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Author(s)	Murakami, Gaku; Inoue, Haruhisa; Tsukita, Kayoko; Asai, Yasuyuki; Amagai, Yuji; Aiba, Kazuhiro; Shimogawa, Hiroki; Uesugi, Motonari; Nakatsuji, Norio; Takahashi, Ryosuke
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1 **Chemical library screening identifies a small molecule that downregulates SOD1**
2 **transcription for drugs to treat ALS**

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4 Gaku Murakami M.D.,¹ +81-75-751-3767, mrkmgk@kuhp.kyoto-u.ac.jp

5 Haruhisa Inoue M.D., Ph.D.,^{2,3} +81-75-366-7036, haruhisa@cira.kyoto-u.ac.jp

6 Kayoko Tsukita,^{2,3} +81-75-366-7087, tsukita@cira.kyoto-u.ac.jp

7 Yasuyuki Asai Ph.D.,⁴ +81-45-475-3887, y-asai@reprocell.com

8 Yuji Amagai Ph.D.,⁵ +81-75-313-9535, amagai@scdi.or.jp

9 Kazuhiro Aiba Ph.D.,⁵ +81-75-313-9535, aiba@scdi.or.jp

10 Hiroki Shimogawa Ph.D.,⁶ +81-774-38-3225, shimogaw@scl.kyoto-u.ac.jp

11 Motonari Uesugi Ph.D.,⁶ +81-774-38-3225, uesugi@scl.kyoto-u.ac.jp

12 Norio Nakatsuji Ph.D.,⁷ +81-75-753-9740, nnakatsu@icems.kyoto-u.ac.jp

13 Ryosuke Takahashi M.D., Ph.D.^{1,3} +81-75-751-3767, ryosuket@kuhp.kyoto-u.ac.jp

14
15 ¹ Department of Neurology, Graduate School of Medicine, Kyoto University

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

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

19 ⁴ ReproCELL, Inc.

20 ⁵ Stem Cell and Drug Discovery Institute

21 ⁶ Institute for Chemical Research, Kyoto University

22 ⁷ Institute for Integrated Cell-Material Sciences, Kyoto University

23
24 Correspondence should be addressed to

25 H. I. (haruhisa@cira.kyoto-u.ac.jp); phone number: +81-75-366-7036, fax number:
26 +81-75-366-7023, at Center for iPS Cell Research and Application (CiRA), Kyoto
27 University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan,

28 or R. T. (ryosuket@kuhp.kyoto-u.ac.jp); phone number: +81-75-751-3770, fax number:
29 +81-75-761-9780, at Department of Neurology, Graduate School of Medicine, Kyoto
30 University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan

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1 **Abstract**

2 Familial amyotrophic lateral sclerosis (fALS) accounts for 10% of ALS cases, and about
3 25% of fALS cases are due to mutations in superoxide dismutase 1 (SOD1). Mutant
4 SOD1-mediated ALS is caused by a gain of toxic function of the mutant protein, and the
5 SOD1 level in non-neuronal neighbors, including astrocytes, determines the progression
6 of ALS (non-cell-autonomous toxicity). Therefore, we hypothesized that small
7 molecules that reduce SOD1 protein levels in astrocytes might slow the progression of
8 mutant SOD1-mediated ALS. We developed and optimized a cell-based,
9 high-throughput assay to identify low molecular weight compounds that decrease SOD1
10 expression transcriptionally in human astrocyte-derived cells. Screening of a chemical
11 library of 9,600 compounds with the assay identified two hit compounds that selectively
12 and partially downregulate SOD1 expression in a dose-dependent manner, without any
13 detectable cellular toxicity. Western blot analysis showed that one hit compound
14 significantly decreased the level of endogenous SOD1 protein in H4 cells, with no
15 reduction in expression of β -actin. The assay developed here provides a powerful
16 strategy for discovering novel lead molecules for treating familial SOD1-mediated ALS.

17

18 **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}

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

6

7 **Materials and methods**

8 *Generation of a SOD1 promoter-luciferase reporter cell line*

9 We used the SOD1 genomic promoter, including 5' and 3' untranslated regions
10 (UTR), in our construct to generate SOD1 transgenic mice. The cassette was identical to
11 that carried by SOD1^{G93A} transgenic mice (gPr^{SOD1}-Luc), to reflect physiological activity
12 of the SOD1 promoter (Fig.1A). A total of 1.2 Kb of human SOD1 (hSOD1)
13 5'-fragment, with 5'-EcoR1 and 3'-Afe1-BamH1 sites, was amplified using PfuUltra™
14 2 Fusion HS DNA Polymerase (Stratagene, Cedar Creek, TX, USA). The following
15 PCR primers were used to amplify the region: forward primer,
16 5'-AAAGAATTCTGCCAACCAAATAAG-3'; reverse primer,
17 5'-TTTGGATCCAGCGCTGAAGCCGGAAAGCGGAG-3'. The fragment was cloned
18 into pKF18k-2 plasmid (Takara, Otsu, Japan). To add Cla1 site and delete the start

1 codon of SOD1 exon1, the cassette was amplified by PfuUltra™2 Fusion HS DNA
2 Polymerase using the following PCR primers: forward primer,
3 5'-GTTATCGATGCGACGAAGGCCGTGT-3'; reverse primer,
4 5'-TCGCTAGGCCACGCCGAGG-3'. The fragment was cut with EcoR1 and Afe1, and
5 cloned into pKF18k-2-hSOD1^{G93A}. The SV40-Neo-Poly(A) was incorporated
6 downstream from the SOD1 gene, between the BamH1 and Sal1 sites. Finally, secreted
7 luciferase gene (MetLuc) from the marine copepod, *Metridia longa* (Clontech,
8 Mountain View, CA, USA), with ATG was added at the Cla1 site.

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

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

3

4 *The compound library*

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

11

12 *High-throughput screening (HTS) assay*

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

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

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

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

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

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

8

9 *Dose-response and cytotoxicity*

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

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

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

10

11 *Secondary assay*

12 Enzyme-linked immunosorbent assays (ELISAs) and western blots were used to
13 determine whether effects observed in the reporter cell line could be reproduced at the
14 level of endogenous SOD1 protein. As in the primary assays, untransfected H4 cells
15 were pre-cultured overnight, and the media were exchanged with hit compounds to give
16 final concentrations of 0-40 μM . The cells were cultured for 48 h, then each well was
17 washed once with 200 μL of PBS, and lysed with 100 μL of 1% Triton-X containing
18 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

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

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

8

9 *Western blot analysis for phosphorylation of transcription factors*

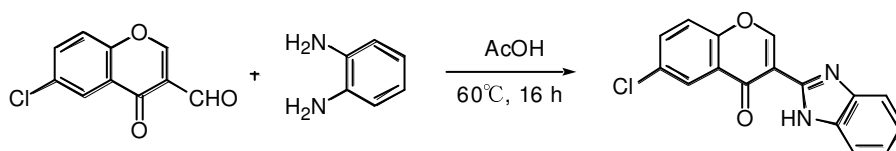
10 Untransfected H4 cells were pre-cultured overnight on 12-well plates at 5.0×10⁴
11 cells. The hit compound was diluted with culture medium to 25μM and 50 μM, and
12 used to replace 0.8 mL of 1 mL per well of cell culture to give a final concentration of
13 20μM and 40 μM, respectively. The cells were then cultured for another 16 h, and then
14 each well was washed once with 2 mL of PBS, and lysed with 100 μL of 1% Triton-X
15 containing protease inhibitors (Roche) and phosphatase inhibitor cocktail (Nacalai
16 Tesque, Kyoto, Japan). Western blotting was performed with antibody specific to
17 Ser⁴⁰-phosphorylated Nrf2 (2500:1, Abcam, #EP1809Y, Cambridge, MA), Nrf2 (500:1,
18 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

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

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

1 4). The structure of this hit compound (Fig. 5) was confirmed by re-synthesis and
2 spectroscopic characterization: the molecule is composed of a benzimidazole ring and
3 a chromen unit, and is not analogous to any of the drugs used in ALS treatment trials to
4 date. Two major transcription factors have been reported to activate the expression of
5 SOD1: NF-E2 (Nrf2) and cAMP response element binding protein (CREB). We
6 examined the effects of 052C9 on the phosphorylation status of these two transcription
7 factors by western blot analysis. The results showed that 052C9 blocked the
8 phosphorylation of NF-E2 (Nrf2) with no reduction of total Nrf2 protein level, whereas
9 052C9 had no detectable effects on the phosphorylation status of CREB (Fig. 6).

10

11 **Discussion**

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

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

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

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

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

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

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

12

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18

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1 **FIGURE LEGENDS**

2

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

13

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

19

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

6
7 **Figure 4.** A) Representative western blot showing the effect of different concentrations
8 of the selected hit compound, 052C9, on expression of SOD1 and β -actin in H4 cells. B)
9 Band density of SOD1 relative to β -actin. Values are means \pm SEM (n=5). Difference
10 between relative abundance at 0 and 40 μ M was significant at $P<0.05$ (one-way
11 ANOVA followed by the Bonferroni post hoc test).

12
13 **Figure 5.** The chemical structure of the selected hit compound.

14 ¹H NMR (DMSO-d₆, 300 MHz) δ_{-H} 9.38 (d, J=1.1 Hz, 1H), 8.18 (d, J=2.7 Hz, 1H),
15 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),
16 7.36 (dd, J=6.0, 3.0 Hz, 2H)

17 ¹³C NMR (DMSO-d₆, 75 MHz) δ_{-C} 172.9 (s), 160.0 (d), 154.1 (s), 143.5 (s), 135.2
18 (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.

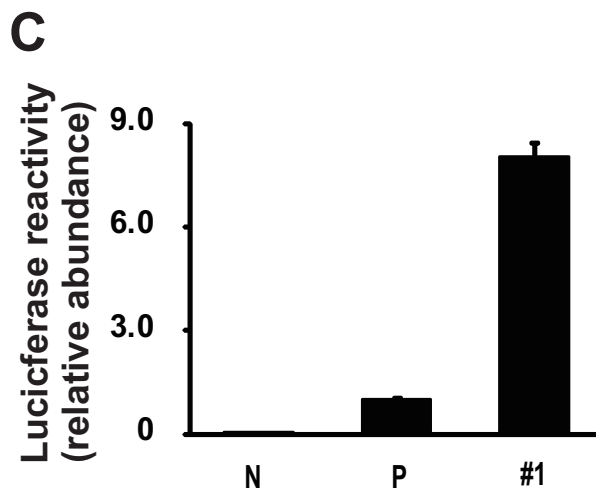
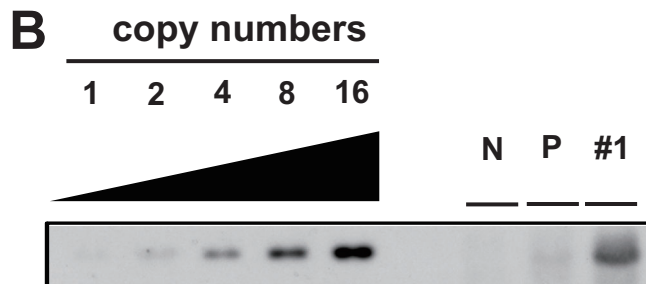
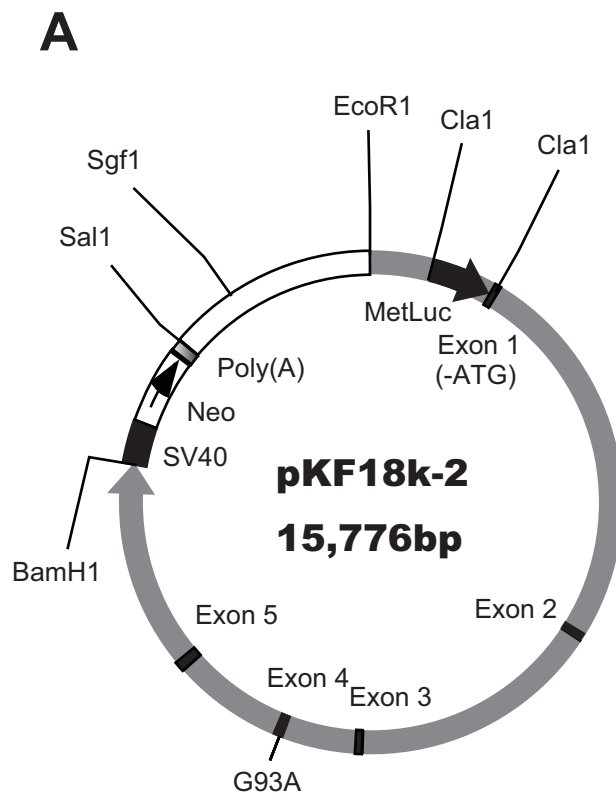
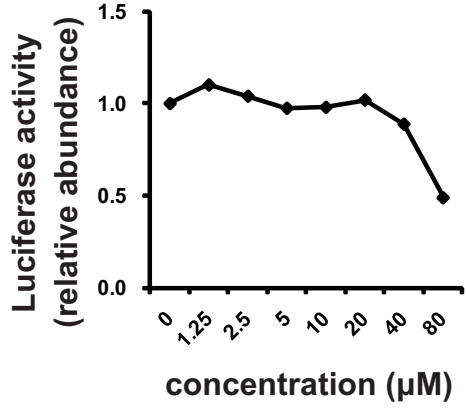
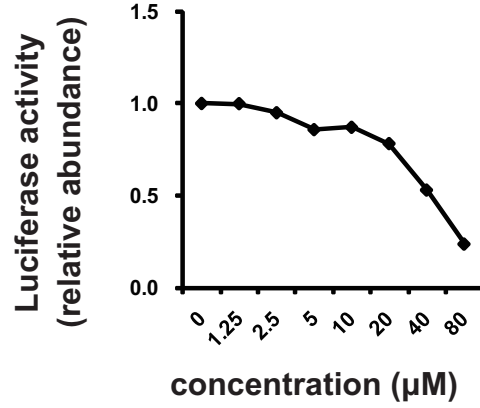
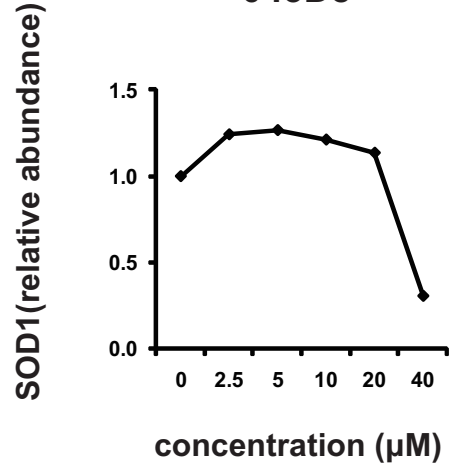
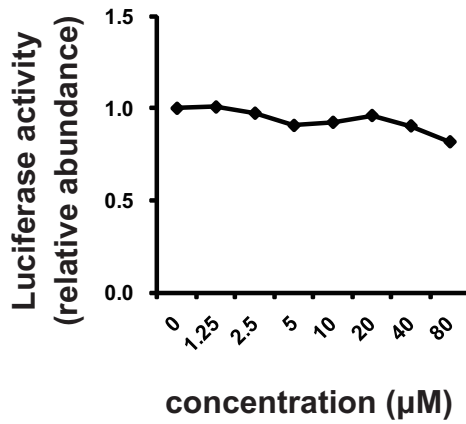
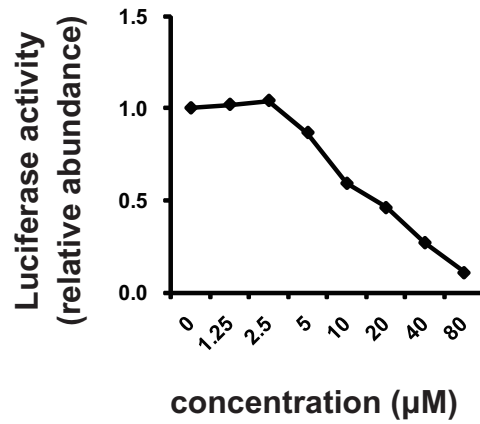
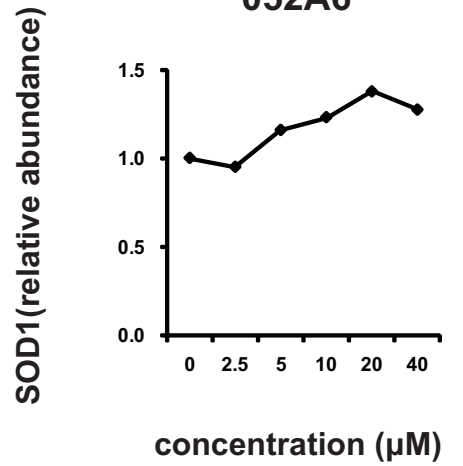
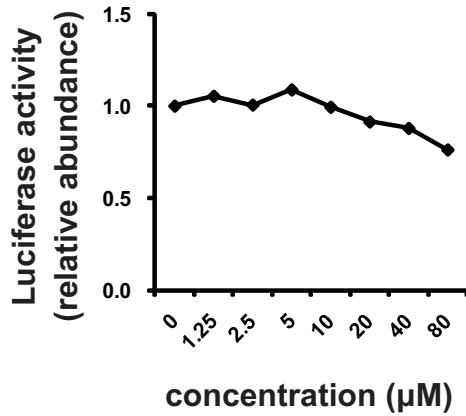
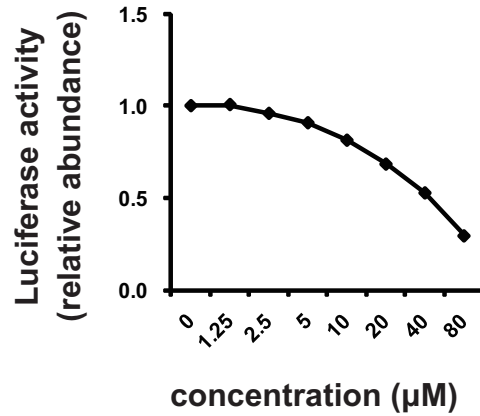
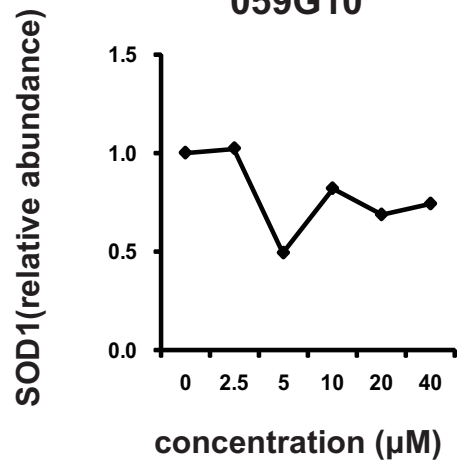
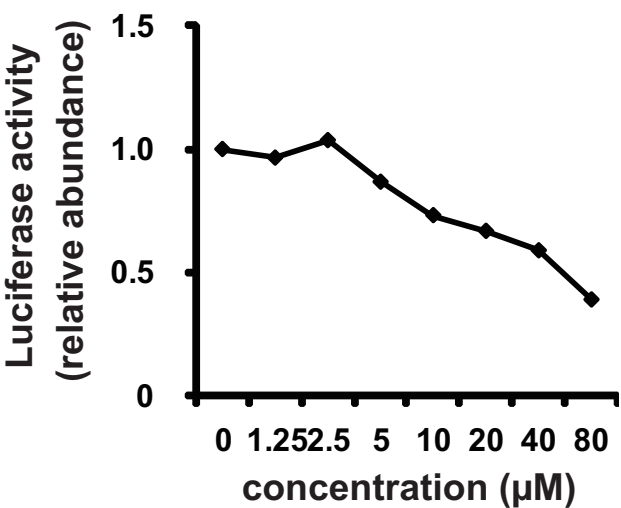
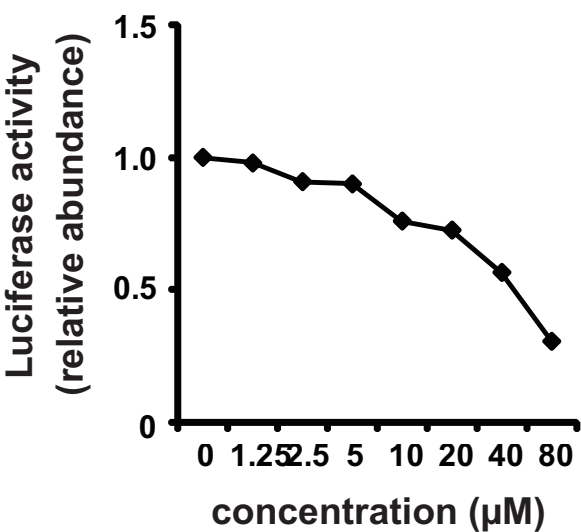
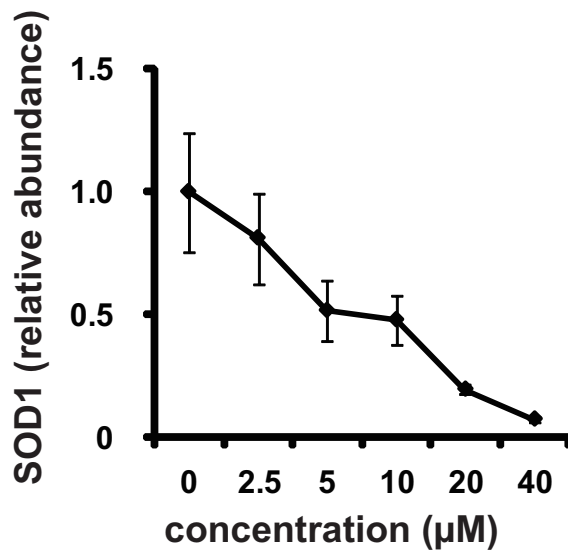
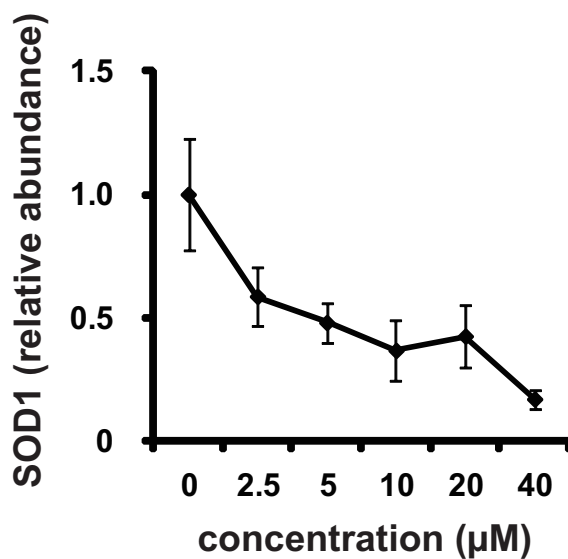


Fig. 1

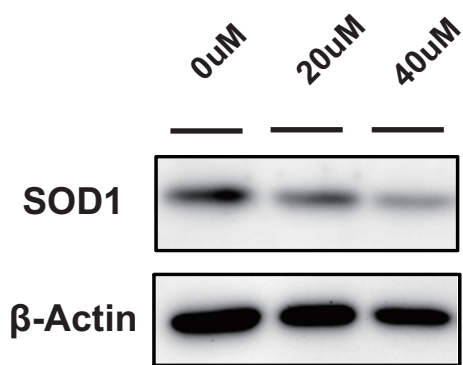
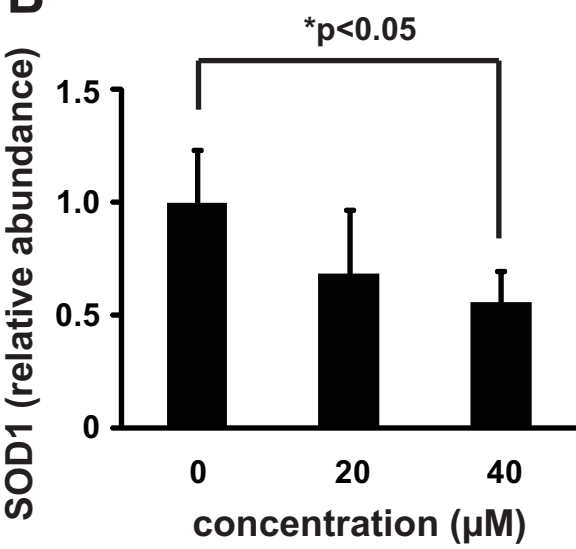
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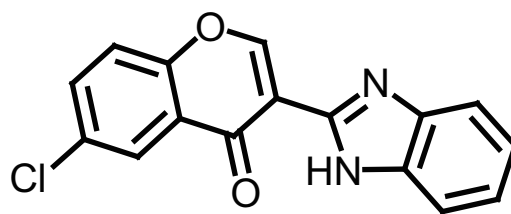
A**052C9****059E11****B****052C9****059E11****C**

Hit Compounds through SOD1-ELISA

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

Fig. 3

A**B****Fig. 4**



NMR H-1 Estimation

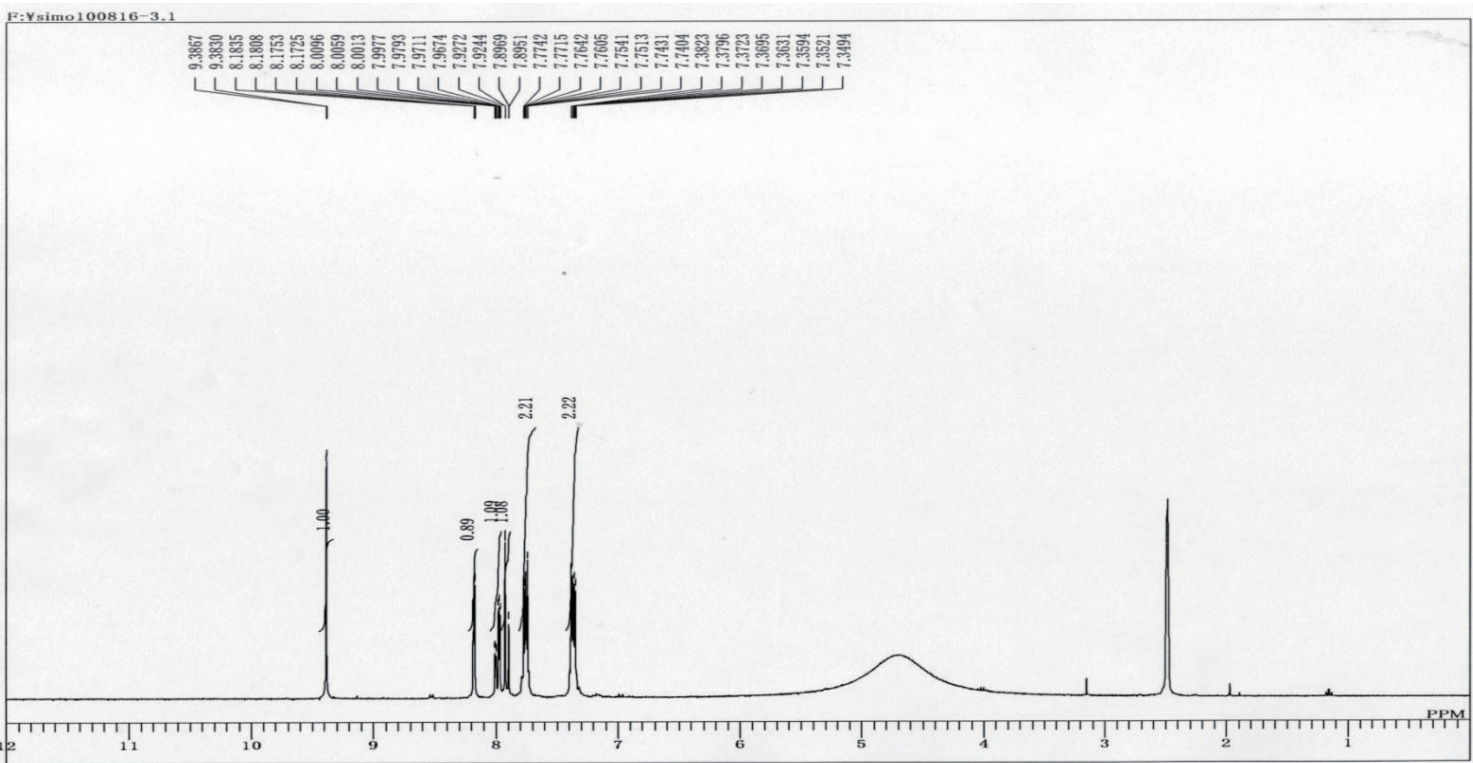
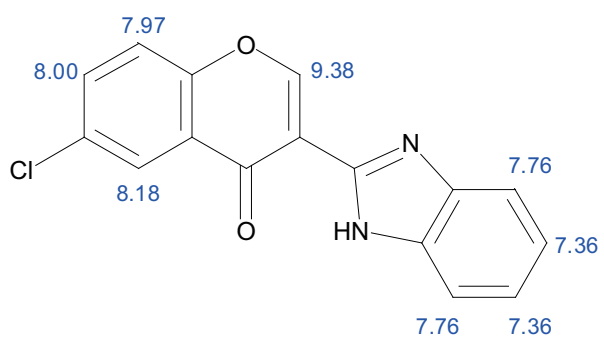


Fig. 5

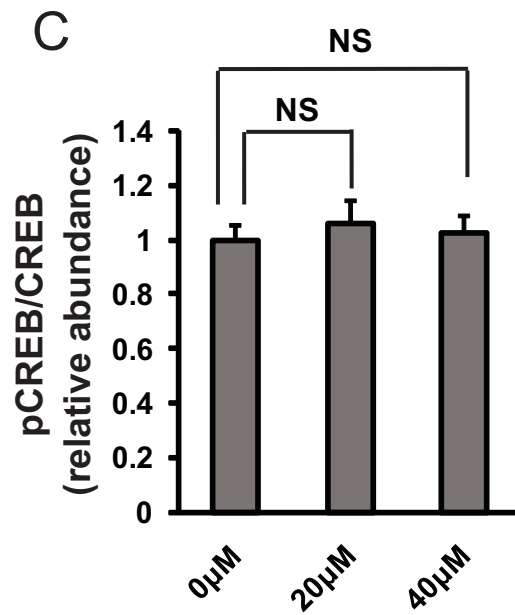
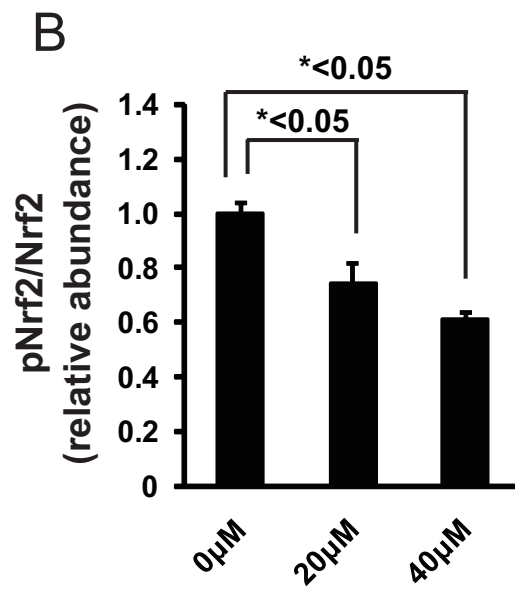
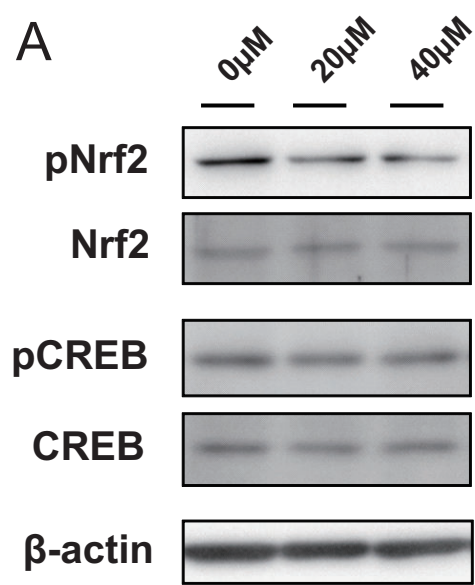


Fig. 6