduce the mutant SOD1-mediated toxicity at all. This suggests that CatB inhibitory activity was also essential for the neuroprotective activity of CysC as well as the induction of autophagy. Moreover, a CatB-specific inhibitor CA-074 did not improve the viability of the N2a cells (Figure 7g), indicating that the CatB inhibitory activity was essential but not sufficient to protect neurons as well as the induction of autophagy.

CysC protects primary cultured motor neurons from mutant SOD1-mediated toxicity. In order to confirm the protective effect of CysC on primary motor neurons, we examined motor neuronal survival in a primary neuron-glia mix culture derived from Hb9-GFP/SOD1^{G85R} mouse embryos. As Hb9-GFP mice have been used to identify motor neurons in mice,[40] the number of GFP-positive cells represents surviving motor neurons (Figure 8a). Mixed culture derived from SOD1^{G85R} embryos showed an accelerated decrease of motor neurons compared with that of the wild-type one and CysC treatment markedly improved the viability of SOD1^{G85R} motor neurons (Figure 8b). This result indicates that CysC exerts a neuroprotective activity against mutant SOD1-mediated toxicity on primary cultured motor neurons as well as N2a cells.

Discussion

In the present study, we demonstrated the neuroprotective activity of CysC against mutant SOD1-mediated toxicity.

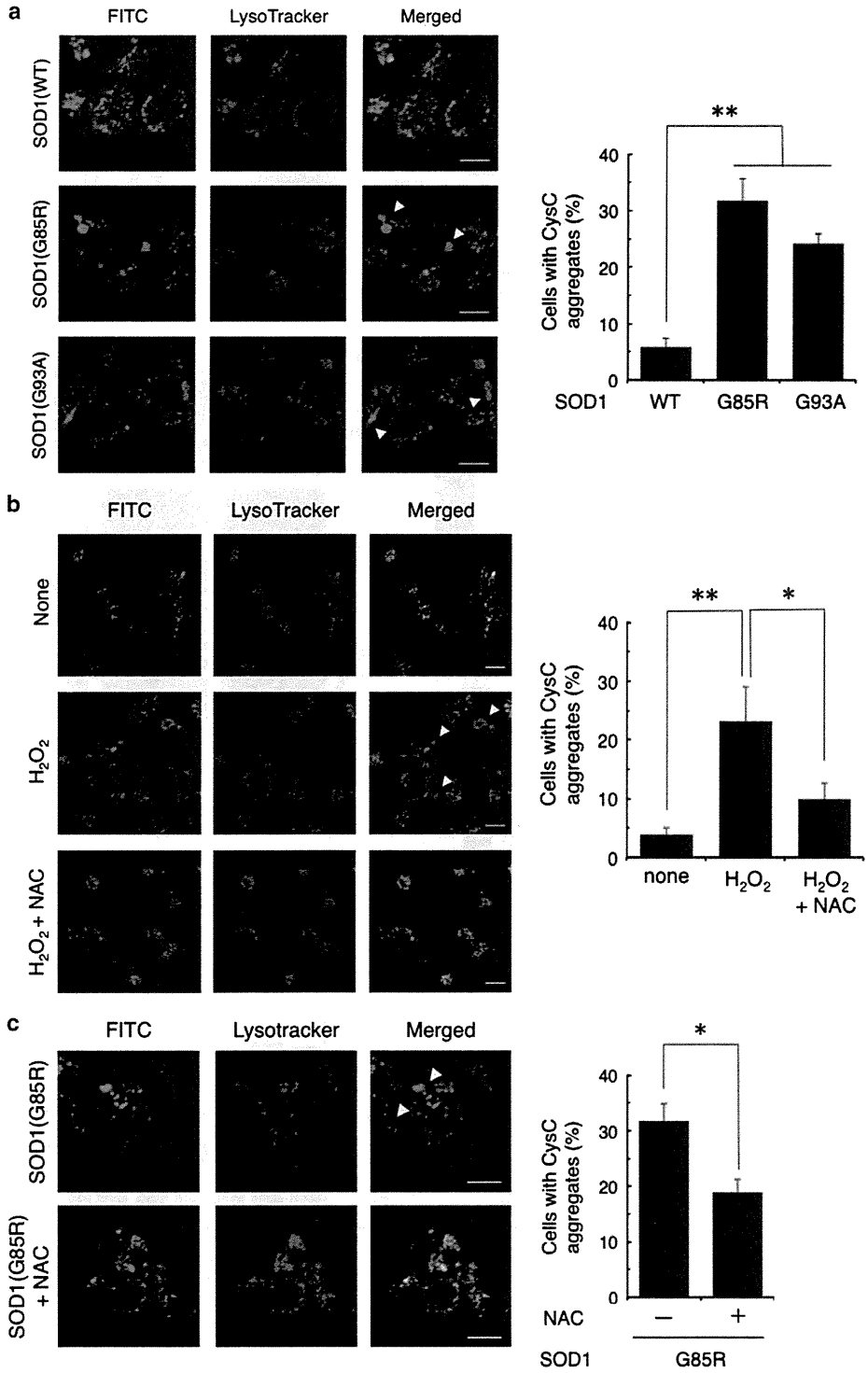

Figure 6 Stress-induced leakage from the lysosomes and aggregation of CysC in N2a cells. (**a**) Leakage and aggregation of CysC caused by mutant SOD1 expression. N2a cells transiently expressing SOD1 were incubated with 0.5 μM FITC-CysC for 24 h, treated with LysoTracker and analyzed by confocal microscopy. (**b**) H_2O_2-induced aggregation of CysC and effects of N-acetyl cysteine (NAC). N2a cells were incubated for 24 h in the medium containing 0.5 μM FITC-CysC and 50 μM H_2O_2 with or without 100 μM NAC. Cells were treated with LysoTracker and observed by confocal microscopy. (**c**) NAC prevented mutant SOD1-induced aggregation of CysC. N2a cells expressing G85R SOD1 mutant were incubated for 24 h in the medium containing 0.5 μM FITC-CysC with or without 100 μM NAC. Cells were treated with LysoTracker and observed by confocal microscopy. The arrowheads indicate the leaked and aggregated CysC in the cytosol. (**a**–**c**, right panel) Cells with CysC aggregates were quantified. $^{**}P<0.01$. $^{*}P<0.05$. Scale bars: 25 μm