A novel compound activating TrkB in mouse brain attenuates depression-like behavior

（マウスの脳内においてTrkBを活性化する新規化合物は、うつ様行動を改善する）

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CHAPTER 1
ABSTRACT

Brain-derived neurotrophic factor (BDNF) and its high affinity receptor tyrosine kinase receptor B (TrkB) are involved in maintaining neuronal survival, differentiation, and synaptic plasticity. In our previous study, *in silico* screening to identify compounds targeting the BDNF binding domain of TrkB led to a hypothesis that the candidate compounds activate TrkB signaling. To test this hypothesis in the present study, we first examined the selected compounds for their proliferative effects in TrkB-expressing SH-SY5Y cells. The two positive compounds, 48 and 56, were then assessed for their ability to induce TrkB phosphorylation *in vitro* and *in vivo*. The elevated phosphorylation of TrkB by the compounds was blocked by the Trk inhibitor, K252a. Because dysfunction of BDNF-TrkB signaling is associated with the pathogenesis of depression, we conducted a forced swim test to confirm the efficacy of the compounds in a mouse model of depression. We found that compound 48 significantly reduced mouse immobility time compared with that following a vehicle injection, suggesting an antidepressant-like effect of the compound *in vivo*. Thus, our present study demonstrated that compound 48, selected through *in silico* screening, is a novel activator of TrkB signaling and a potential antidepressant agent.

CHAPTER 2
INTRODUCTION

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, which contains nerve growth factor, neurotrophin-3, and neurotrophin-4, is a specific ligand for tropomyosin-related kinase receptor B (TrkB). The binding of BDNF to TrkB triggers the dimerization of the receptor via conformational changes and the autophosphorylation of tyrosine residues in the intracellular domain of the receptor, leading to activation of the downstream signaling pathways involving extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase, and phospholipase C-gamma (Numakawa *et al*, 2013). BDNF-TrkB signaling contributes to neuronal survival, differentiation, and synaptic plasticity in the central nervous system. Accumulating evidence suggests that dysregulation of the BDNF-TrkB signaling pathway is associated with several neurodegenerative diseases, including Huntington disease (Ferrer *et al*, 2000), Alzheimer disease (Durany *et al*, 2000), amyotrophic lateral sclerosis (Ekestern, 2004), and depression (Lindholm and Castrén, 2014; Hashimoto *et al*, 2004; Nestler *et al*, 2002). Among these disorders, a decreased level of BDNF expression is reportedly associated with the pathogenesis of depression (Karege *et al*, 2005; Shimizu *et al*, 2003). BDNF and TrkB are considered attractive therapeutic targets because of their important role in the efficacy of antidepressants (Shirayama *et al*, 2002). However, obtaining pharmacological agents that activate BDNF-TrkB signaling in brain will require developing new compounds that can penetrate the blood-brain barrier. We previously conducted *in silico* screening to find low molecular weight compounds interacting with the BDNF binding domain of TrkB (Nakamura *et al*, 2014). Among the candidate

chemicals, we identified two compounds that function as TrkB antagonists. In the present study, we hypothesize that other compounds selected by the screening contained potential TrkB activators or agonists. To test this hypothesis, we assessed these other compounds for their ability to induce activation of TrkB signaling *in vitro* and *in vivo.* TrkB activation has been reported in rat and mouse brain tissues after antidepressant treatment (Nibuya *et al*, 1995; Saarelainen *et al*, 2003). Therefore, we also conducted the forced swim test (FST) using mice to assess the pharmacological effects of the compounds against depression-like behavior.

CHAPTER 3
MATERIALS AND METHODS

3-1. Reagents, cells, and mice

Small molecules identified during *in silico* screening were purchased from Namiki Shoji Co. Ltd. (Tokyo, Japan). Human neuroblastoma-derived SH-SY5Y/TrkB cells were a gift from Dr. Angelika Eggert (University Children's Hospital Essen, Essen, Germany) and grown in RPMI 1640 medium (Sigma, Tokyo, Japan). A mouse neuroblastoma cell line, Neuro2a, was maintained in Dulbecco's modified Eagle's medium (Sigma)

3-2. Cell growth/survival assays

SH-SY5Y/TrkB and Neuro2a cells were seeded with media containing 2% and 1% FBS, respectively. After overnight culture, the cells were treated with DMSO, the selected compounds (0.06–2.0 μM or 0.1–3.0 μM), or BDNF (1–50 ng/mL, Wako, Osaka, Japan), as indicated. The plate was incubated and monitored for 9 days in an IncuCyte live-cell imaging system (Essen Bioscience, Michigan, USA) according to the manufacturer's instructions. SH-SY5Y/TrkB cells were pre-treated with the Trk receptor inhibitor K252a (Sigma) (200 nM) for 30 min after pre-culture in serum-free medium for 2.5 h. Cells were then treated with DMSO, BDNF (1 ng/mL), or compounds 48 or 56 (0.3 μM) for 1 h.

For the TrkB inhibition assay, SH-SY5Y/TrkB cells were seeded with medium containing 2% FBS and allowed to attach overnight. Cells were then treated with BDNF (5 ng/mL) or compound (1 μM) in the presence of K252a (100 nM). Detailed methods are provided in supplemental information.

3-3. Immunoblotting

Cells were pre-cultured in serum-free medium for 2.5 h and then treated with DMSO, compounds, or BDNF for the indicated times. The total cell lysates were subjected to immunoblotting analyses using the following primary antibodies: anti-TrkB antibody (1:1000 dilution), anti-TrkB antibody (1:1000), anti-AKT antibody (1:1000), anti-ERK antibody (1:1 000), anti-β tubulin (1:10 000), anti-phospho-TrkB (1:1 000), anti-phospho-AKT (1:1000), anti-phospho-ERK (1:1000).

3-4. Treatment of mice with compounds

Eight-week-old male C57BL/6J mice (CREA Japan Inc., Tokyo, Japan) were maintained in a temperature-controlled environment for 1 week prior to the start of experiments. All animal experiments were performed in compliance with the regulations for animal experiments at the Chiba Cancer Center Research Institute after obtaining approval by the Animal Ethics Committee of Chiba Cancer Center. The mice were intraperitoneally administered DMSO or the compounds (3 or 15 mg/kg body weight). After 3 h, the mice were killed, and cerebral cortical and hippocampal samples were collected. Tissue lysates were analyzed by immunoblotting with antibodies against phospho- (p)TrkB, TrkB, pAKT, AKT, pERK and ERK. The remaining half of the tissue was immediately fixed in 4% paraformaldehyde and embedded in OCT compound (Sakura, Tokyo, Japan). Frozen sections were cut using a cryostat, and the sections were subjected to immunohistochemistry assays using the anti-pTrkB antibody visualized with Alexa 488-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA). Images were captured using a confocal microscope (Leica), and

the signal intensities were quantified in nine randomly selected fields on three slides using WinROOF software (version 7.0, Mitani Corp., Fukui, Japan).

3-5. Forced swim test

Male C57BL/6J mice were intraperitoneal injected with DMSO or compounds (3 mg/kg) for 21 days and then subjected to a forced swim test. They were then placed in a clear glass cylinder with a diameter of 20 cm and height of 30 cm filled with clear water of approximately 25°C. Their mobility was recorded for 10 min, and the time spent immobile was analyzed for the last 4 min of the test after a 3 min pre-swim as well as for the entire 10 min without a pre-swim.

3-6. Statistical analysis

One-way analysis of variance followed by Dunnett's test was used for multiple group comparisons in the cell proliferation assay. Student's *t* tests were performed for analysis of the differences between two groups in the immunohistochemistry and FST assays. *P* values less than 0.05 were considered statistically significant. All values are expressed as the means ± S.E.

CHAPTER 4
RESULTS

4-1. Identification of chemical compounds promoting cell growth by activating the TrkB pathway

Our previous study identified two compounds (out of three million) that induce cell death in neuroblastoma cells and are novel candidate antagonists of TrkB (Nakamura *et al*, 2014). In that study, we also conducted *in silico* screening to identify compounds with the potential of interacting with the BDNF binding domain of TrkB. We hypothesized that molecules positive for TrkB interactions may act as TrkB agonists. Therefore, in the present study, we first investigated whether 11 compounds selected by the *in silico* screening assay in that previous study (Nakamura *et al*, 2014) enhanced cell proliferation in TrkB-expressing cells. Human neuroblastoma SH-SY5Y cells stably expressing TrkB were treated with 10 μM of the compounds and subjected to a cell proliferation assay. The number of cells was counted 5 days after the treatment (Supplementary Figure S1). Based on the results, we selected the commercially available compounds 48 and 56 for further evaluation (Supplementary Figure S2). To confirm their ability to promote cell growth in TrkB-expressing cells, we performed an *in vitro* cell growth assay in SH-SY5Y/TrkB cells cultured with a medium containing a low percentage of FBS (2%). As expected, the treatment with BDNF markedly enhanced cell proliferation compared with the DMSO treatment, in which no viable cells were detected 9 days after the treatment (Figure 1a). Compound 48 at concentrations above 0.2 μM and compound 56 at 0.6 and 2 μM significantly enhanced cell proliferation (Figure 1a). We also conducted *in vitro* cell growth assays using

mouse neuroblastoma Neuro2a cells, which endogenously express TrkB. The treatment of BDNF at concentrations above 10 ng/mL tended to enhance the growth of Neuro2a cells comparable to that in SH-SY5Y/TrkB cells, although no statistically significant difference was found between DMSO and 50 ng/mL of BDNF. Treatment with compound 48 also showed a tendency to restore cell growth to a level similar to that produced by BDNF (Figure 1b). However, the proliferative effect of compound 56 was marginal in Neuro2a cells.

We next performed western blot analysis to investigate the activation of TrkB and its downstream signaling cascades after treatment with the compounds. In SH-SY5Y/TrkB cells, TrkB and its downstream molecules AKT and ERK were phosphorylated by BDNF treatment (Figure 1c and d). In compound-treated cells at a concentration of 0.3 μM, TrkB phosphorylation was gradually increased and peaked approximately 30 min after treatment, whereas phosphorylation of AKT and ERK was minimal compared with that following BDNF treatment (Figure 1c). In concentration-response experiments, the compounds induced TrkB phosphorylation 10 min after treatment, with the highest phosphorylation of TrkB at 0.1 μM of the compounds (Figure 1d). ERK phosphorylation was also elevated by treatment with the compounds in a concentration-dependent manner (Figure 1d). We also performed western blot analysis to examine the phosphorylation of TrkB by compound 48 in Neuro2a cells. Compared with that following DMSO treatment, TrkB and AKT phosphorylation were slightly increased after compound 48 treatment in a time-dependent manner, whereas ERK phosphorylation was markedly increased at 30 min, with the increase lasting until 60 min after treatment. (Figure 1e). These results suggest that compounds 48 and 56

enhance phosphorylation of TrkB and its downstream molecules in TrkB-expressing cells.

4-2. Proliferative effects of compounds 48 and 56 require the activation of TrkB

The Trk tyrosine kinase inhibitor K252a was used to confirm the involvement of TrkB activity in the compound 48- and 56-dependent cell proliferation. SH-SY5Y/TrkB cells pretreated with K252a were exposed to 0.3 μM of compound 48 or 56 and subjected to western blot analysis for TrkB activation. As shown in Figure 2a, phosphorylation of TrkB induced by BDNF treatment was completely blocked by K252a treatment. Similar reductions in TrkB phosphorylation were observed in compound 48- and 56-treated cells, suggesting that these compounds induce TrkB phosphorylation in a manner similar to that induced by BDNF. Indeed, the cell growth restored by treating cells maintained in a medium having a low percentage of FBS with the compounds was completely inhibited by the same co-treatment with K252a that significantly suppressed the BDNF-induced cell growth (Figure 2b and c). However, treatment with compounds 48 and 56 failed to rescue the cell growth of SH-SY5Y/TrkA cells (Supplementary Figure S3), suggesting that compounds 48 and 56 function to upregulate cell proliferation in a TrkB-dependent manner.

4-3. Compounds 48 and 56 induce TrkB phosphorylation *in vivo*

To investigate whether compounds 48 and 56 activate endogenous TrkB *in vivo*, we injected C57BL/6J mice intraperitoneally with 3 mg/kg body weight of the compounds. Three hours after the injection, the whole brain was collected and tissue lysates were prepared. Western blot analysis showed that TrkB phosphorylation was slightly elevated

by treatment with compounds 48 and 56 (Supplementary Figure S4). Because the phosphorylation of AKT and ERK showed a tendency to increase, we injected the mice with compound 48 at a higher dose (15 mg/kg body weight). No phosphorylation of TrkB, AKT, and ERK was detected in the tissue lysates collected from the cerebral cortex and hippocampus of mice injected with DMSO or negative control compound 37 (Figure 3a). By contrast, the compound 48-treated cerebral cortex and hippocampus showed high levels of phosphorylation for TrkB, AKT, and ERK. Immunohistochemistry assays using the antibody against phosphorylated TrkB also revealed increased elevated phosphorylation of TrkB in the hippocampus of compound 48-treated mice (Figure 3b and c) compared with that in negative controls. These results indicate that the intraperitoneally administered compound 48 crosses the blood-brain barrier and stimulates the TrkB signaling pathway *in vivo*.

4-4. Compound 48 produces antidepressant-like effects in mice

Because previous reports have suggested that the impaired function of the BDNF-TrkB signaling pathway is related to depressive behavior (Groves, 2007; Nibuya *et al*, 1995), we next conducted a forced swim test (FST), which is generally used to screen potential antidepressants. Male C57BL/6J mice were intraperitoneally administered compound 48 at a dose of 3 mg/kg body weight per day for 21 days. Three hours after the last administration, the FST was conducted and the time spent immobile was recorded. The immobility time during the last 4 min of the test after a 3-min pre-swim was significantly decreased in the mice injected with compound 48 compared with those injected with DMSO (Figure 4a, $p = 0.02$). The immobility time for the total 10 min of

the FST without pre-swim was also significantly lower in mice injected with compound 48 compared with that in control (Figure 4b, $p = 0.016$).

Compound 48 treatment for 21 days did not affect body weight gain (Figure 4c). Furthermore, acute *in vivo* toxicity testing of compound 48 in ICR mice showed no adverse effects (data not shown) or body weight changes (Supplementary Figure S5). These data suggest that compound 48 has an antidepressant-like action with limited toxicity in mice.

CHAPTER 5
DISCUSSION

In the present study, we demonstrated that compound 48 was a novel inducer of phosphorylation of the neurotrophic receptor TrkB and that compound 48 exhibited antidepressant effects in a mouse model of depression.

The compound was selected through an *in silico* analysis that also identified two chemical agents that induce apoptosis in neuroblastoma cells (Nakamura *et al*, 2014). We report here on two compounds among the previously selected molecules that enhanced TrkB activity in a manner similar to the TrkB ligand BDNF. Compounds 48 and 56 rescued cell survival in TrkB-expressing SH-SY5Y cells cultured with low FBS and activated the TrkB downstream signal cascade, including the AKT and ERK signaling pathways.

BDNF and TrkB are widely expressed in various brain regions (Adachi, 2014; Autry and Monteggia, 2012). The BDNF-TrkB pathway is an attractive therapeutic target of depression (Li *et al*, 2008; Nestler and Carlezon 2006; Zhang *et al*, 2014; Cazorla *et al*, 2011). To date, several TrkB agonists have been identified, including 7,8-dihydroxyflavone (Jang *et al*, 2010b; Liu *et al*, 2010), deoxygedunin (Jang *et al*, 2010a) and amitriptyline (Jang *et al*, 2009). Our present results demonstrated that compounds 48 and 56 were unique among previously reported TrkB ligands because these compounds strongly elicited TrkB phosphorylation *in vivo* despite their moderate effect *in vitro*. One reason for this apparent disparity in efficacy may be the different treatment times used for the *in vitro* and *in vivo* experiments. In the *in vitro* assays, we observed the elevation of TrkB phosphorylation within 60 min. In the *in vivo* study, we

examined TrkB phosphorylation 3 h after compound administration. Thus, the compounds might have been given adequate time to induce strong phosphorylation *in vivo* but not *in vitro*.

We found that compounds 48 and 56 induced Tyr706 phosphorylation of TrkB, which is responsible for initiating TrkB autophosphorylation (Cunningham *et al*, 1997). Upregulation of downstream signals AKT and ERK and inhibition of TrkB phosphorylation by K252a are suggestive of the direct activation of TrkB by the compounds. Furthermore, because the compounds did not enhance cell proliferation in TrkA-expressing cells (Figure S3), the binding affinity of the compounds to TrkB might be stronger than that to TrkA, which has nerve growth factor as a natural ligand. However, it remains unclear whether the compounds specifically bind to the BDNF binding domain of TrkB. Further experiments will be required to elucidate the precise molecular mechanism of action for the compounds, including an analysis of surface plasmon resonance to determine their binding affinity to TrkB. Several studies have reported that drugs effective against depression require the expression and activation of brain TrkB to exert their antidepressant effect in mouse models (Hajszan *et al*, 2005; Monteggia *et al*, 2007). Interestingly, the intraperitoneal administration of compounds 48 and 56 elicited a relatively higher level of TrkB phosphorylation *in vivo* than *in vitro*. In addition, a higher level of phosphorylation was also detected in the downstream factors AKT and ERK in the brain tissues of compound-treated mice. These data indicated the blood-brain permeability of these compounds, although the pharmacokinetics of the compounds has not yet been determined. The strong induction of TrkB signaling molecules in mouse brain tissue also indicated potential metabolic activation of the compounds before they were distributed to TrkB-expressing cells.

Further investigation will be necessary to determine the pharmacokinetics of the compounds *in vitro* and *in vivo*. Overall, compound 48 is a promising small molecule that may be developed as a novel drug against depression as well as for other BDNF-TrkB-associated neurodegenerative diseases, including Alzheimer and Parkinson diseases.

CHAPTER 6
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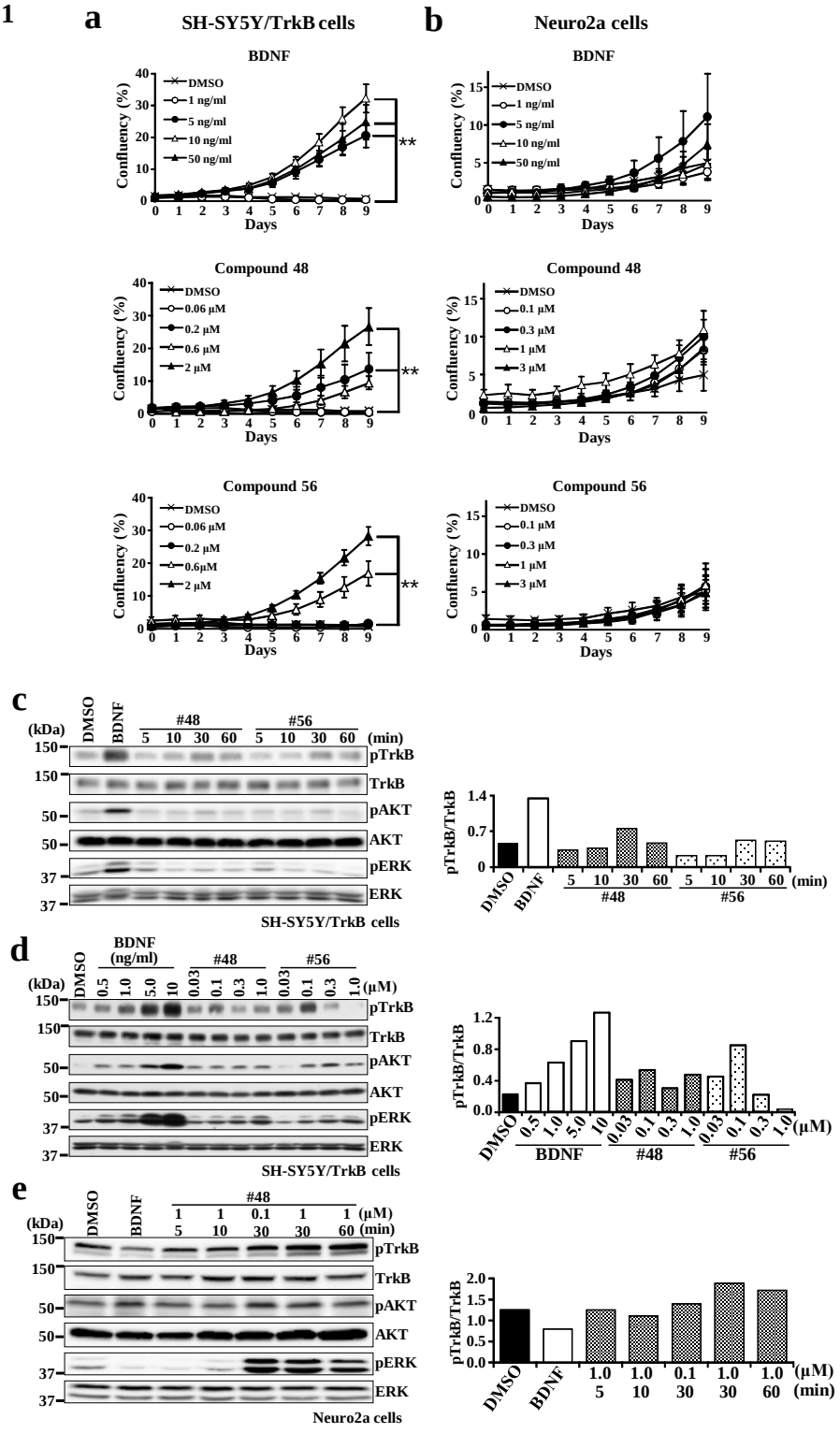
FIGURES

Figure 1



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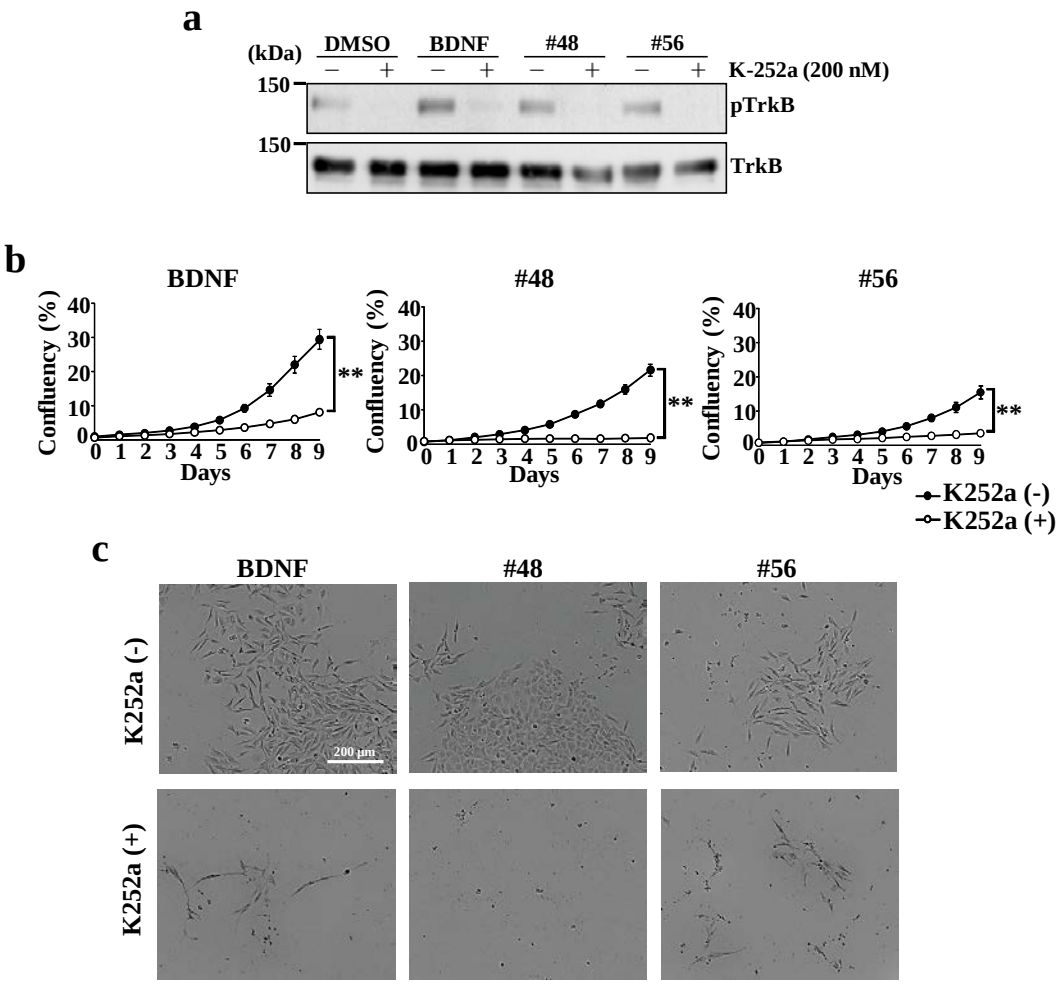
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Figure 3

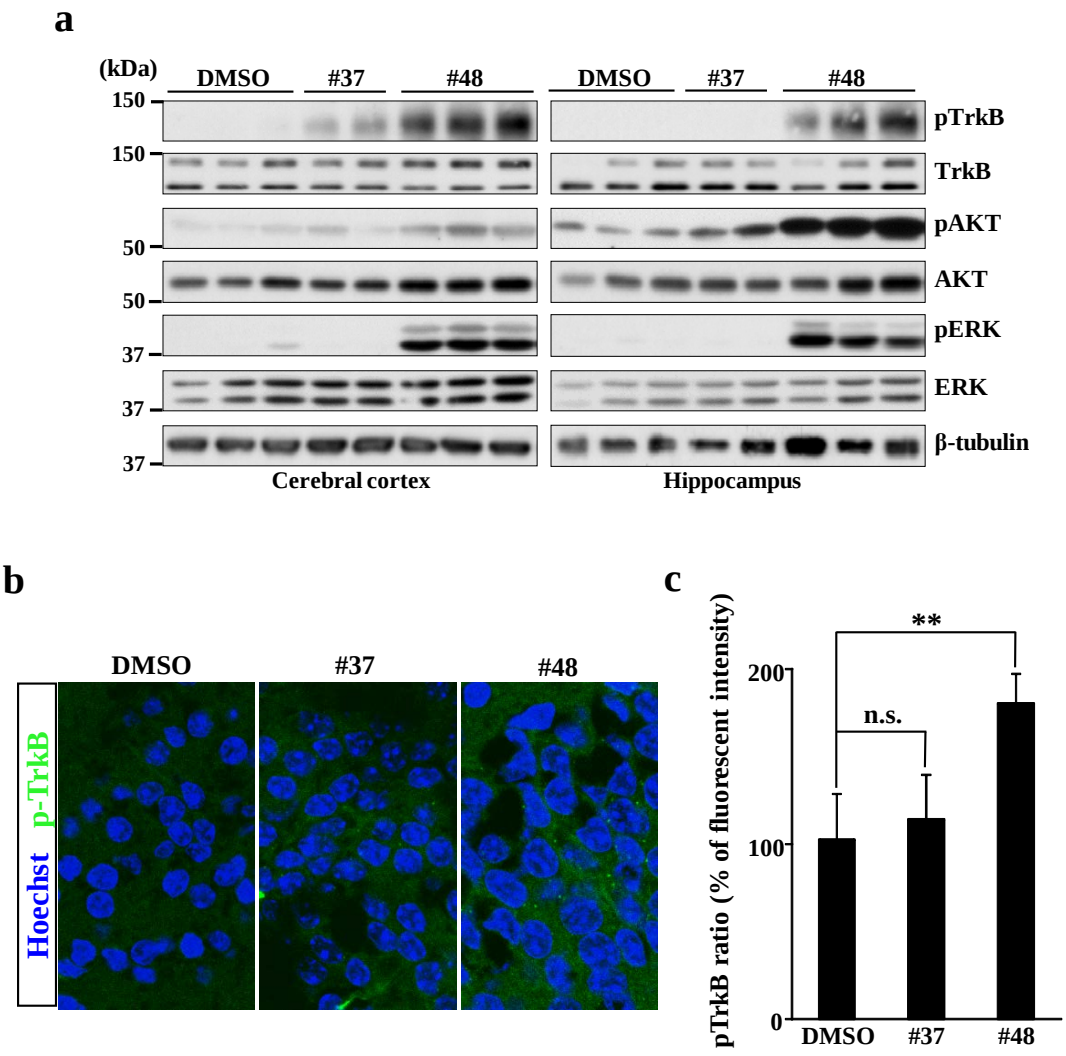
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Figure 4

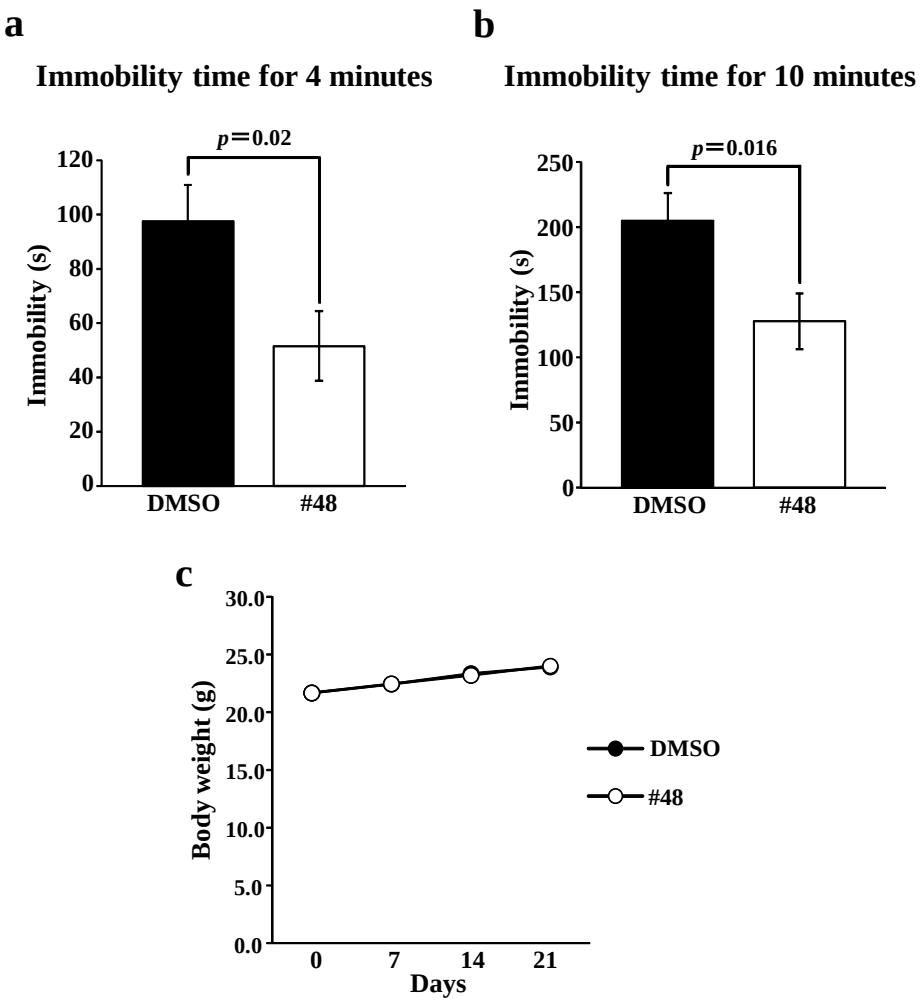
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FIGURE LEGENDS

Figure 1. Compounds 48 and 56 induce cell proliferation and activate TrkB signaling pathways in SH-SY5Y/TrkB and Neuro2a cells.

(a) Growth curves for SH-SY5Y/TrkB cells treated with DMSO or increasing concentrations of BDNF or compound 48 or 56. SH-SY5Y/TrkB cells were cultured in 2% FBS-containing medium for 9 days and treated as follows: "×", DMSO; open circles, BDNF (1 ng/mL) or compound (0.06 μM); filled circles, BDNF (5 ng/mL) or compound (0.2 μM); open triangles, BDNF (10 ng/mL) or compound (0.6 μM); filled triangles BDNF (50 ng/mL) or compound (2 μM). Data represent mean ± S.E. $**p < 0.05$, $*p < 0.01$ compared with DMSO. (b) Growth curves for Neuro2a cells treated with DMSO, or increasing concentrations of BDNF or compounds 48 or 56. Neuro2a cells were cultured in 2% FBS-containing medium for 9 days and treated as follows: "×", DMSO; open circles, BDNF (1 ng/mL) or compound (0.1 μM); filled circles, BDNF (5 ng/mL) or compound (0.3 μM); open triangles, BDNF (10 ng/mL or 1 μM); filled triangles, BDNF (50 ng/mL) or compound (3 μM). Data represent mean ± S.E. (c) Time-course analysis for compounds 48 and 56 in SH-SY5Y/TrkB cells. The cells were treated with 0.3 μM of compounds 48 and 56 for the indicated times (5–60 min). DMSO was used as a negative control and BDNF was used as a positive control. The cell lysates were analyzed by immunoblotting using antibodies against TrkB and downstream molecules, as indicated. The results were quantified by chemiluminescence imaging, and the ratio of phospho-TrkB to total TrkB was calculated and is shown in the graph. (d) Concentration response of TrkB phosphorylation in SH-SY5Y/TrkB cells treated with compounds 48 and 56. The cells were treated with increasing

concentrations (0.03–1.0 μM) of compounds 48 and 56 for 30 min. (e) Western blot analysis for TrkB phosphorylation in Neuro2a cells treated with compound 48. The cells were treated with 0.1 or 1 μM of compound 48 for the indicated times (5–60 min). DMSO was used as a negative control, and BDNF was used as a positive control.

Figure 2. Effects of compounds 48 and 56 depend on TrkB activation.

(a) Immunoblot analysis of SH-SY5Y/TrkB cells pre-treated with the Trk inhibitor K252a followed by treatment with compounds 48 and 56. (b) Cell growth assay of SH-SY5Y/TrkB cells treated with 1 μM of compounds 48 and 56 in the presence or absence of K252a (100 nM). BDNF (5 ng/mL) was used as a positive control. Data represent mean ± S.E. $^{**}p < 0.05$, $^{*}p < 0.01$ compared with DMSO. (c) Morphological changes of SH-SY5Y/TrkB cells treated with 1 μM of compounds 48 and 56 in the presence or absence of K252a (100 nM). BDNF (5 ng/mL) was used as a positive control. Scale bar; 200 μm.

Figure 3. Compounds 48 and 56 induce TrkB phosphorylation in cortex and hippocampus.

(a) Male C57BL/6J mice (9 weeks old) were intraperitoneally administered 15 mg/kg of compound 48 or 56. DMSO and compound 37 were used as negative controls. Three hours after the injections, tissue lysates were prepared and analyzed by western blotting. (b) Immunohistochemical staining for phosphorylated TrkB in hippocampal tissues of C57BL/6J mice. Mice were intraperitoneal injected with 3 mg/kg of compound 48. DMSO and compound 37 were used as negative controls. (c) Quantification of

phospho-TrkB levels in hippocampus. The signal intensities from three mice were measured in nine fields at high magnification using WinROOF software.

Figure 4. Effects of compound 48 on immobility time in the forced swim test (FST). (a) Male C57BL/6J mice were intraperitoneally administered 3 mg/kg/day of compound 48 or DMSO for 21 days and subjected to the FST. The FST was performed for 10 min. The immobility time was recorded for 4 min after a pre-swim for 3 min. (b) Immobility time in the same FST as that for panel (a) was analyzed for 10 min. Data are indicated as mean ± S.E. of eight mice/group (*p* values are against the DMSO control group, one-way ANOVA followed by *post hoc* Dunnett's test). (c) Body weight changes in C57BL/6J mice injected daily with 3 mg/kg of compound 48 for 21 days. Data are presented as mean ± S.E.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIALS AND METHODS

Reagents, cells, and mice

Small molecules identified during *in silico* screening were purchased from Namiki Shoji Co. Ltd. (Tokyo, Japan). Details of the *in silico* screening are described in our previous study (Nakamura *et al*, 2014). All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Nakalai, Kyoto, Japan) to a final concentration of 10 mM and kept at −20°C. Human neuroblastoma-derived SH-SY5Y/TrkB cells were a gift from Dr. Angelika Eggert (University Children's Hospital Essen, Essen, Germany) and grown in RPMI 1640 medium (Sigma, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, CA, USA), 50 U/mL penicillin, 50 µg/mL streptomycin (Gibco), and 40 µg/mL G418 (Sigma). A mouse neuroblastoma cell line, Neuro2a, was maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco). The cells were cultured at 37°C in the humidified atmosphere of a 5% CO_2 incubator.

In vitro compound screening

SH-SY5Y/TrkB cells were seeded at a density of 1.0×10^4 cells/well in 24-well plates with medium containing 10% fetal bovine serum (FBS). After overnight culture, the cells were treated with dimethyl sulfoxide (DMSO) and 10 µM of the various compounds (#19, #30, #31, #36, #45, #48, #49, #50, #56, and #59). The plate was

incubated and monitored for 5 days. Images of the cells were captured and the cell number was counted on the fifth day after compound treatment.

Cell growth/survival assay

For the cell proliferation assay, SH-SY5Y/TrkB and Neuro2a cells were seeded at a density of 0.5×10^3 cells/well in 96-well plates with media containing 2% and 1% FBS, respectively. After overnight culture, the cells were treated with DMSO, the selected compounds (0.06–2.0 μM or 0.1–3.0 μM), or BDNF (1–50 ng/mL, Wako, Osaka, Japan), as indicated. The plate was incubated and monitored for 9 days in an IncuCyte live-cell imaging system (Essen Bioscience, Michigan, USA) according to the manufacture's instructions. Images were captured in nine fields per well every 6 h to monitor cell confluency. SH-SY5Y/TrkB cells were pre-treated with the Trk receptor inhibitor K252a (Sigma) (200 nM) for 30 min after pre-culture in serum-free medium for 2.5 h to investigate whether the activities of compounds 48 and 56 were mediated through TrkB receptors. Cells were then treated with DMSO, BDNF (1 ng/mL), or compounds 48 or 56 (0.3 μM) for 1 h. The cells were collected, and the proteins were analyzed by immunoblotting.

For the TrkB inhibition assay, SH-SY5Y/TrkB cells were seeded at a density of 0.5×10^4 cells/well in 96-well plates with medium containing 2% FBS and allowed to attach overnight. Cells were then treated with BDNF (5 ng/mL) or compound (1 μM) in the presence of K252a (100 nM). The plate was incubated and monitored for 9 days using IncuCyte. Images were captured in nine fields per well every 6 h to monitor cell morphology.

SH-SY5Y/TrkA cells were seeded at a density of 0.5×10^3 cells/well in 96-well plates with medium containing 2% FBS. After overnight culture, the cells were treated with DMSO and increasing concentrations of the compounds (0.03–1.0 μM). The plate was incubated and monitored for 9 days in an IncuCyte live-cell imaging system (Essen Bioscience, Michigan, USA) according to the manufacture's instructions. Images were captured in three fields per well every 6 h to monitor cell confluency.

Immunoblotting

The effects of the compounds *in vitro* were examined in cells plated in 6-cm diameter dishes and cultured for 2 days before the experiments. Cells were pre-cultured in serum-free medium for 2.5 h and then treated with DMSO, compounds, or BDNF for the indicated times. Cells were washed with cold phosphate-buffered saline (PBS), lysed on ice for 30 min in a CHAPS lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM CHAPS, a protease inhibitor cocktail and a phosphatase inhibitor cocktail [Roche]) and centrifuged at 4°C at 12100 g for 20 min. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). The proteins were separated using SDS-PAGE and transferred onto Immobilon-P transfer membrane filters (Millipore). After being blocked with skim milk, the membranes were incubated with the following primary antibodies overnight at 4°C: anti-TrkB antibody (1:1 000 dilution; catalog #4603, Cell signaling, MA, USA), anti-TrkB antibody (1:1000; #610101, BD Biosciences, CA, USA), anti-AKT antibody (1:1 000; #9272, Cell signaling), anti-ERK antibody (1:1 000, #9102S, Cell signaling), anti-β tubulin (1:10 000, MMS-435P, BioLegend, CA, USA), anti-phospho-TrkB (1:1 000, #4621, Cell signaling), anti-phospho-AKT (1:1 000, #9271, Cell signaling),

anti-phospho-ERK (1:1 000, #9101, Cell signaling). Immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (34080, Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

Compound administration in mice

Eight-week-old male C57BL/6J mice (CREA Japan Inc., Tokyo, Japan) were maintained in a temperature-controlled environment for 1 week prior to the start of experiments. All animal experiments were performed in compliance with the regulations for animal experiments at the Chiba Cancer Center Research Institute after obtaining approval by the Animal Ethics Committee of Chiba Cancer Center. The mice were intraperitoneally administered DMSO or the compounds (3 mg/kg or 15 mg/kg body weight). After 3 h, the mice were killed, and cerebral cortical and hippocampal samples were collected. Tissue lysates were extracted from half of the collected tissues in homogenization buffer (20 mM Tris at pH 8.0, 137 mM NaCl, 1% Igepal, 10% glycerol) on ice and analyzed by immunoblotting with antibodies against phospho-(p)TrkB, TrkB, pAKT, AKT, pERK and ERK. The remaining half of the tissue was immediately fixed in 4% paraformaldehyde and embedded in OCT compound (Sakura, Tokyo, Japan). Frozen sections were cut using a cryostat, and the sections were subjected to immunohistochemistry assays using the anti-pTrkB antibody visualized with Alexa 488-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA). Images were captured using a confocal microscope (Leica), and the signal intensities were quantified in nine randomly selected fields on three slides using WinROOF software (version 7.0, Mitani Corp., Fukui, Japan).

Single compound administration test

Male ICR mice (5 weeks old) were maintained in a temperature-controlled environment for 1 week before experiments. Animal experiments were performed in compliance with the regulations for animal experiments of the Chiba Cancer Center Research Institute after approval by the Animal Ethics Committee of the Chiba Cancer Center. Mice were orally administered a solution of 0.5% methylcellulose (Wako, Osaka, Japan) and 20, 200, and 2000 mg/kg of compound 48 or intravenously injected with a solution of 10% DMSO (Wako, Osaka, Japan) and 0.75, 1.5 and 3 mg/kg of the compound. The body weights of mice were measured for 15 days after compound administration. Methylcellulose and DMSO were used as controls.

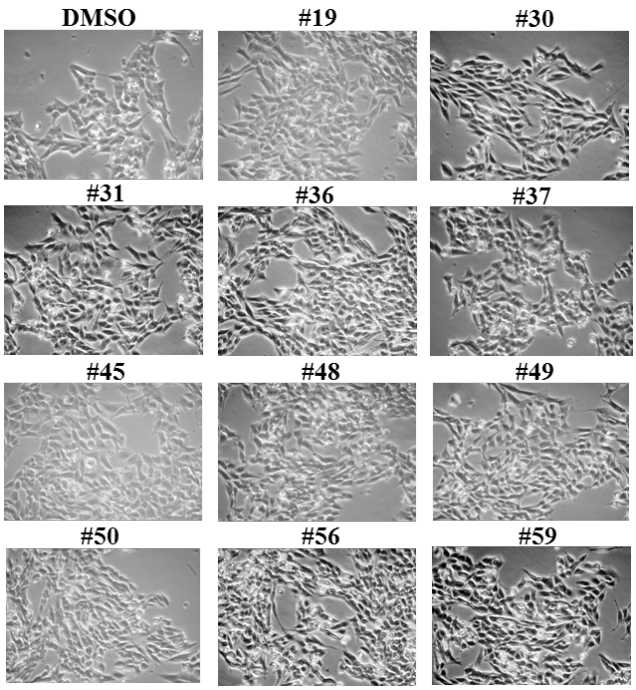
Forced swim test

Male C57BL/6J mice were intraperitoneal injected with DMSO or compounds (3 mg/kg) for 21 days and then subjected to a forced swim test. The mice were allowed to adapt to the test room for 3 h after the injection. They were then placed in a clear glass cylinder with a diameter of 20 cm and height of 30 cm filled with clear water of approximately 25°C (a water depth of 20 cm did not allow the mice to touch the bottom of the cylinder). Their mobility was recorded for 10 min, and the time spent immobile was analyzed for the last 4 min of the test after a 3 min pre-swim as well as for the entire 10 min without a pre-swim.

SUPPLEMENTARY FIGURES

Supplementary Figure S1

a



SH-SY5Y/TrkB cells

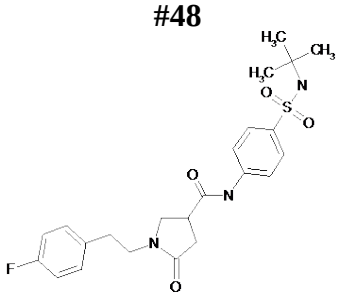
b

Compound	Cell No. (x10^4)
DMSO	13.20
19	20.15
30	19.79
31	19.78
36	22.25
37	19.81
45	19.62
48	26.13
49	21.71
50	23.76
56	21.94
59	23.09

Supplementary Figure S2

a

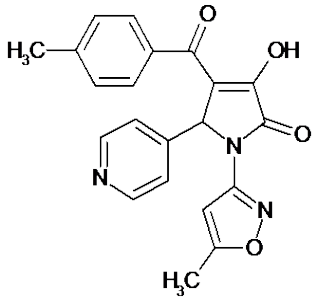
#48



1-[2-(2-Fluorophenyl)ethyl]-1-(1-O,2-O-isopropylidene-5-deoxy-α-D-xylofuranose-5-yl)-3-(5-methyl-2-pyridyl)thiourea

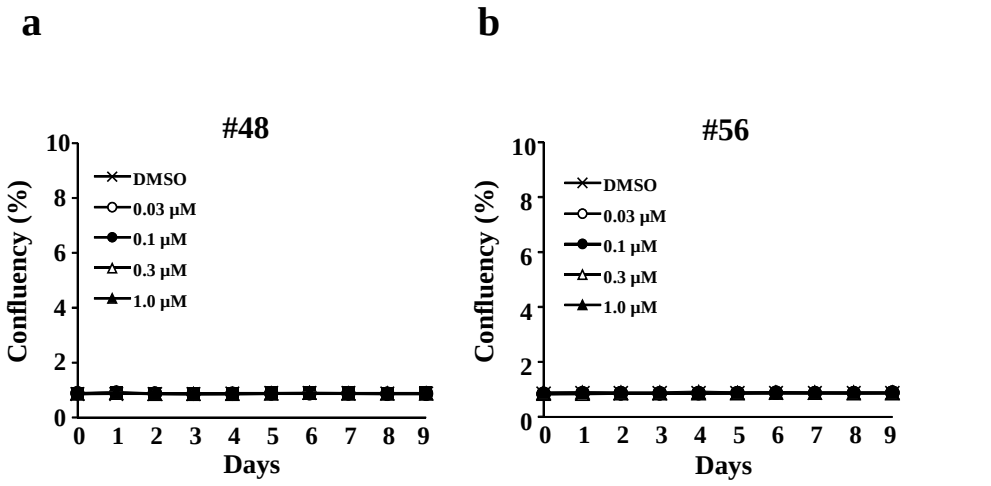
b

#56



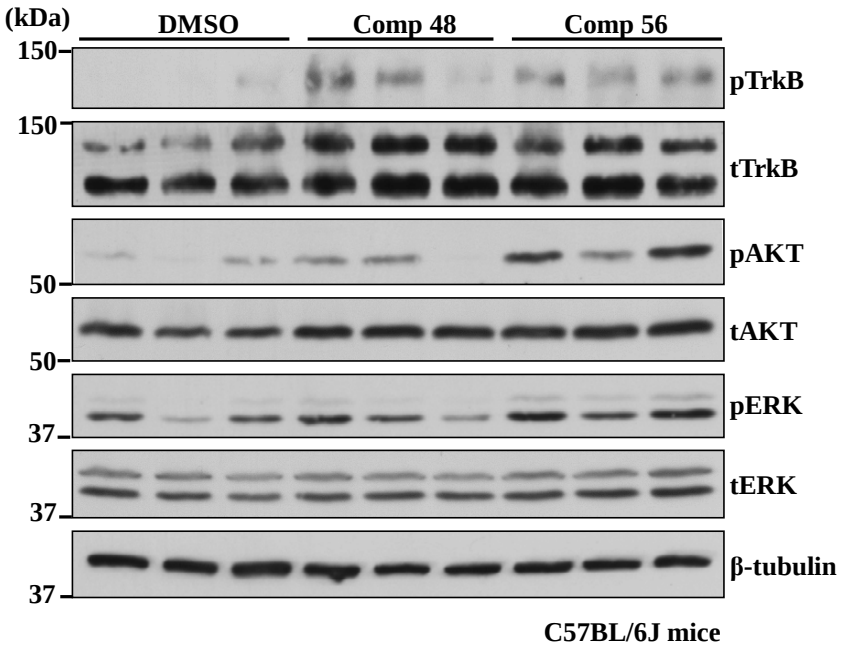
2-(4-Aminophenylamino)-α-cyano-1,4-dihydro-1,4-dioxonaphthalene-3-acetic acid ethyl ester

Supplementary Figure S3

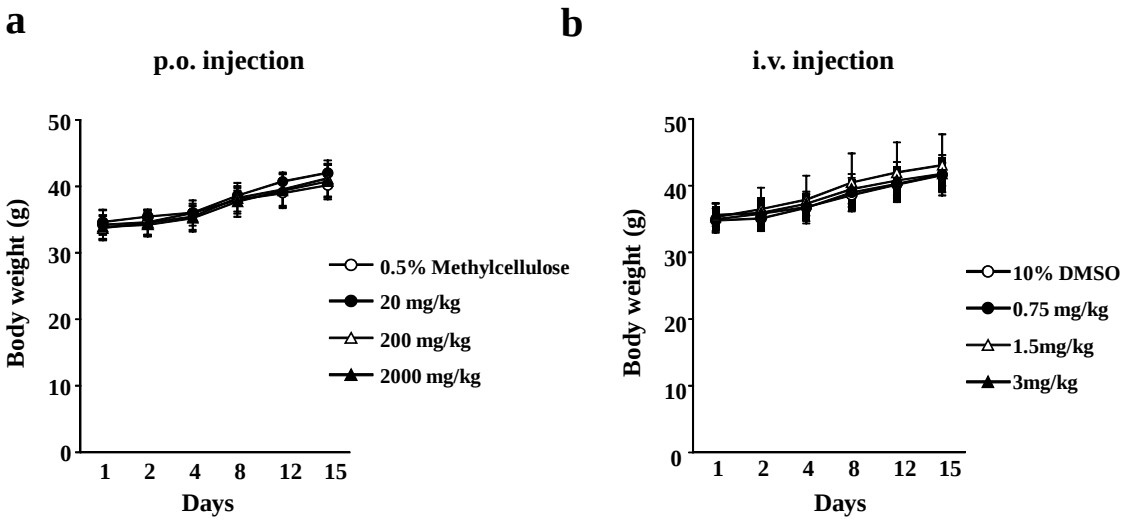


SH-SY5Y/TrkA cells

Supplementary Figure S4



Supplementary Figure S5



SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Initial screening of compounds selected through *in silico* screening in TrkB-expressing SH-SY5Y cells.

(a) Cell proliferation changes in SH-SY5Y/TrkB cells treated with 11 candidate compounds selected from previously screened compounds numbered 19 to 59. SH-SY5Y/TrkB cells were grown in normal culture medium containing 10% FBS for 9 days. DMSO was used as a control. Images were captured 9 days after compound treatment. (b) Number of live SH-SY5Y/TrkB cells treated with the 11 compounds. SH-SY5Y/TrkB cells were treated with the 11 compounds, and the cell number was determined 5 days after a single treatment.

Supplementary Figure S2. Chemical structures of two candidate molecules.

(a) Structure and name of compound 48. (b) Structure and name of compound 56.

Supplementary Figure S3. **Compounds 48 and 56 do not affect cell proliferation in SH-SY5Y/TrkA cells.**

(a) Growth curves of SH-SY5Y/TrkA cells treated with compound 48. SH-SY5Y/TrkA cells were cultured in 2% FBS-containing medium for 9 days as follows: "×", DMSO; open circles, compound (0.03 μM); filled circles, compound (0.1 μM); open triangles, compound (0.3 μM); filled triangles, compound (1 μM). Data represent mean ± S.E. $^{**}p < 0.05$, $^{*}p < 0.01$ compared with DMSO. (b) Growth curves of SH-SY5Y/TrkA cells treated with compound 56. SH-SY5Y/TrkA cells were cultured in 2% FBS-containing medium for 9 days as follows: "×", DMSO; open circles, compound

(0.03 μM); filled circles, compound (0.1 μM); open triangles, compound (0.3 μM); filled triangles, compound (1 μM). Data represent mean ± S.E. $^{**}p < 0.05$, $^{*}p < 0.01$ compared with DMSO.

Supplementary Figure S4. **TrkB phosphorylation in whole brain induced by compounds 48 and 56.**

Male C57BL/6J mice (9 weeks old) were intraperitoneally administered 3 mg/kg of compounds 48 and 56. DMSO was used as negative control. Three hours after injection, tissue lysates were prepared and analyzed by western blotting.

Supplementary Figure S5. **Changes in body weights of ICR mice in single-dose toxicity tests.**

Male ICR mice (5 weeks old) were divided into two groups. (a) Mice in one group were orally administered 20, 200, 2000 mg/kg of compound 48. A solution of 0.5% methylcellulose was used as a control. (b) Mice in the other group were intravenously injected with the compound. The body weights of the mice were measured for 15 days. Data represent mean ± S.D. $^{**}p < 0.05$, $^{*}p < 0.01$ compared with DMSO.

FUNDING AND DISCLOSURE

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