A small-molecule inhibitor of SOD1-Derlin-1 interaction ameliorates pathology in an ALS mouse model

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder. Despite its severity, there are no effective treatments because of the complexity of its pathogenesis. As one of the underlying mechanisms of Cu, Zn superoxide dismutase (SOD1) gene mutation-induced ALS, SOD1 mutants (SOD1mut) commonly interact with an endoplasmic reticulum-resident membrane protein Derlin-1, triggering motoneuron death. However, the importance of SOD1-Derlin-1 interaction in in vitro human model and in vivo mouse model remains to be elucidated. Here, we identify small-molecular-weight compounds that inhibit the SOD1-Derlin-1 interaction by screening approximately 160,000 compounds. The inhibitor prevents 122 types of SOD1mut from interacting with Derlin-1, and significantly ameliorates the ALS pathology both in motoneurons derived from patient induced pluripotent stem cells and in model mice. Our data suggest that the SOD1-Derlin-1 interaction contributes to the pathogenesis of ALS and is a promising drug target for ALS treatment.
myotrophic lateral sclerosis (ALS) is a progressive, late-onset neurodegenerative disorder characterized by the selective loss of both upper and lower motor neurons. Riluzole and Edaravone are the only two available treatments for ALS that provide a modest improvement in survival. Although some clinical trials have been conducted over the past 20 years, most of them have failed, at least in part because of the poor understanding of the pathogenesis of ALS. Multiple motor neuron toxicities have been proposed, such as oxidative stress, excitotoxicity, proteasome dysfunction, endoplasmic reticulum (ER) stress, abnormal mitochondrial function, and altered axonal transport, but no consensus or precise mechanisms have been elucidated. Thus, there remains strong demand for finding new and effective ALS treatments based on the molecular mechanisms of ALS pathogenesis.

Mutations in the Cu, Zn superoxide dismutase (SOD1) gene are frequently found in familial ALS (FALS) patients. Although SOD1 is a well-known antioxidant enzyme, mutant SOD1 (SOD1mut)-mediated FALS is now considered to result from disruption of the SOD1mut-Derlin-1 interaction by expressing SOD1mut. Therefore, further investigation of SOD1mut-induced toxic mechanisms is required for managing this fatal neurodegenerative disease.

We have previously reported that over 100 types of ALS-related SOD1mut but not wild-type SOD1 (SOD1WT) commonly expose the Derlin-1-binding region (termed the DBR) and interact via the DBR with Derlin-1, one of the critical components of the ER-associated degradation (ERAD) machinery. Moreover, disruption of the SOD1mut-Derlin-1 interaction by expressing Derlin-1-derived peptide (termed Derlin-1 (CT4)), which corresponds to the binding site for the DBR and competes for binding to SOD1mut, has been shown to be protective against SOD1mut-dependent motor neuron loss in the mouse primary spinal cord culture. These findings suggest that the SOD1mut-Derlin-1 interaction is a common feature of SOD1mut-caused FALS pathology and that inhibition of this interaction may be a potential target for ALS treatment.

Protein–protein interaction (PPI) is an attractive target for drug development because it plays central roles in large parts of biological processes, and in pathological conditions. Thus, small molecules that inhibit PPIs have potential as therapeutic medicines themselves and also as useful experimental tools to elucidate pathological mechanisms. Although it is considered as challenging to identify small molecules that modulate PPIs, several reports provide encouraging evidence for finding such compounds.

Here, we developed a high-throughput screening (HTS) system to find small-molecular-weight compounds that would function as SOD1mut-Derlin-1 interaction inhibitors. By utilizing time-resolved fluorescence resonance energy transfer (TR-FRET) technology, we successfully established a high-throughput, robust assay system to measure the SOD1-Derlin-1 interaction. We performed an HTS of 160,000 compounds in the public chemical library (at the Drug Discovery Initiative (DDI), The University of Tokyo) and found that an analog of one of the hit compounds prevent 122 types of SOD1mut from interacting with Derlin-1. The inhibitor significantly ameliorates the ALS pathology in motor neuron cells derived from patient-induced pluripotent stem cells (iPSCs) and delays the onset and prolongs the survival (14.5% and 14.2% improvement, respectively) of ALS model mice expressing SOD1G93A. Our data emphasize the importance of the SOD1-Derlin-1 interaction as a common mechanism of motor neuron toxicity in SOD1mut, and we provided a potential mechanism-based ALS treatment.

Results

TR-FRET-based HTS assay for the PPI inhibitors. To identify the inhibitors of the SOD1-Derlin-1 interaction, we first established an HTS system. We employed a TR-FRET-based assay for the detection of SOD1-Derlin-1 interaction by using antibodies labeled with either a donor or an acceptor fluorophore (Fig. 1a). Theoretically, when SOD1 and Derlin-1 are interacting, a donor fluorophore (Europium cryptate: Eu) and an acceptor fluorophore (d2) are in close proximity and generate the FRET signal. In contrast, there is no FRET signal when the small molecules inhibit the SOD1-Derlin-1 interaction. We previously mapped each binding site: SOD1 (5-18), the amino-terminal 14 amino acids of SOD1WT, was identified as the minimum essential DBR, and the cystolic carboxyl-terminal 12 amino acids, Derlin-1 (CT4), specifically interacts with SOD1mut. To identify the compounds that efficiently generate FRET signal, lysates from HEK293A cells transfected with various combinations of SOD1 and Derlin-1 constructs including each binding site were mixed with the antibodies labeled with either Eu or d2. Among the over 1000 combinations of constructs and antibodies tested, we obtained a robust signal from the cell lysate expressing Flag-SOD1G93A and Derlin-1-HA using an anti-Flag antibody labeled with Eu (Flag-Eu) and an anti-HA antibody labeled with d2 (HA-d2), which was sufficient for compound screening (Z’-factor >0.9) (Fig. 1b). To confirm that the FRET signal was derived from the interaction of SOD1G93A with Derlin-1, we performed the competition assay using each binding-site peptide. Both the DBR-containing peptide (SOD1 (5-20); amino-terminal 16 amino acids of SOD1) and the Derlin-1 (CT4) peptide attenuated the FRET signal in a concentration-dependent manner, clearly showing the requirement of the SOD1-Derlin-1 interaction for generating the FRET signal (Fig. 1c).

We screened a total of approximately 160,000 small molecules using the inhibition of FRET signal as a readout. The Z’-factor of all plates in the first screening was set to be over 0.75, suggesting that this assay system was sufficiently robust and reliable (Supplementary Figure 1a). In this stage, 1460 compounds that showed over 15% inhibition were selected for further validation in the second screening (Fig. 1d and Supplementary Figure 1b). The decrease in FRET signal may occur not only because of the inhibition of SOD1-Derlin-1 interaction but also because of the inhibition of interactions between the tags and the corresponding antibodies. Alternatively, compounds may also target the energy transfer itself by interacting with the emission or excitation processes. To exclude such false-positive compounds, we prepared the monomolecular FRET assay using HA-SOD1WT, Flag with Flag-Eu and HA-d2 as a counter assay (Supplementary Figure 1c). In the second screening, we reassessed the inhibition of the SOD1-Derlin-1-derived FRET signal of 1460 positive compounds in the first screening and simultaneously checked the effect of each compound on the monomolecular FRET signal of HA-SOD1WT-Flag. Many of the compounds that were confirmed to inhibit the SOD1-Derlin-1-derived FRET signal also inhibited the monomolecular FRET signal (Fig. 1e, lower panel, region A). However, some compounds attenuated only the SOD1-Derlin-1-derived FRET signal with little effect on monomolecular FRET, suggesting that these compounds inhibit the SOD1-Derlin-1 interaction (Fig. 1e, lower panel, region B). Therefore, 131 compounds were selected, each showing over 15% inhibition of the SOD1-Derlin-1-derived FRET signal without inhibiting the monomolecular FRET signal by more than 15% (Fig. 1e, lower panel, region B and Supplementary Figure 1b).
**Fig. 1** Screen of small molecules for SOD1-Derlin-1 interaction inhibitors. **a** TR-FRET-based interaction assay model showing the case of tagged SOD1 as a target of the Eu-labeled antibody and tagged Derlin-1 as a target of the d2-labeled antibody. **b** FRET signals with Flag-Eu and HA-d2 shown as fold changes from the FRET signal in non-transfected lysate. The data are shown as the mean ± s.d. **c** FRET signal using lysates from HEK293A cells transfected with plasmids as indicated (n = 4). **d** Competition assay of FRET signal generated by Flag-SOD1G93A and Derlin-1-HA using indicated concentration of SOD1 (5-20) and Derlin-1 (CT4) peptide. Lysates from HEK293A cells transfected with Flag-SOD1G93A and Derlin-1-HA were incubated with the indicated concentration of each peptide for 16 h (n = 3). Light gray: with Derlin-1 (CT4) peptide; dark gray: with SOD1 (5-20) peptide. **e** Result of the first screening. The compounds are presented in order of inhibition rate. **f** Blue: inhibition (%) against FRET signal generated by Flag-SOD1G93A and Derlin-1-HA; red: inhibition (%) against FRET signal generated by HA-SOD1WT-Flag. **g** Result of #56 in the third screening. The data are shown as the mean ± s.d. (n = 4). **h** Chemical structure of #56.
Using a titration assay as the third screening, 44 compounds that inhibited the SOD1-Derlin-1-derived FRET signal in a concentration-dependent manner were further selected as positive compounds (Supplementary Figure 1b, d).

**In vitro compound validation identified #56 analogs.** In the FRET assay, positive compounds may still include compounds that alter only the conformation of SOD1 or Derlin-1 and thereby disrupt FRET signal without dissociation between the two proteins. Thus, we performed an in vitro co-immunoprecipitation assay by adding the compounds to the purified Flag-SOD1G93A-Derlin-1-HA complex. Twelve compounds out of 44 became positive in this assay (Supplementary Figure 2a–e). Among them, we focused on one of the most prominent inhibitors, named compound #56, because of its dose-dependency in the FRET screening and its drug-like chemical structure defined according to the Lipinski’s rule of five, the absence of reactive functional groups, and the exclusion of promiscuous hitters26,27. The easiness to speculate the pharmacophore and to synthesize the analogs was also taken into consideration (Fig. 1f, g and Supplementary Figure 2d).

Structural analogs of compound #56 were commercially available. More than 200 of those compounds were examined for potential inhibitory effects on SOD1-Derlin-1 interaction. Some of the #56 analogs that showed over 15% inhibition of the SOD1-Derlin-1-derived FRET signal also inhibited the interaction between SOD1 and Derlin-1 in in vitro immunoprecipitation assay (Fig. 2a). Moreover, we found inhibitors that showed lower half-maximal inhibitory concentration (IC50) values than #56 itself (Fig. 2b), including #56-20 and #56-26 (Figs. 2c, d, respectively). These data suggest that the basic core structure of #56 analogs has an inhibitory effect on the SOD1-Derlin-1 interaction.

**#56-59 disrupts all SOD1mut-Derlin-1 interactions analyzed.** Unfortunately, none of the #56 analogs tested above showed inhibitory activities in the cell-based co-immunoprecipitation assay. To evaluate the possibility that the undesired interaction with serum-derived substances was the cause of the inactivation, we assessed the activity in serum-starved condition. Within the serum-depleted medium, some of the compounds showed the inhibition activity, suggesting that the undesired interaction in the culture medium was one of the causes of the inactivity (Supplementary Figure 3a). In addition, #56-26 was relatively stable and showed low permeability, suggesting that the inability might also be due to the low permeability of the plasma membrane (Supplementary Figure 3b).

Although there were some compounds that inhibited the SOD1-Derlin-1 interaction in serum-depleted condition, we have previously reported that the serum depletion causes the conformational change in SOD114,28. Thus, we synthesized new #56 derivatives to obtain novel inhibitors that are active in the

![Fig. 2 Compound validation via in vitro assays. a Inhibition of SOD1G93A-Derlin-1 interaction by #56 analogs in the in vitro immunoprecipitation (IP) assay. Purified SOD1G93A-Derlin-1 complex form HEK293A cell lysates transfected with Flag-SOD1G93A and Derlin-1-HA were incubated with the indicated compounds for 16 h. Then, the complex was immunoprecipitated using anti-Flag beads, followed by immunoblotting (IB) analysis with the indicated antibodies. b IC50 values of notable #56 analogs and #56 in the in vitro assay. IC50 values were calculated by generating standard curves. c, d Chemical structure of #56-20 and #56-26.](image-url)
culture medium containing serum. In an in vitro immunoprecipitation assay, two (#56-40, #56-41) out of six new compounds worked as well as #56 itself, correlating with the result of FRET-based assay (Fig. 3a, b and Supplementary Figure 3c). Moreover, #56-40 showed an improved permeability and inhibitory effect on the SOD1-Derlin-1 interaction even in a cell-based co-immunoprecipitation assay (Fig. 3c and Supplementary Figure 3b). However, this compound has the inconsistent effect, suggesting that the effective concentration depends on cellular conditions. Therefore, we synthesized additional compounds and found a

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**Fig. 3** Identification of cell-permeable compounds. **a** Chemical structure of newly synthesized #56 analogs. #56-43 is the resynthesized #56-10. **b** Inhibition of SOD1<sup>G93A</sup>-Derlin-1 interaction by new #56 analogs in in vitro IP assay. The SOD1<sup>G93A</sup>-Derlin-1 complex purified from lysates transfected with Flag-SOD1<sup>G93A</sup> and Derlin-1-HA was incubated with the indicated compounds for 16 h and analyzed by IP-IB with the indicated antibodies. **c, d** Inhibition of SOD1<sup>G93A</sup>-Derlin-1 interaction by #56 analogs in a cell-based IP assay. HEK293A cells transfected with indicated plasmids were treated with the indicated compounds for 24 h, and lysates were analyzed by IP-IB with the indicated antibodies. **e** Inhibition of SOD1<sup>G93A</sup>-Derlin-1 interaction by the same compounds as in the in vitro assay **b** in a cell-based assay. Compound #66 is used as a positive control of a cell-permeable inhibitor, although the resynthesized #66 does not show any activities. **d** Two cell-permeable SOD1<sup>G93A</sup>-Derlin-1 interaction inhibitors in a cell-based IP assay. **e, f** Chemical structure of #56-40 and #56-59. **g** Inhibitory activities of other compounds containing tertiary amide groups, #56-102-105, in the cell-based IP assay as in **c, d, h** Chemical structure of #56-102, 103, 104, and 105.
more stable inhibitor in cell-based assay, #56-59 (Fig. 3d–f). Through the further evaluation of newly synthesized #56 derivatives, we found that the character of potent inhibitors in cell-based assay is provided by the replacement of hydrogen with alkyl groups at the amide bond, turning that amide into a tertiary amide (Fig. 3g, h). Testing several derivatives with a tertiary amide, we found even more potent inhibitor, #56-111 (Supplementary Figure 4a, b).

SOD1G93A, which we used for the above screening steps, is a representative SOD1mut among 122 types of ALS-related SOD1mut that interact with Derlin-1. Thus, we next examined whether #56-59 could also inhibit the interactions of Derlin-1 with 122 types of SOD1mut in the cell-based co-immunoprecipitation assay. #56-59 clearly attenuated the interactions between Derlin-1 and all tested SOD1mut (Supplementary Figure 5a–h). These results suggest that ALS-related SOD1mut commonly possess a similar mechanism of interaction with Derlin-1, consistent with our previous findings of the importance of DBR exposure in SOD1 for the interaction with Derlin-114,28,29. SOD1WT is known to form homodimers, and Derlin-1 forms homomeric and heteromeric complexes with Derlin family proteins and with other ERAD components such as Hrd130. Under the same conditions as those in which #56-59 inhibited the SOD1-Derlin-1 interaction, these interactions were not affected, suggesting that #56-59 inhibits the SOD1-Derlin-1 interaction with some specificity (Supplementary Figure 6).

#56 analogs target SOD1 DBR. There are two putative modes of action of PPI inhibitors: intervention by direct binding to the interface or allosteric interference. To reveal the mechanism of #56-mediated PPI inhibition, we evaluated the inhibition activity of #56 analogs against the SOD1-Derlin-1 interaction using full-length proteins and/or fragments containing binding sites in the in vitro assay. Due to the low activity of #56-59 in vitro, two prominent in vitro inhibitors, #56-20 and #56-26, were assessed. Both of them inhibited all combinations of the interaction (Fig. 4a). The inhibition of the interaction between SOD1 (1-20) and Derlin-1 (CT4) suggested that #56 analogs exert their activities by interacting with the binding region of either SOD1 or Derlin-1. To identify the direct binding target of #56 analogs, we tried the surface plasmon resonance (SPR) method with biotinylated peptide as a ligand and #56 analogs as an analyte. However, the interaction could not be detected probably due to the relatively weak interaction and low solubility of the compounds (Supplementary Figure 7a). Thus, we next performed fluorescence polarization (FP) assays in the presence of SOD1 (5-20) peptide or Derlin-1 (CT4) peptide, utilizing the fluorescence properties of #56-20 per se. The degree of FP increased in the presence of SOD1 (5-20) peptide rather than the Derlin-1 (CT4) peptide, suggesting that #56-20 preferentially and directly interacts with the SOD1 DBR (Fig. 4b). We also compared the binding ability of #56-20 to the full-length recombinant SOD1WT and SOD1G93A. The preferential interaction of #56-20 with SOD1G93A was observed ($K_d = 81.1 \mu M$ and Supplementary Figure 7b). This is consistent with our model in which SOD1mut possess a DBR-exposed conformation and that #56-20 interacts with the DBR14,29.

Next, to confirm the target of the compounds within the cells, we embedded benzophenone as a photocrosslinkable moiety, and a biotin as a tag to #56-59, a potent inhibitor in cell-based assay. We named this compound Photo-Biotin-PGE2-#56-59, PB56 for short (Fig. 4c, right). Although it was less potent than the original compound (#56-59), PB56 also inhibited the SOD1-Derlin-1 interaction in a dose-dependent manner (Supplementary Figure 7c). In the crosslinking and pull-down assays using SOD1G93A-transfected or Derlin-1-transfected cells treated with PB56, we clearly observed the biotin-labeled band at the same position of monomer PEG-SOD1G93A and high-molecular smear bands probably representing sodium dodecyl sulfate (SDS)-resistant SOD1G93A aggregates, suggesting that PB56 selectively binds to SOD1 and not Derlin-1 (Fig. 4c, left). Pre-treatment with #56-59 competitively displaced the binding of PB56 to SOD1G93A, indicating that the common basic structure of #56-59 in PB56 mediated the interaction with SOD1 (Fig. 4d). Moreover, we confirmed that PB56 interacted with SOD1G93A more strongly than SOD1WT within the cells (Fig. 4e).

Additionally, we evaluated the effects of #56 analogs to SOD activity in vitro. Both #56-20 and #56-26, which possess strong inhibitory activity on the SOD1-Derlin-1 interaction in vitro, showed no effects on SOD activity (Supplementary Figure 7d). Taken together, these data suggest that #56 analogs might specifically inhibit the interaction with Derlin-1 by directly binding to the SOD1 DBR without affecting the enzymatic activity of SOD1.

#56-59 ameliorates the pathology of ALS. Finally, we evaluated the therapeutical effects of #56 analogs in vitro human model utilizing ALS patient iPSCs as well as in vivo ALS mouse model. Recently, we have characterized and established the in vitro ALS model system by utilizing patient iPSCs31. Therefore, we treated #56-40 and #56-59 to motoneurons derived from ALS patient iPSCs with L144FVX mutation in SOD1 (ALSI). Although #56-40 was less effective, probably because of the narrow effective dose, treatment with #56-40 and #56-59 increased the survival of ALS motoneurons (Fig. 5a). Then, we further investigated the effect of #56-59 to other iPSC-derived motoneurons. The number of motoneurons generated from healthy control-derived iPSC was not increased in the presence of #56-59, indicating that the increase of ALS motoneurons with #56-59 was not due to the improvement of differentiation efficiency or survival independent of ALS pathology (Supplementary Figure 8a). Although #56-59 did not improve the viability of motoneurons generated from ALS3 (SOD1G93S), it restored the motoneuron survival of ALS2 (SOD1L144FVX) (Supplementary Figure 8a). These data suggested that #56-59 could ameliorate SOD1mut toxicity in in vitro human ALS model. The conformational changes in SOD1WT have been reported in FALS with other ALS-causative gene mutations, including FUS and TDP-4332. Thus, we examined the effect of #56-59 on motoneuron toxicity with TDP-43 mutant; however, #56-59 failed to improve the survival of ALS4 motoneurons (TDP-43M337V) at least in this condition (Supplementary Figure 8a).

Next, we performed continuous delivery of the compounds to SOD1G93A transgenic male mice by using osmotic pumps. Because we were concerned that the efficacy of the compounds might be compromised by a limited ability to cross the blood–brain barrier and to access the target motoneurons, intracerebroventricular (i.c.v.) cannulation was chosen as the method for delivery. The start point of administration was set at 22 weeks of age, approximately 6 weeks before the usual onset timing as defined in our previous study13. We infused the mice with dimethyl sulfoxide (DMSO) as control, 1 mM #56-40, and 3 mM #56-59 at a flow rate of 0.15 $\mu l$ h$^{-1}$. The onset, defined as motor function deficit observed in rotarod performance, and the survival time were monitored. While mice infused with #56-40, which might be out of effective dose, were comparable to control mice, mice infused with #56-59 showed significantly delayed onset, by a median of 4.5 weeks (14.5% improvement), and also showed significantly prolonged survival, by a median of 5 weeks (14.2% improvement) (Fig. 5b, Supplementary Figure 8b, and
**Fig. 4** #56 analogs target SOD1 DBR. **a** In vitro IP assay with various SOD1-Derlin-1 complexes. SOD1-Derlin-1 complexes purified from lysates transfected with the indicated plasmids were incubated with #56-20 or #56-26 for 16 h and analyzed by IP-IB with the indicated antibodies. **b** FP analysis of #56-20 in the presence of indicated concentration of SOD1 (5-20) and Derlin-1 (CT4) peptide. Here, 250 nM #56-20 was incubated with the indicated concentration of each peptide for 6 h, and FP was measured. Blue: Derlin-1 (CT4); red: SOD1 (5-20). The data are shown as the mean ± s.d. (n = 3). **c** Pull-down assay using PB56. HEK293A cells transfected with indicated plasmid were treated with 200 μM PB56 for 24 h followed by UV light irradiation for 1 h. Lysates were analyzed by IP-IB with the indicated antibodies. **d** Pull-down assay by Flag-SOD1G93A or Derlin-1-Flag. Chemical structure shows PB56 (blue: #56-59 (active part); black: benzophenone with polyethylene glycol (PEG) as spacers; red: biotin). **e** Competition assay with the pre-treatment of 20 μM #56-59 for 12 h. **e** Pull-down assay by Flag-SOD1G93A or Flag-SOD1WT.
Supplementary Table 2). Consistent with these results, the number of motoneurons detected by Nissl staining of lumbar spinal cord sections at 31 weeks of age was significantly increased in #56-59-treated ALS model mice (Fig. 5c, d). These data clearly show that the SOD1-Derlin-1 interaction inhibitor can ameliorate ALS pathology both in in vitro human model and in vivo mouse model, demonstrating the importance of the SOD1-Derlin-1 interaction in the pathogenesis of SOD1mut-induced FALS and the potential of the SOD1-Derlin-1 interaction as a therapeutic target in ALS.

Discussion
In the present study, we designed and developed a high-throughput, robust screening assay system for measuring the #56-40 (#μM) Probability n.s.

| Survival motoneurons (%) | #56-40 (μM) | #56-59 |
|--------------------------|-------------|--------|
| 0.1                      | *           |        |
| 0.3                      | *           |        |
| 1                        | *           |        |
| 3                        |             | *      |

Survival (median) Onset (median)

- DMSO: 31.0 weeks
- #56-40: 31.0 weeks
- #56-59: 35.5 weeks

Survival (median) Onset (median)

- DMSO: 35.0 weeks
- #56-40: 35.0 weeks
- #56-59: 40.5 weeks

**Fig. 5** Inhibitor of SOD1-Derlin-1 interaction, #56-59, ameliorates ALS pathology. **a** Effects of #56 analogs on motoneurons derived from ALS patient iPSCs with L144FVX mutation in SOD1 (ALS1). The ratios of surviving motoneurons (day 14/day 7 (%)) are shown as the mean ± s.d. (n = 6; *P < 0.05, also see Methods for statistical test). **b** Analysis of onset (DMSO: n = 10, #56-40: n = 11, #56-59: n = 12) and survival (DMSO: n = 14, #56-40: n = 11, and #56-59: n = 15) in SOD1G93A transgenic mice (SOD1 G93A Tg) continuously infused with #56 analogs by i.c.v. cannulation. Onset was determined by motor function deficit seen in rotarod performance. Black: DMSO treatment (control); green: #56-40 treatment; red: #56-59 treatment. Mice were followed in this survival study for 58 weeks, and the survival time of one mouse that did not show paralysis is shown as a tick on the line. Statistical analysis was performed using the Kaplan–Meier method followed by the log-rank test and the Gehan–Breslow–Wilcoxon test (*P < 0.05, n.s.: not significant).

**c** Representative Nissl stain image of the lumbar spinal cord section (L2–L5) of indicated mice. All motoneuron counts were performed in a blinded fashion. Scale bar = 20 μm. **d** Quantification of motoneuron counts in **c**. Five sections per mouse were counted, and the data are shown as the mean ± s.e.m. WT mice: n = 4; DMSO-treated and #56-59-treated SOD1 G93A Tg: n = 8 in each group, *P < 0.05: significance was calculated using Student’s t test.
interaction between two proteins, SOD1 and Derlin-1 (Fig. 1a–c). We screened approximately 160,000 compounds and selected one potential scaffold, #56 (Fig. 1d–g). We found that some analogs of #56 also possessed inhibitory activities in vitro (Fig. 2a–c). Moreover, newly synthesized #56 analogs inhibited the SOD1-Derlin-1 interaction in cell-based assays (Fig. 3c, d, g). One of these inhibitors, #56-59, exerted its activity on all types of SOD1mut-Derlin-1 interaction that we previously reported [14] (Supplementary Figure 5a–h). Furthermore, we show that the SOD1-Derlin-1 interaction inhibitor can ameliorate ALS pathology both in vitro in human model and in vivo mouse model (Fig. 5).

We used two inhibitors, #56-40 and #56-59, to assess the effect to the ALS pathology. However, unlike #56-59, #56-40 showed only modest effects to the ALS pathology (Fig. 5a, b). Our concern was that the effective concentration of #56-40 was in a very narrow range. Thus, we assume that the dose of #56-40 might be insufficient to show a therapeutic effect on ALS model mice under these conditions. Moreover, ALS4 motoneurons showed even a vulnerability to #56-59. The ALS motoneurons showed vulnerability, and the effective dose of #56-59 might be different among iPSC lines. The failure of improvement in ALS3 and the significant reduction in ALS4 could be caused by the toxicity of #56-59.

The detected concentration of #56-59 in the brain and spinal cord of the mice were very low (Supplementary Figure 8b and Supplementary Table 2). In addition, we could not evaluate the inhibition activity in vivo, because the level of the SOD1-Derlin-1 interaction varies even in the non-treated ALS model mice (Supplementary Figure 9a). Taking account of the unbound fractions within the serum, the effective concentration of #56-59 could be estimated between 0.25 to 1 μM in cell-based immunoprecipitation assay (Supplementary Figure 4a and Supplementary Figure 8c). One of the possible reasons of this discrepancy is the modification of #56-59. #56-40 showed a relatively unstable character (Supplementary Figure 3b). Although HEK293A cells and the cells in central nervous system do not express typical metabolizing enzymes compared to hepatic cells, #56-40 and probably #56-59 could be metabolized in cells and in vivo. In the present method that we measured the concentration of #56-59, the modified compounds could not be estimated. Therefore, it is possible that the modified #56-59, which we could not identify in this study, contributed to the inhibition of SOD1-Derlin-1 interaction in the present assays.

Another possibility is the difference of the treatment course. In the long-term treatment in vivo, #56-59 could occupy the DBR of newly synthesized SOD1mut before its interaction with Derlin-1. Thus, #56-59 would be able to efficiently inhibit the SOD1-Derlin-1 interaction compared to the cell-based immunoprecipitation assay, where #56-59 might dissociate the pre-formed complexes. It is also possible that as we could find the increase of #56-59 in spinal cord at 5 weeks of infusion (Supplementary Figure 8b and Supplementary Table 2), the longer continuous infusion would reach higher concentration.

Notably, one ALS model mouse treated with #56-59 did not show any symptoms until 58 weeks of age. Although it is unclear whether #56-59 could really suppress the pathology completely in some population or whether additional factor(s) might exist, further improvement of the administration method, including doses, timing of initial administration, and pharmacokinetics, should provide us with more effective treatment for ALS pathologies.

In the present study, we administered the compounds before the appearance of defects in motor function. Our compound delayed the onset of the disease, suggesting that the SOD1-Derlin-1 interaction is involved in the onset of ALS, and earlier treatment might improve the phenotype more effectively. The early diagnosis of conformation-disordered SOD1 using our ELISA system that can easily detect the DBR-exposed SOD1 may enable us to perform a differential diagnosis of the applicable patients before onset and to provide pre-onset treatment [14, 26].

We have previously reported that ER stress evoked by the SOD1-Derlin-1 interaction activates ASK1, and ASK1 deficiency prolonged the survival of ALS model mice by improving the disease progression but not onset [23]. Moreover, the administration of ASK1 inhibitors showed the reduction of the glial cell activity [31], which is known to contribute to the progression of the pathology [34, 35]. On the other hand, the #56-59 treatment had little effect on the progression and did not show significant suppression of gliosis (Supplementary Figure 9b). Thus, we estimate that the SOD1-Derlin-1 interaction may primarily target the signaling pathways that contribute to the disease onset independent of ASK1 pathway. It would be an important issue to reveal the detailed signaling pathways affected in the present treatment protocol and the role of ASK1 in non-cell autonomous motoneuron death, as well as the effects of #56-59 on the ASK1 pathway in vitro and in vivo.

#56 analogs preferentially interact with SOD1mut rather than SOD1WT. Although we cannot exclude the possibility that #56 analogs inhibit the interaction of SOD1mut through the DBR not only with Derlin-1 but also with other proteins, the SOD1-Derlin-1 interaction is the most likely and promising target for ALS treatment. It is also important to carefully interpret the specificity of #56 analogs in cells or in vivo because of the very weak Kd value of them (Supplementary Figure 7a). Further improvements of #56 analogs’ affinity to SOD1mut would be required. The elucidation of compound-SOD1 complex atomic structure should resolve these questions and facilitate further optimization of the compounds.

We also found that zinc deficiency caused a conformational change of SOD1WT to a mutant-like form (exposure of the DBR), resulting in SOD1-Derlin-1 interaction [28]. Recently, it was reported that conformationally disordered SOD1WT was observed in SOD1 mutation-negative sporadic ALS (SALS) patients [36]. SOD1WT has also been shown to be required for non-cell autonomous motoneuron toxicity in astrocytes or oligodendrocytes derived from SALS patients [37, 38]. These data suggest that under certain conditions, including genetic factors and environmental factors, SOD1WT may also serve as the cause of ALS pathogenesis and that inhibition of the SOD1-Derlin-1 interaction may be a potential target for ALS therapy development not only for SOD1mut-caused FALS but also for a subset of SOD1 mutation-negative FALS and SALS, in which conformationally disordered SOD1WT is proposed to be involved. To approach this possibility, we investigated the effect of #56-59 on the iPSC-derived motoneurons with TDP-43 mutant. However, consistent with our previous observation that the exogenously expressed TDP-43 mutants could not alter the SOD1WT conformation [29], administration of #56-59 did not improve motoneuron viability (Supplementary Figure 8a). Since we cannot exclude the possibility that the vulnerability masked the positive effect of #56-59 in ALS4, it is important to evaluate the involvement of the SOD1-Derlin-1 interaction by using multiple inhibitors, different iPSC-derived motoneurons, and/or other methods.

In summary, we succeeded in providing a proof-of-concept regarding our SOD1-Derlin-1 interaction hypothesis by ameliorating the disease both in vitro human model and in vivo mouse model by using a developed PPI inhibitor. These data re-emphasize the importance of the SOD1-Derlin-1 interaction as a common mechanism of motoneuron toxicity in SOD1mut, and we provided a potential approach for a molecular mechanism-based ALS treatment.
Eagle to the high ef

Cell cultures

concentrations of 50 µM in 0.25% DMSO by applying 250 nL of 2 mM stock replicates.

SOD1G93A and Derlin-1-HA or HA-SOD1WT-Flag were lysed in FRET buffer TR-FRET-based interaction assay electroblotted onto polyvinylidene diextracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and 10 (NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-05127-2 | www.nature.com/naturecommunications)

Flag, pcDNA3.0-Derlin-2-Flag, pcDNA3.0-Derlin-3-Flag, pcDNA3.0-Flag-HRD1, pcDNA3.0-Derlin-SOD11, pcDNA3.0-Flag-SOD1, and pcDNA3.0-Derlin-1-(2-10)-CFP plasmids were constructed in previous studies. Transfection was performed using Polyethyleneimine-Max (Polysciences) according to the manufacturer’s instructions.

Antibodies and peptides. Anti-tag antibodies labeled with either Eu or d2 (Supplementary Table 1c) were purchased from Cibio. Horse radish peroxidase (HRP)-linked anti-biotin antibody (7075, Cell Signaling Technology, dilution 1:10000) and anti-Flag tag (F3165, Sigma, dilution 1:100) and HA tag (11867431001, Roche, dilution 1:10,000), BSA–tubulin (MR3-455, Covance, dilution 1:2000), GFAP (N1506, DAKO, dilution 1:10), and Iba1 (1901-19741, Wako, dilution 1:500), SOD1 (ADI-SOD1-100, Enzo Life Science, dilution 1:5000), and Derlin-1 (SAB4200148, Sigma, 1.5 µg for immunoprecipitation) were pur chased from the indicated suppliers. The Derlin-1 antibody has been generated previously (dilution 200 ng ml⁻¹). The biotinylated SOD1 (5-20) peptide (biotinVCLVKGDPVQVGIIPF and biotinylated Derlin-1 (CT4) peptide (biotinCFLYRLWLPSPRRGG) were purchased from Eurofin.

Cell cultures. HEK293A cells were purchased from Invitrogen and were used to for all experiments. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and maintained in an atmosphere of 5% CO₂ at 37 °C. HEK293A cells were used for the present of mycoplasma and identified by morphology and efficient production of adenovirus.

TR-FRET-based interaction assay. HEK293A cells transfected with Flag-SOD11, and Derlin-1-HA or HA-SOD1WT-Flag were lysed in FRET buffer containing 25 mM phosphate buffer pH 7.0, 400 mM potassium fluoride, 0.1% bovine serum albumin (BSA), 0.5% Triton X-100, 5 µg ml⁻¹ leupeptin, 1 mM phenylmethylsulfonyl fluoride. In competition assay using biotinylated SOD1 (5-20) peptide and Derlin-1 (CT4) peptide, lysates from HEK293A cells were transfected with Flag-SOD1 and Derlin-1-HA were incubated with the indicated concentration of each peptide for 16 h. Then, the fluorescence-labeled antibodies were mixed, and FRET signals were measured. Antibody elution buffer (377 nm excitation filter/665 nm emission filter) and donor intensity (377 nm excitation filter/620 nm emission filter) were measured using a FRET analysis. FRET signal was calculated as FRET intensity/donor intensity. FRET signal is described in Supplementary Methods.

Compound treatment for immunoprecipitation assay. In vitro immunoprecipitation, compounds were added to purified SOD1-Derlin-1 complexes from HEK293A cells at 4 °C for 1 h. For the cell-based immunoprecipitation, compounds were added to the culture medium of HEK293A cells for 24 h.

Chemical compounds. Compound #56 (InterBioScreen Ltd), #56-20, and #56-26 (Vitas-M laboratory) were purchased. Other compounds were synthesized as described in Supplementary Methods.

Permeability assay and metabolic stability assay. The permeability and metabolic stability of the compounds were determined commercially using the CEREP in vitro drug absorption and in vitro drug metabolism service, respectively.

Surface plasmon resonance. SPR was analyzed with Biacore T100 (GE Healthcare).

Pull-down assay for target identification. HEK293A cells transfected with Flag-SOD1WT, Flag-SOD11, or Derlin-1.Flag were treated with 100 µM PBS 24 h and then irradiated with 365 nm ultraviolet (UV) light for 1 h on ice. The competition assay, HEK293A cells transfected with Flag-SOD1WT or Flag-SOD11 were treated with 200 µM PBS 24 h and then incubated with 365-nm UV light for 1 h on ice. In the competition assay, HEK293A cells transfected with Flag-SOD1WT or Flag-SOD11 were treated with 200 µM PBS 24 h and then irradiated with 365-nm UV light for 1 h. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5 µg ml⁻¹ leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysozyme was immunoprecipitated with anti-Derlin-1 antibody with protein G-Sepharose (GE Healthcare).

Pull-down assay for target identification. HEK293A cells transfected with vector, Flag-SOD1WT, Flag-SOD11, or Derlin-1.Flag were treated with 200 µM PBS 24 h and then irradiated with 365-nm UV light for 1 h on ice. The competition assay, HEK293A cells transfected with Flag-SOD1WT or Flag-SOD11 were treated with 200 µM PBS 24 h and then irradiated with 365-nm UV light for 1 h. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 5 µg ml⁻¹ leupeptin, and 1 mM phenylmethylsulfonyl fluoride.

Recombinant proteins and SOD activity assay. The cDNAs encoding GST-SOD1WT and GST-SOD11 were constructed in the pGEX-6P-1 vector (GE Healthcare), and each plasmid was transformed into Escherichia coli (BL21). The transformed cells were cultured in Luria Broth medium containing 50 µg ml⁻¹ ampicillin at 37 °C for 16 h. Isopropyl β-D-1-thiogalactopyranoside was then added at a final concentration of 0.1 mM, and the cells were incubated at 30 °C for an additional 2 h. Cells were lysed with an antibiotic and chloroform (PBS) containing 10 mM EDTA and 1% Triton X-100. After centrifugation at 17,700 × g, the supernatant was incubated with Glutathione Sepharose 4B (GE Healthcare) at 4 °C for more than 2 h. After three washes with PBS containing 10 mM EDTA, proteins were eluted using the elution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% glutathione). The proteins were incubated with 100 µM CuSO₄ and ZnCl₂, dialyzed using PBS, and incubated with DMSO and 50 µM #56-20 or #56-26 at 4 °C for 16 h. SOD activity was measured using a SOD Assay Kit
according to the manufacturer's instructions (Dojindo). The experiment was repeated three times on three separate days.

**Motoneuron survival assay using iPSCs.** Use of human iPSCs was approved by the Ethics Committees of the respective departments at Kyoto University. All methods were performed in accordance with approved guidelines. Informed consent was obtained from all subjects. Motoneurons were generated from previously established iPSCs by introducing transcription factors Lhx3, Ngn2, and Isl1 under the control of a retrovirus template. Some iPSC clones, Control 1, ALS1, ALS2, and ALS4, are deposited into RIKEN BioResource Center (depository numbers are HPS0063, HPS0251, HPS0252, and HPS0292, respectively). The compound screen was previously established. The iPSCs were dissociated to single cells using Accutase (Innovative Cell Technologies, San Diego, CA, USA) and plated onto Matrigel-coated 96-well plates (Corning, Corning, NY, USA) with neuronal medium (DMEM/F12 (Thermo Fisher Scientific), 100 μg ml⁻¹ apotransferrin (Sigma), 5 μg ml⁻¹ insulin (Sigma), 30 nM selenite (Sigma), 20 nM progesterone (Sigma), 100 nM putrescine (Sigma)) containing 1 μM retinoic acid (Sigma), 1 μM thiotretinoin (Sigma), 10 ng ml⁻¹ brain-derived neurotrophic factor (R&D Systems, Minneapolis, MN, USA), 10 ng ml⁻¹ nerve growth factor (R&D Systems), and 10 ng ml⁻¹ NT-3 (R&D Systems) with 1 μg ml⁻¹ doxycycline (TAKARA), and cultured for 7 days. Compounds were added to cells on day 7, and cultures were maintained for 14 days. Cells were fixed and stained on days 7 and 14. The number of surviving motoneurons stained with βIII-tubulin (Covance, Princesdon, NJ, USA) was identified by IN Cell Analyzer 6000 (GE Healthcare, Chicago, IL, USA) and IN Cell Developer tool software 1.9 (GE Healthcare).

**Animals.** All experiments were performed in accordance with protocols approved by the Animal Research Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan). The mouse genotypes were verified by PCR at weaning and before the administration of compounds. At 22 weeks of age, approximately 6 weeks before the usual onset timing as described in our previous study, male mice with the human SOD1(G93A) transgene (G93A line, backcrossed to C57BL/6) were randomly separated into three groups and continuously infused with DMSO as control, with 1 mM #56-40, or with 3 mM #56-40 using osmotic pumps by i.c.v. cannulation. Onset was determined at the time point when the average of week 15 body weight was increased by 3% compared to the start value. The investigators were not blinded to allocation during experiments and outcome assessment.

**Measurement of #56-59 concentration.** Male mice (C57BL/6) were continuously infused with 3 mM #56-59 using somatic pumps by i.c.v. cannulation. The brain and spinal cord were excised at 1, 3, and 5 weeks after the start of the infusion and homogenized with phosphatase buffer. The tissue homogenates were deproteinized with acetonitrile containing the internal standard (methyltestosterone) and homogenized with phosphate buffer. The tissue homogenates were continuously infused with 3 mM #56-59 using osmotic pumps (flow rate 0.15 μl h⁻¹, Alzet Mode 2006) by i.c.v. cannulation with the Brain infusion kit 3 (Alzet). Pumps were placed on the back of the mouse and were replaced every 6 weeks until the mouse showed paralysis. Onset was determined at the time point when the average of week's rotarod performance (three times per week) began to decline at an accelerated speed to 40 rpm for 5 min. The genotype of the mouse that did not show any symptoms was again confirmed by PCR, and the treatment trial of this mouse was stopped at 58 weeks of age. The investigators were not blinded to allocation during experiments and outcome assessment.

**Nissl staining and immunohistochemistry.** Mice were perfused with PBS and then with 4% paraformaldehyde (PFA) in PBS and the spinal cords (L2-L5) were fixed with 4% PFA in PBS for 1 day. The lumbar spinal cords were then incubated in 30% sucrose for 24 h, embedded in CryoMount (Muto PureChemicals), and frozen sections (40 μm) were produced. Nissl staining was performed using 0.1% cresyl violet acetate (WALDECK). Motoneurons showing a clear nucleolus and their nonneuronal neighbors were included in cell counts. All images were obtained using a Leica TCS SP5 confocal laser scanning microscope.

**Statistical analysis.** No statistical methods were used to predetermine sample size. The number of surviving motoneurons stained with βIII-tubulin (Covance, Princeton, NJ, USA) was identified by IN Cell Analyzer 6000 (GE Healthcare, Chicago, IL, USA) and IN Cell Developer tool software 1.9 (GE Healthcare).

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Author contributions

N.T. and K.H. performed most of the experiments. K.I. and H.I. performed the iPSCs experiments. T.H., A.B., H.Y., N.S., S.N., S.-I., N.U., N.K., S.Y., and M.S. designed and synthesized the chemical compounds. H.I. conceived the idea. N.T., K.H., T.F., and H.I. designed the experiments with critical assistance from M.S., H.K., T.O., T.N., I.N., N.T., K.H., and H.I. mainly wrote the manuscript. All authors commented on the manuscript.

Additional information

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Competing interests: The University of Tokyo and Public University Corporation Nagoya City University are applying for a patent of #56 analogs. The inventors are N.T., K.H., M.S., H.K., T.O., T.N., T.F., H.I., N.S., T.H., S.N., H.N., and S.I. The application number is PCT/JP2017/021895 and the status is patent-pending. The patent covers the effect of #56–59 analogs to ALS pathology in model mouse. The other authors declare no competing interests.

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