TITLE:

Chemical library screening identifies a small molecule that downregulates SOD1 transcription for drugs to treat amyotrophic lateral sclerosis.

AUTHOR(S):

Murakami, Gaku; Inoue, Haruhisa; Tsukita, Kayoko; Asai, Yasuyuki; Amagai, Yuji; Aiba, Kazuhiro; Shimogawa, Hiroki; Uesugi, Motonari; Nakatsuji, Norio; Takahashi, Ryosuke

CITATION:

Murakami, Gaku ...[et al]. Chemical library screening identifies a small molecule that downregulates SOD1 transcription for drugs to treat amyotrophic lateral sclerosis.. Journal of biomolecular screening 2011, 16(4): 405-414

ISSUE DATE:

2011-04

URL:

<http://hdl.handle.net/2433/156968>

RIGHT:

© 2011 Society for Laboratory Automation and Screening; この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。 ; This is not the published version. Please cite only the published version.

Chemical library screening identifies a small molecule that downregulates SOD1 transcription for drugs to treat ALS

Gaku Murakami M.D.,¹ +81-75-751-3767, mrkmgk@kuhp.kyoto-u.ac.jp
Haruhisa Inoue M.D., Ph.D.,^{2,3} +81-75-366-7036, haruhisa@cira.kyoto-u.ac.jp
Kayoko Tsukita,^{2,3} +81-75-366-7087, tsukita@cira.kyoto-u.ac.jp
Yasuyuki Asai Ph.D.,⁴ +81-45-475-3887, y-asai@reprocell.com
Yuji Amagai Ph.D.,⁵ +81-75-313-9535, amagai@scdi.or.jp
Kazuhiro Aiba Ph.D.,⁵ +81-75-313-9535, aiba@scdi.or.jp
Hiroki Shimogawa Ph.D.,⁶ +81-774-38-3225, shimogaw@scl.kyoto-u.ac.jp
Motonari Uesugi Ph.D.,⁶ +81-774-38-3225, uesugi@scl.kyoto-u.ac.jp
Norio Nakatsuji Ph.D.,⁷ +81-75-753-9740, nnakatsu@icems.kyoto-u.ac.jp
Ryosuke Takahashi M.D., Ph.D.^{1,3} +81-75-751-3767, ryosuket@kuhp.kyoto-u.ac.jp

¹ Department of Neurology, Graduate School of Medicine, Kyoto University

² Center for iPS Cell Research and Application (CiRA), Kyoto University

³ Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation

⁴ ReproCELL, Inc.

⁵ Stem Cell and Drug Discovery Institute

⁶ Institute for Chemical Research, Kyoto University

⁷ Institute for Integrated Cell-Material Sciences, Kyoto University

Correspondence should be addressed to

H. I. (haruhisa@cira.kyoto-u.ac.jp); phone number: +81-75-366-7036, fax number: +81-75-366-7023, at Center for iPS Cell Research and Application (CiRA), Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan, or R. T. (ryosuket@kuhp.kyoto-u.ac.jp); phone number: +81-75-751-3770, fax number: +81-75-761-9780, at Department of Neurology, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan

Word count: 4,748

Abstract

Familial amyotrophic lateral sclerosis (fALS) accounts for 10% of ALS cases, and about 25% of fALS cases are due to mutations in superoxide dismutase 1 (SOD1). Mutant SOD1-mediated ALS is caused by a gain of toxic function of the mutant protein, and the SOD1 level in non-neuronal neighbors, including astrocytes, determines the progression of ALS (non-cell-autonomous toxicity). Therefore, we hypothesized that small molecules that reduce SOD1 protein levels in astrocytes might slow the progression of mutant SOD1-mediated ALS. We developed and optimized a cell-based, high-throughput assay to identify low molecular weight compounds that decrease SOD1 expression transcriptionally in human astrocyte-derived cells. Screening of a chemical library of 9,600 compounds with the assay identified two hit compounds that selectively and partially downregulate SOD1 expression in a dose-dependent manner, without any detectable cellular toxicity. Western blot analysis showed that one hit compound significantly decreased the level of endogenous SOD1 protein in H4 cells, with no reduction in expression of β -actin. The assay developed here provides a powerful strategy for discovering novel lead molecules for treating familial SOD1-mediated ALS.

Key words: amyotrophic lateral sclerosis, superoxide dismutase 1, high-throughput

1 screening, cell-based assay

1 Introduction

2 Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that
3 selectively involves motor neurons in the brain and spinal cord. ALS leads to muscle
4 weakness, paralysis, and respiratory failure within five years of onset. Familial ALS
5 (fALS) accounts for about 10% of all ALS cases, and approximately 25% of fALS cases
6 are due to mutations in superoxide dismutase [Cu-Zn] (SOD1).¹

7 Some evidence suggests that mutant SOD1 protein has neurotoxic properties and
8 leads to ALS via a gain of toxic function. Mice carrying a high copy number of the
9 mutant SOD1 gene suffer more severe muscle weakness and death than mice carrying a
10 low copy number.² SOD1 knockout mice do not develop the motor neuron disease
11 phenotype at all.³ In rats, only strains with the highest level of mutant SOD1 expression
12 develop an ALS phenotype.⁴

13 Previous studies reported that the SOD1 level in neurons and in non-neuronal
14 neighbors, including astrocytes and microglia, determines the onset and progression of
15 motor neuron disease.^{5,6} Therefore, we hypothesized that reduction of SOD1 expression
16 in astrocytes might ameliorate mutant SOD1-mediated ALS. This hypothesis is
17 supported by prolonged survival of ALS model mice, following application of RNA
18 interference or antisense oligonucleotide, which reduced SOD1 protein levels.^{7,8}

Furthermore, inactivation of a mutant allele reversed the phenotypes in other neurodegenerative disease models, such as Huntington's disease and Alzheimer's disease, even after onset.^{9,10} The present study developed and optimized a high-throughput screening (HTS) system to identify compounds that downregulate the transcription of SOD1.

Materials and methods

Generation of a SOD1 promotor-luciferase reporter cell line

We used the SOD1 genomic promoter, including 5' and 3' untranslated regions (UTR), in our construct to generate SOD1 transgenic mice. The cassette was identical to that carried by SOD1^{G93A} transgenic mice (gPr^{SOD1}-Luc), to reflect physiological activity of the SOD1 promoter (Fig.1A). A total of 1.2 Kb of human SOD1 (hSOD1) 5'-fragment, with 5'-EcoR1 and 3'-Afe1-BamH1 sites, was amplified using PfuUltra™ 2 Fusion HS DNA Polymerase (Stratagene, Cedar Creek, TX, USA). The following PCR primers were used to amplify the region: forward primer, 5'-AAAGAATTCTGCCAACCAAATAAG-3'; reverse primer, 5'-TTTGGATCCAGCGCTGAAGCCGGAAAGCGGAG-3'. The fragment was cloned into pKF18k-2 plasmid (Takara, Otsu, Japan). To add Cla1 site and delete the start

codon of SOD1 exon1, the cassette was amplified by PfuUltra™2 Fusion HS DNA Polymerase using the following PCR primers: forward primer, 5'-GTTATCGATGCGACGAAGGCCGTGT-3'; reverse primer, 5'-TCGCTAGGCCACGCCGAGG-3'. The fragment was cut with EcoR1 and Afe1, and cloned into pKF18k-2-hSOD1^{G93A}. The SV40-Neo-Poly(A) was incorporated downstream from the SOD1 gene, between the BamH1 and Sal1 sites. Finally, secreted luciferase gene (MetLuc) from the marine copepod, *Metridia longa* (Clontech, Mountain View, CA, USA), with ATG was added at the Cla1 site.

Human astrocytoma-derived H4 cells,¹¹ which are frequently used for research on neurodegenerative diseases,¹² were used for transfection, to mimic the transcription of SOD1 in astrocytes. The cell lines were cultured at 37°C in DMEM (Sigma, St. Louis, MO, USA), containing 10% (v/v) fetal bovine serum (FBS), 50 U ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin, and 200 µg ml⁻¹ G418 (Nacalai, Kyoto, Japan). The cells were stably transfected with the SOD1 genomic construct cut by Sgf1, using FuGENE 6 Transfection Reagent (Roche, Basel, Switzerland). Clonal cell lines were selected based on high levels of secreted luciferase genes, and reactivity was confirmed by southern blotting and luciferase reporter assay (Fig. 1B, C). For the southern blotting, 15 µg of DNA, cut at EcoR1 and BamH1, were loaded, and the probe was made from the

following primers: forward primer, 5'-ATCTGGGAGACCATGGAAGT-3'; reverse primer, 5'-TTCTTTGAAGCCGCTGATCTC-3'.

The compound library

HTS assays using the gPr^{SOD1}-luciferase cell line were performed to screen a library of 9,600 compounds provided by the Institute for Chemical Research, Kyoto University. The library was delivered in 96-well racks, with each compound dissolved in DMSO at 5 mM. The extreme right and left well contained DMSO without any compound, leaving the corresponding well on assay plates available for controls. All compounds were stored at -20°C.

High-throughput screening (HTS) assay

Luciferase expression by the gPr^{SOD1}-luciferase cells after exposure to various small compounds was assayed in white, flat-bottomed, 96-well plates (Costar, Bethesda, MD, USA). The cells were precultured overnight at 3.0×10^4 cells well⁻¹ and 37°C. The compound to be tested was preplated, diluted with culture medium to 50 μM, and used to replace 80 μL of 100 μL per well of cell culture to give a final concentration of 40 μM. The cells were then cultured for another 16 h at 37°C.

SOD1 gene expression by cells exposed to each compound was determined by measuring activity of luciferase proteins secreted by the cells. The cell culture in each well was transferred to the corresponding well on a 96-well assay plate, using a Multifunction Tabletop Dispenser EDR-384S2 (Biotec, Tokyo, Japan). Ready-To-GlowTM Secreted Luciferase Reporter System (Clontech) was added, and luciferase activity was measured as emission at 450 nm, using a 1420 VICTOR 3 Multilabel Plate Reader with optional dispenser (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). The ratios of the vehicle-treated samples were used to correct for spontaneous decay of the signal.

Assay performance was determined by calculating the Z factor (Z'), using the following equation:

$$Z' = 1 - \frac{3 \times (\sigma_{C+} + \sigma_{C-})}{(\mu_{C-} - \mu_{C+})}$$

where μ_{C+} and σ_{C+} are the mean and standard deviation (SD), respectively, of the positive control; μ_{C-} and σ_{C-} are the mean and SD of the negative control. The positive control assays treated cells with 10 $\mu\text{g mL}^{-1}$ mitomycin-C (Wako, Osaka, Japan).¹³ The negative control assays treated cells with vehicle (DMSO). The Z' value indicates the quality of an assay by describing the magnitude of the signal window ($\mu_{C-} - \mu_{C+}$) and the

precision of the assay ($\sigma_{C+} + \sigma_{C-}$). A compound was selected as a hit when it decreased luciferase activity less than mean minus 3SD of negative controls. In each run, four or five library plates were applied to the screening assay with an individual control plate for calculating Z' value as well as an average and SD of luciferase activity for negative control. Hits were not selected from runs with Z' value less than zero. The effect of hit compounds on the SOD1 expression was confirmed when it also decreased luciferase activity less than mean minus 3SD of negative controls in duplicate by another assay.

Dose-response and cytotoxicity

Dose-response analysis was carried out using the gPr^{SOD1}-luciferase cell line to confirm that the hit compounds reduced SOD1 expression in a dose-dependent manner. As in the primary assays, the cells were precultured overnight, then the media were exchanged to give a 0-80 μ M range of compound concentrations. The cells were incubated for another 16 h, and luciferase activity was measured. Only compounds that resulted in greater than -3SD inhibition of SOD1 expression at 40 μ M were included in further analyses, because the concentration was also adapted for HTS assay selection.

Toxicity assays identified compounds that produced a non-specific decrease in luciferase activity, due to cellular toxicity. Toxicity analysis was performed on

untransfected H4 cells, using the tetrazolium salt, WST-1 (Roche). In this assay, cleavage of WST-1 to formazan by mitochondrial dehydrogenases causes a color change from red to yellow. As in the primary assays, untransfected H4 cells were precultured overnight in a 96-well plate, and then the media were exchanged to give a 0-40 μM range of compound concentrations. The cells were incubated for 16 h, then WST-1 was added at 10 $\mu\text{L well}^{-1}$, and the cells were incubated for 1 h at 37°C. Absorbance at 450 nm was compared to that of cells that were not treated with the compound. Compounds were considered to have significant cellular toxicity, if cells treated with 40 μM showed greater than a -2SD decrease in fluorescence compared to untreated cells.

Secondary assay

Enzyme-linked immunosorbent assays (ELISAs) and western blots were used to determine whether effects observed in the reporter cell line could be reproduced at the level of endogenous SOD1 protein. As in the primary assays, untransfected H4 cells were pre-cultured overnight, and the media were exchanged with hit compounds to give final concentrations of 0-40 μM . The cells were cultured for 48 h, then each well was washed once with 200 μL of PBS, and lysed with 100 μL of 1% Triton-X containing protease inhibitors (Roche).

1 ELISAs were performed to quantify differences in SOD1 protein levels, and EC₅₀
2 values were calculated using a two-antibody sandwich ELISA for human SOD1.
3 Polystyrene, enzyme-linked, immunosorbent, 96-well assay plates (Greiner Bio-one,
4 Frickenhausen, Germany) were coated with 0.02 µg 0.1 mL⁻¹ well⁻¹ of rabbit anti-SOD1
5 antibody (1:5,000, cat. #SOD100, Stressgen, Ann Arbor, MI, USA) in 50 mM sodium
6 carbonate buffer at pH 9.4. The plates were incubated overnight at 4°C. The wells were
7 washed with PBS and blocked for 2 h with 3% bovine serum albumin (BSA) in wash
8 buffer (PBS containing 0.05% Tween 20). The blocking solution was discarded; 50 µL
9 of cell lysate diluted 1:100 in 3% BSA in wash buffer was added to each well, along
10 with recombinant SOD1 protein¹⁴ (standard curve); and the plates were incubated
11 overnight at 4°C. The wells were washed with PBS, 100 µL of mouse anti-SOD1
12 antibody (1:1,000, cat. #S2147, Sigma) were added, and the plates were incubated for 1
13 h at room temperature (RT). The wells were washed with PBS, and the bound mouse
14 antibody was detected with 100 µL per well of HRP-conjugated goat anti-mouse IgG
15 antibody (1:5000, cat. #NA9310V, GE Healthcare, Buckinghamshire, UK). The plate
16 was incubated for 1 h at RT, and then reacted for 30 min with OptEIA™ TMB Substrate
17 Reagent Set (BD Biosciences, San Jose, CA, USA). The reaction was stopped by adding
18 100 µL of 1 M sodium phosphate. The rate of change in absorbance at 450 nm was

measured with a ThermoFischer Scientific Multiskan JX (Thermo Electron Corporation, Waltham, MA, USA). The concentration of SOD1 in the cell lysates was derived from a standard curve with a linear concentration range of 1.0-125 ng mL⁻¹.

The cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes. Membranes were blocked in 3% BSA in TBS and probed with anti-SOD1 antibody (1:1,000, Stressgen), and then reprobed with an anti-β-actin antibody (1:5,000, cat. #A1978, Sigma), as an internal control.

Western blot analysis for phosphorylation of transcription factors

Untransfected H4 cells were pre-cultured overnight on 12-well plates at 5.0×10⁴ cells. The hit compound was diluted with culture medium to 25μM and 50 μM, and used to replace 0.8 mL of 1 mL per well of cell culture to give a final concentration of 20μM and 40 μM, respectively. The cells were then cultured for another 16 h, and then each well was washed once with 2 mL of PBS, and lysed with 100 μL of 1% Triton-X containing protease inhibitors (Roche) and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Western blotting was performed with antibody specific to Ser⁴⁰-phosphorylated Nrf2 (2500:1, Abcam, #EP1809Y, Cambridge, MA), Nrf2 (500:1, H-300, Santa Cruz Biotechnology Inc., #sc13032, Santa Cruz, CA),

1 Ser¹³³-phosphorylated CREB (500:1, Millipore, #06-519, Billerica, MA), CREB
2 (1000:1, Cell Signaling, #9197, Salem, MA) and β -actin (1:5,000, Sigma).

3

4 *Synthesis of 052C9*

5 6-Chloro-3-formylchromone (0.20 g, 0.96 mmol) and o-phenylene diamine (0.10 g,
6 0.96 mmol) were dissolved in acetic acid (5 mL). The reaction mixture was stirred at 60
7 °C for 16 h and then diluted with an aqueous solution of NaHCO₃ (20 mL). The
8 resulting precipitate was filtered and washed with water. The residue was dissolved in
9 trifluoroacetic acid (1 mL) and then concentrated in vacuum. To the residue was added
10 EtOAc (3 mL), and the resulting suspension was filtered to give 052C9 (72 mg, 18%) as
11 a trifluoroacetic acid salt.

14 **Results**

15 *HTS assays using the gPr^{SOD1}-Luc cell line*

16 As evidenced by the reduction in luciferase activity (Fig. 1B,C), the stable
17 gPr^{SOD1}-Luc cell line expresses secreted luciferase under the control of a genomic SOD1

promoter and, therefore, is useful for identifying compounds that decrease SOD1 expression transcriptionally. The HTS assays using the gPr^{SOD1}-Luc cell line exhibited good reproducibility, with an average Z' value of 0.39 (range, -0.23-0.75). There was only two runs that had Z' values below zero (Table 1). We did not select hit compounds from these two runs. The effect of each compound was represented as the degree of inhibition of luciferase activity compared to vehicle-treated cells (Supplemental Fig.).

Using the HTS assay, duplicate assay, and dose-dependent testing, we identified 120 hit compounds that significantly inhibited SOD1 transcription (Table 2). We excluded the compounds with poor dose-dependent responses (Fig. 2A). WST-1 assays indicated that five of these hit compounds had non-specific cell toxicity (data not shown). ELISA results showed that two of the remaining 115 compounds reduced the level of endogenous SOD1 protein in a dose-dependent manner. We did not employ the compounds with no significant decline of SOD1 protein by ELISA (Fig. 2B, C). One of the compounds, 052C9, was selected for western blot analysis, based on its downregulation of SOD1 expression, determined by the reporter assay (Fig. 3A) and by ELISA (Fig. 3B) with the lower 50% effective concentration (EC₅₀) compared to the other compound (Fig. 3C). The selected compound significantly decreased the level of endogenous SOD1 protein in H4 cells, with no reduction in expression of β-actin (Fig.

4). The structure of this hit compound (Fig. 5) was confirmed by re-synthesis and spectroscopic characterization: the molecule is composed of a banzoimidazole ring and a chromen unit, and is not analogous to any of the drugs used in ALS treatment trials to date. Two major transcription factors have been reported to activate the expression of SOD1: NF-E2 (Nrf2) and cAMP response element binding protein (CREB). We examined the effects of 052C9 on the phosphorylation status of these two transcription factors by western blot analysis. The results showed that 052C9 blocked the phosphorylation of NF-E2 (Nrf2) with no reduction of total Nrf2 protein level, whereas 052C9 had no detectable effects on the phosphorylation status of CREB (Fig. 6).

Discussion

In a recent article, Broom WJ et al. developed HTS assays to identify compounds that downregulate SOD1 expression.¹⁹ Based on this previous study, we executed the present study targeting the transcription of SOD1 with a different compound library and a modified reporter construct of SOD1 promoter. The HTS system using astrocytoma-derived H4 cells successfully identified a number of hit compounds that decrease the expression of SOD1 protein. The HTS assays exhibited good reproducibility, with an average Z' value of 0.39 (range, -0.23-0.75). This variability

might be due to the manual preplating of the cells for screening, or due to the instability of the secreted luciferase. Although the assay results had a high coefficient of variation, this could be attributed to the relatively high abundance of hit compounds (3.39%, Table 2). Because this hit percentage may partially reflect gaussian statistics, we confirmed the significant efficacy of the hit compounds on SOD1 expression through another duplicate assay and dose-dependent analysis. This process would allow us to rule out the effect of gaussian statistics on the hit selection. Although most of the hit compounds failed to decrease endogenous SOD1 protein level by ELISA in a dose-dependent manner, we suppose that this may be due to direct inhibition of luciferase reaction, or to the difference between temporal patterns of the transcription and translation of SOD1. At least one of the hit compounds, 052C9, significantly downregulated SOD1 protein levels in a dose-dependent manner. It is unlikely that the effect reflects non-specific cellular toxicity, because the WST-1 assay showed no significant effects at the concentrations at which the compound exerted the downregulation of SOD1. It is also unlikely that the hit compound represses transcription generally, as there was no corresponding reduction in expression of β -actin.

The mode of action of 052C9 remains unclear at the moment. Nevertheless, our analysis suggests that 052C9 directly or indirectly blocks the phosphorylation of Nrf2.

Transcription factor Nrf2 binds to the antioxidant response element (ARE) in the promoter region of detoxifying genes.¹⁵ Phosphorylation of Nrf2 promotes its translocation into the nucleus where it activates the transcription of antioxidant genes.¹⁶ Since SOD1 gene also contains ARE,¹⁷ the hit compound, 052C9, may downregulate the transcription of SOD1 by inhibiting phosphorylation of Ser⁴⁰ of Nrf2. 052C9 had no detectable effects on the Ser¹³³ phosphorylation of CREB in the present study. Since protein kinase C (PKC) phosphorylates both of the two transcription factors,^{16,18} it is likely that 052C9 inhibits the activity of an unidentified Nrf2-selective kinase or its activation. 052C9 or its analogs may serve as a powerful tool for exploring the molecular mechanism of SOD1 expression.

The hit compounds identified in the present study cause a partial reduction in SOD1 expression. Although the effects on ALS-model mice have not yet been examined, partial downregulation of SOD1 expression may be desirable. SOD1-knockout mice do not develop the motor neuron disease phenotype³, but do show modest vulnerability to axotomy³, and pathological degeneration of neuromuscular junctions and axons.²⁰

Decreasing wild type SOD1 by a small molecule may prove to alleviate the disease phenotype in ALS-model mice, and even in sporadic ALS patients. A previous study showed that wild type (WT) SOD1 transgenic mice have pathological changes similar

to those in mutant SOD1 mice, and that WT SOD1 aggravates the ALS phenotype in double-transgenic mice with both WT-SOD1 and mutant SOD1.²¹ Moreover, the mutation in the SOD1 promoter reduces SOD1 gene expression and may correlate with a delay in the onset of sporadic ALS.²² Indeed, Zhong Z et al. reported that administration of activated protein C (APC) to mutant SOD1 mice, which decreases the expression of SOD1 protein *in vivo*, ameliorates the ALS phenotype.²³ Based on these findings, toxicity of mutant SOD1 may not be explained by "a gain of toxic function", but "an increased" toxicity of wild-type SOD1. Direct reduction of the transcription of pathogenic SOD1 protein may provide a new therapeutic strategy for SOD1-mediated ALS, and that similar strategies may be used to treat other neurodegenerative diseases mediated by aberrant proteins.

Acknowledgements

This work was supported by research grants from the Ministry of Health and Labour (R.T., H.I.), the New Energy and Industrial Technology Development Organization (NEDO) (N.N.), JSPS (21591079) (H.I.), and JST (M.U.). We thank Ryoichi Nakano for providing the pHGSOD-Svneo plasmid, and Kazumi Murai for editing the manuscript.

REFERENCES

- 1 1. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al.:
2 Mutations in Cu/Zn superoxide dismutase gene are associated with familial
3 amyotrophic lateral sclerosis. *Nature* 1993;362:59-62
- 4 2. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, et al. :
5 Motor neuron degeneration in mice that express a human Cu/Zn superoxide
6 dismutase mutation. *Science* 1994;264:1772-1775
- 7 3. Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, et al.:
8 Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but
9 exhibit enhanced cell death after axonal injury. *Nat Genet* 1996;13:43-47
- 10 4. Nagai M, Aoki M, Miyoshi I, Kato M, Pasineli P, Kasai N, et al.: Rats expressing
11 human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic
12 lateral sclerosis: associated mutations develop motor neuron disease. *J Neurosci*
13 2001;21:9246-9254
- 14 5. Yamanaka K, Chun SJ, Boillee S, Fujimoto-Tonou N, Yamashita H, Gutmann DH,
15 et al.: Astrocytes as determinants of disease progression in inherited amyotrophic
16 lateral sclerosis. *Nat Neurosci* 2008;11:251-253
- 17 6. Boillee S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, et
18 al.: Onset and progression in inherited ALS determined by motor neurons and

- 1 microglia. *Science* 2006;312:1389-1392
- 2 7. Saito Y, Yokota T, Mitani T, Ito K, Anzai M, Miyagishi M, et al.: Transgenic small
- 3 interfering RNA halts amyotrophic lateral sclerosis in a mouse model. *J Biol Chem*
- 4 2005;280:42826-42830
- 5 8. Smith RA, Miller TM, Yamanaka K, Monia BP, Condon TP, Hung G, et al.:
- 6 Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest*
- 7 2006;116:2290-2296
- 8 9. Yamamoto A, Lucas JJ, and Hen R: Reversal of neuropathology and motor
- 9 dysfunction in a conditional model of Huntington's disease. *Cell* 2000;101:57-66
- 10 10. SantaCruz K, Lewis J, Spires T, Paulson J, Kotilinek L, Ingelsson M, et al.: Tau
- 11 suppression in a neurodegenerative mouse model improves memory function.
- 12 *Science* 2005;309:476-481
- 13 11. Krex D, Mohr B, Hausen M, Ehninger G, Schackert HK and Schackert G:
- 14 Identification of uncommon chromosomal aberrations in the neuroglioma cell line
- 15 H4 by spectral karyotyping. *J Neuro-oncol* 2001;52:119-128
- 16 12. Kukar TL, Ladd TB, Bann MA, Fraering PC, Narlawar R, Maharvi GM, et al.:
- 17 Substrate-targeting c-secretase modulators. *Nature* 2008;453:925-930
- 18 13. Broom WJ, Ay I, Pasinelli P, and Brown Jr. RH: Inhibition of SOD1 expression by

- 1 mitomycin C is a non-specific consequence of cellular toxicity. *Neurosci Lett*
- 2 2006;393:184-188
- 3 14. Urushitani M, Kurisu J, Tateno M, Hatakeyama S, Nakayama K, Kato S, et al.:
- 4 CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by
- 5 ubiquitinating Hsp/Hsc70. *J Neurochem* 2004;90:231-244
- 6 15. Nguyen T, Nioi P, and Pickett CB: The Nrf2-antioxidant response element signaling
- 7 pathway and its activation by oxidative stress. *J Biol Chem* 2009;284:13291-13295.
- 8 16. Huang HC, Nguyen T, and Pickett CB: Phosphorylation of Nrf2 at Ser40 by protein
- 9 kinase C regulates antioxidant response element-mediated transcription. *J Biol*
- 10 *Chem* 2002;277:42769-42774.
- 11 17. Park EY and Rho HM: The transcriptional activation of the human copper/zinc
- 12 superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-p-dioxin through two
- 13 different regulator sites, the antioxidant responsive element and xenobiotic
- 14 responsive element. *Mol Cell Biochem* 2002;24:47-55.
- 15 18. Johannessen M and Moens U: Multisite phosphorylation of cAMP response
- 16 element-binding protein (CREB) by a diversity of protein kinases. *Front Biosci*
- 17 2007;12:1814-1832.
- 18 19. Broom WJ, Auwarter KE, Ni J, Russel DE, Yeh L, Maxwell MM, et al.: Two

- 1 approaches to drug discovery in SOD1-mediated ALS. *J Biomol Screen*
- 2 2006;11:729-735.
- 3 20. Flood DG, Reaume AG, Gruner JA, Hoffman EK, Hirsch JD, Lin Y, et al.:
- 4 Hindlimb motor neurons require Cu/Zn superoxide dismutase for maintenance of
- 5 neuromuscular junctions. *Am J Pathol* 1999;155:663-672
- 6 21. Jaarsma D, Haasdijk ED, Grashorn JAC, Hawkins R, Duijn W, Verspaget HW, et
- 7 al.: Human Cu/Zn superoxide dismutase (SOD1) overexpression in mice causes
- 8 mitochondrial vacuolization, axonal degeneration, and premature motoneuron death
- 9 and accelerates motoneuron disease in mice expressing a familial amyotrophic
- 10 lateral sclerosis mutant SOD1. *Neurobiol Dis* 2000;7:623-643
- 11 22. Broom WJ, Greenway M, Sadri-Vakili G, Russ C, Auwarter KE, Glajch KE, et al.:
- 12 50bp deletion in the promoter for superoxide dismutase 1 (SOD1) reduces SOD1
- 13 expression in vitro and may correlate with increased age of onset of sporadic
- 14 amyotrophic lateral sclerosis. *Amyotroph Lateral Scler* 2008;9:229-237
- 15 23. Zhong Z, Ilieva H, Hallagan L, Bell R, Singh I, Paquette N, et al.: Activated protein
- 16 C therapy slows ALS-like disease in mice by transcriptionally inhibiting SOD1 in
- 17 motor neurons and microglia cells. *J Clin Invest* 2009;119:3437-3449

FIGURE LEGENDS

Figure 1. The stable gPr^{SOD1}-Luc cell line used for the high-throughput screening assay of compounds that downregulate SOD1 transcription. A) Diagram of the superoxide dismutase gene (SOD1) promoter-luciferase reporter plasmid, which encodes a secreted luciferase incorporated into the human SOD1 gene, including the 5'- and 3'- untranslated region and introns. B) Southern blotting analysis of the gPr^{SOD1}-Luc cell line. Clone #1 was the stable clone used in HTS assays. N = negative control, non-transfected H4 cells. P = positive control, H4 cells transiently transfected with the same cassette as clone #1. Copy number indicates the number of the transgene loaded. C) Results of the luciferase reporter assay. Clone #1 has a relatively high number of copies of the transgene and, therefore, high luciferase activity. Values are mean ± SEM.

Figure 2. A) Representatives of dose-dependent effects of the excluded compounds on luciferase activity in gPr^{SOD1}-Luc cells. B) Representatives of dose-dependent effects of compounds excluded by ELISA on luciferase activity in gPr^{SOD1}-Luc cells. C) Representatives of ELISA results of compounds excluded by ELISA on SOD1 abundance in H4 cells. Each point is the mean of duplicate measurements.

Figure 3. A) Dose-dependent effects of the hit compounds on luciferase activity in gPr^{SOD1}-Luc cells. Each point is the mean of duplicate measurements. B) ELISA results showing effects of different concentrations of the two hit compounds on SOD1 expression. Values are means $\pm$ SEM (n=5). C) Hit compounds identified through SOD1-ELISA. EC₅₀=50% effective concentration. TC₅₀=50% toxic concentration.

Figure 4. A) Representative western blot showing the effect of different concentrations of the selected hit compound, 052C9, on expression of SOD1 and β -actin in H4 cells. B) Band density of SOD1 relative to β -actin. Values are means $\pm$ SEM (n=5). Difference between relative abundance at 0 and 40 μ M was significant at $P<0.05$ (one-way ANOVA followed by the Bonferroni post hoc test).

Figure 5. The chemical structure of the selected hit compound.

¹H NMR (DMSO-d₆, 300 MHz) δ -H 9.38 (d, J=1.1 Hz, 1H), 8.18 (d, J=2.7 Hz, 1H), 7.99 (dd, J=9.1, 2.7 Hz, 1H), 7.91 (dd, J=9.1, 1.1 Hz, 1H), 7.76 (dd, J=6.0, 3.0 Hz, 2H), 7.36 (dd, J=6.0, 3.0 Hz, 2H)

¹³C NMR (DMSO-d₆, 75 MHz) δ -C 172.9 (s), 160.0 (d), 154.1 (s), 143.5 (s), 135.2 (s), 134.4 (s $\times$ 2), 131.3 (s), 124.4 (d), 124.3 (d $\times$ 2), 124.3 (d), 121.4 (d), 114.8 (d), 111.7

1 (s)

2 MS (ESI) mass calcd for $C_{16}H_9ClN_2O_2 + H$ requires m/z 297 Found m/z 297

3

4 **Figure 6.** A) Representative western blot showing the effect of different concentrations

5 of the selected hit compound, 052C9, on phosphorylation of Nrf2 and CREB in H4 cells.

6 B) Band density of pNrf2 relative to Nrf2. Values are means $\pm$ SEM (n=7). Differences

7 between relative abundance both at 0 and 20 μ M and at 0 and 40 μ M were significant at

8 $P < 0.05$ (one-way ANOVA followed by the Bonferroni post hoc test). C) Band density of

9 pCREB relative to CREB. Values are means $\pm$ SEM (n=7). Differences between relative

10 abundance both at 0-20 μ M and at 0-40 μ M were not significant (one-way ANOVA

11 followed by the Bonferroni post hoc test).

1 **Table 1.** Z' of all the runs

Run No.	Z'	Run No.	Z'
1	0.69	14	0.38
2	0.39	15	0.32
3	0.35	16	0.40
4	0.55	17	0.75
5	0.60	18	0.52
6	0.38	19	0.33
7	0.42	20	0.12
8	0.38	21	0.55
9	0.37		
10	-0.04		
11	0.47		
12	0.46		
13	0.51		

2

3

4

5

6

1 **Table 2.** Hit compounds identified using the HTS assay.

Screened compounds	9,600	
Total	325	(3.39%)
Duplicate	141	(1.47%)
Dose dependency	120	(1.25%)
Toxic hits	5	
Analysis continuing	115	(1.20%)

2 Hits with dose-dependency were defined as compounds that reduced luciferase activity in a
3 dose-dependent manner, with a significant effect at least at 40 μ M. Toxic hit compounds also decreased
4 reporter activity in the WST-1 assay.

5

6

7 **Supplemental Figure 1.** Luciferase activity of 9,600 compounds in the screened
8 library, determined by HTS assay, relative to activity of vehicle-treated cells. The
9 coefficient of variation for all compounds tested was 15.0.

Fig. 1

Fig. 2

A

B

C

Hit Compounds through SOD1-ELISA

Compound	EC ₅₀ (μM)	TC ₅₀ (μM)
052C9	7.3	>40
059E11	19.6	>40

Fig. 3

Fig. 4

NMR H-1 Estimation

Fig. 5

Fig. 6