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Running Title: Neuroprotection and Neurite Outgrowth Promotion by T-817MA

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Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid-β peptides; Aβ(1–42), amyloid-β(1–42); H₂O₂, hydrogen peroxide; CNS, central nervous system; GAP-43, growth-associated protein 43; DMEM, Dulbecco’s modified Eagle medium; PBS, phosphate-buffered saline with neither Ca²⁺ nor Mg²⁺; DNase I, deoxyribonuclease I; FBS, fetal bovine serum; EMEM, Eagle’s minimum essential medium; AraC, cytosine arabinoside; MAP2, microtubule-associated protein 2; GSH, reduced glutathione; IGF-1, insulin-like growth factor-1

Section: Neuropharmacology
ABSTRACT

Progressive neuronal loss in Alzheimer’s disease (AD) is considered to be a consequence of the neurotoxic properties of amyloid-β peptides (Aβ). T-817MA (1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidinol maleate) was screened as a candidate therapeutic agent for the treatment of AD, based on its neuroprotective potency against Aβ-induced neurotoxicity and its effect of enhancing axonal regeneration in the sciatic nerve axotomy model. The neuroprotective effect of T-817MA against Aβ(1–42) or oxidative stress-induced neurotoxicity was assessed using a coculture of rat cortical neurons with glia. T-817MA (0.1 and 1 µM) was strongly protective against Aβ(1–42)-induced (10 µM for 48 h) or hydrogen peroxide (H₂O₂)-induced (100 µM for 24 h) neuronal death. T-817MA suppressed the decrease of reduced glutathione (GSH) levels induced by H₂O₂ exposure (30 µM for 4 h) in cortical neuron culture, therefore T-817MA was likely to alleviate oxidative stress. Besides the neuroprotective effect, T-817MA (0.1 and 1 µM) promoted neurite outgrowth in hippocampal slice cultures and reaggregation culture of rat cortical neurons. T-817MA also increased the growth-associated protein-43 (GAP-43) content in the reaggregation culture of cortical neurons. These findings suggest that T-817MA exerts neuroprotective effect and promotes neurite outgrowth in rat primary
cultured neurons. Based on these neurotrophic features, T-817MA might have a potential for disease modification and to be useful for patients with neurodegenerative diseases, such as AD.
Introduction

Pharmacotherapy of Alzheimer’s disease (AD) is restricted to symptomatic treatment, and has not been helpful in improving the deterioration of this disease (Tariot and Federoff, 2003). AD is a neurodegenerative condition that is characterized by progressive neuronal loss. There are many biological and cellular alterations in patients with AD; many aspects are involved in the pathogenesis of AD (Mattson et al., 2001). One of the most convincing hypothesis states that the conditions of AD may be a consequence of the neurotoxic properties of amyloid-β peptides (Aβ), although this hypothesis is still being argued (Naslund et al., 2000; Rottkamp et al., 2002, Selkoe, 1991). Aβ is considered to cause progressive synaptic degeneration and neuronal loss, thereby result in cognitive dysfunction and behavioral abnormalities in AD (Stepanichev et al., 2004). There is also extensive evidence indicating that oxidative stress might be responsible for dysfunction or death of neuronal cells in AD (Butterfield et al., 2002; Markesbery et al., 2001, Mattson et al., 2001). The molecular mechanisms of Aβ toxicity remain unclear. However many studies supported the idea that an oxidative event is critical for Aβ toxicity (Rottkamp et al., 2002). For example, Aβ toxicity is considered to be caused by unregulated reactive oxygen species such as hydrogen...
peroxide (H$_2$O$_2$) (Barnham et al., 2004). From this point of view, preventing oxidative stress might protect the remaining neurons from A$\beta$ insult during disease progression.

Neurotrophic factors have been studied as one of the potential future therapies for AD (Tariot and Federoff, 2003). Neurotrophic factors can support the remaining neurons and protect them against disease progression in animal and cell culture models of neurodegenerative diseases (Mattson et al., 2001). In the adult nervous system, neurotrophic factors can also regulate neuronal plasticity by promoting nerve growth following injury (Gillespie, 2003) and thereby promote functional restoration (Lim et al., 2003). These features suggest that activation of neurotrophic pathways can contribute to the modification and prevention of disease progression in patients with AD.

T-817MA (1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3- azetidinol maleate) is a newly synthesized agent, which was screened as a candidate therapeutic agent for the treatment of AD. Screening was carried out based on the neuroprotective potency against A$\beta$-induced neurotoxicity and enhancing effect on axonal regeneration in the rat sciatic nerve axotomy model (daily treatment with T-817MA for 14 days enhanced the maximal regeneration distance of sciatic nerve axons measured using an electrophysiological analysis; in house preliminary data), in the expectation of obtaining a neurotrophic agent. In the present study, in order to determine whether T-817MA
exerts neurotrophic potency on central nervous system (CNS), we evaluated its neuroprotective effect and neurite outgrowth promoting effect. Neuroprotective effect of T-817MA was assessed in Aβ(1–42) or H₂O₂-induced neuronal damages by using a coculture of rat cortical neurons with glia. The neurite outgrowth promoting effect was assessed by using a hippocampal slice culture and cultured reaggregates of rat cortical neurons. The therapeutic potential of T-817MA in AD is also discussed.

**Materials and Methods**

**Animals**

Pregnant female Wistar/ST rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and kept in individual aluminum cages with laboratory bedding in an air-conditioned room on a 12h light/dark cycle. The animals were given free access to a commercial diet (MF, Oriental Yeast Co., Ltd. Tokyo, Japan) and water. Neonatal rats were housed with their mother rat. All the experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals at Toyama Chemical Co., Ltd. and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Materials**
T-817MA (1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidinol maleate) was synthesized at Toyama Chemical Co., Ltd. (Toyama, Japan). The chemical structure of T-817MA is shown in Fig. 1. T-817MA was dissolved in distilled water at a concentration of 10 mM and diluted to 1, 0.1 and 0.01 µM with Dulbecco’s modified Eagle medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) on the day of use.

Aβ(1–42): β-amyloid(1–42)-HCl was purchased from AnaSpec Inc. (San Jose, CA). Aβ(1–42) was sonicated in distilled water at a concentration of 250 µM and then incubated at 37°C for 24 h. Fetal bovine serum (FBS) was purchased from JRH Biosciences, Inc. (Lenexa, KA). FBS was heat-inactivated at 56°C for 30 min.

Monoclonal anti-MAP2 antibody (Clone HM-2, Mouse Ascites Fluid) and anti-glia fibrillary acidic protein were purchased from Sigma-Aldrich Co. (Saint Louis, MO). Goat polyclonal anti-GAP-43 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). VECTASTAIN® ABC-PO Goat IgG Kit and VECTASTAIN® ABC-PO Mouse IgG Kit were purchased from Vector Laboratories Inc. (Burlingame, CA). Recombinant human insulin like growth factor-1 (IGF-1) was purchased from PeproTec, Inc. (Rocky Hill, NJ).

Neuroprotective effect against Aβ(1–42)-induced toxicity
Neuron/Glia coculture. We prepared primary cortical neurons plated onto glia monolayer cultures with referring to a previously reported method (Pike et al., 1993). Primary cultures of cortical glial cells were prepared from 1- or 2-day-old neonatal rats. Neonatal rats were decapitated and their whole brains were isolated. Cortices were dissected under a microscope and incubated at 37°C for 20 min with phosphate-buffered saline devoid of Ca\(^{2+}\) and Mg\(^{2+}\) (PBS: Nissui Pharmaceuticals) containing 0.25% trypsin (Invitrogen Corp., Carlsbad, CA) and 40 units/ml deoxyribonuclease I (DNase I: Sigma-Aldrich). Trypsinization was terminated by addition of 25% FBS. The dissociated cells were suspended in Eagle’s minimum essential medium (EMEM: Nissui Pharmaceuticals) containing 10% FBS, 2 mM glutamine and 25 µg/ml gentamicin sulfate. The cells were cultured in 75-cm\(^2\) flasks in a humidified CO\(_2\) incubator with 5% CO\(_2\)/95% air at 37°C for 3 weeks in order to form a monolayer. The glia cells were then harvested and replated on 24-well plates at a density of 400 cells/mm\(^2\) and then maintained for another 1 week. Cortical neurons were prepared from rat embryos (day 18 gestation). Cortices were dissected under the microscope and incubated with PBS containing 0.25% trypsin and 40 units/ml DNase I at 37°C for 20 min. Trypsinization was terminated by the addition of 25% FBS. Dissociated cortical cells were suspended in DMEM containing 10% FBS, 2 mM glutamine and 25 µg/ml gentamicin sulfate.
They were subsequently seeded onto the cortical glial monolayer culture. Three or four days after plating cortical cells, whole medium was replaced with DMEM containing 1% (v/v) N-2 Supplement (Invitrogen Corp.) and added 10 µM cytosine arabinoside (AraC: Nacalai Tesque, Inc. Kyoto, Japan) in order to halt glial proliferation. The cells were then incubated for 7 days in this medium. Aβ(1–42) was added to the coculture at a concentration of 10 µM. T-817MA was added simultaneously with Aβ(1–42) application at concentrations of 0 (Control), 0.01, 0.1 and 1 µM, and the cells were further incubated for 48 h. For the “Normal” group, the preparations were maintained in the medium with neither T-817MA nor Aβ(1–42).

**Assay for the viability of neurons.** Neuronal cell viability was quantified by measuring microtubule-associated protein 2 (MAP2)-immunoreactivity. This method is favorable for measuring neuronal survival in the presence of glia cells without resorting to counting of neurons (Brooke et al., 1999). Measurement was performed using monoclonal anti-MAP2 antibody and VECTASTAIN® ABC-PO Mouse IgG Kit in accordance with the manufacturer’s manual. In brief, the cultured cells were fixed with ice-cold methanol for more than 30 min and washed with PBS three times. Following the blocking procedure with horse serum for 20 min, the cells were incubated for 30 min with anti-MAP2 antibody (1:3,000 dilution). The cells were then incubated with
biotinylated secondary antibody (included in the kit) for 30 min, following by incubation with VECTASTAIN® ABC Reagent for 30 min. Peroxidase activity was estimated using o-phenylenediamine and H$_2$O$_2$ (Maus et al., 2002). The cells were incubated with o-phenylenediamine solution (10 mg/ml o-phenylenediamine, 0.03% hydrogen peroxide, 0.05 mM citric acid and 0.1 mM disodium hydrogenphosphate, Wako Pure Chemical Industries, Ltd. Osaka, Japan) for 3 min. Subsequently the reaction was terminated using 0.05 M sulfuric acid (Wako Pure Chemical Industries). Each solution was diluted with 1 ml of distilled water. The absorbance of each reaction solution was measured at 490 nm. All the procedures were performed at room temperature.

**Neuroprotective effect against H$_2$O$_2$-induced toxicity**

**Assessment in neuron/glia coculture.** A cortical neuron/glia coculture was prepared as described above. T-817MA was added to the cocultures at concentrations of 0 (Control), 0.01, 0.1 and 1 µM, and the cells were subsequently incubated for 5 min or 24 h. H$_2$O$_2$ was then added to the coculture at a concentration of 100 µM, and the cells were incubated for another 24 h. For the “Normal” group, the preparations were maintained
in the medium with neither T-817MA nor H₂O₂. Neuronal cell viability was quantified by measuring the MAP2-immunoreactivity, as described above.

**Assessment in cortical neuron culture.** Primary cultures of cortical neurons were prepared from rat embryos (day 18 gestation). Dissociated cortical cells were suspended in DMEM containing 10% FBS, 2 mM glutamine and 25 µg/ml gentamicin sulfate. The cells were seeded onto 24-well plates precoated with poly-L-lysine (molecular weight: 30,000–70,000, Sigma-Aldrich) at a density of 1,000 cells/mm². Forty eight hours after cell seeding, AraC was added to the cultures (10 µM) and removed by medium exchange after 24 h. In this condition, as shown by immunocytochemical studies using anti-MAP2 monoclonal antibody, the cultures were highly enriched in neurons and less than 10 % of the cells exhibited immunoreactivity with a rabbit antibody raised against glial fibrillary acid protein (data not shown). T-817MA was added to the cultures at concentrations of 0 (Control), 0.01, 0.1 and 1 µM. Twenty four hours following T-817MA application, H₂O₂ was added at a concentration of 30 µM, and the cells were incubated for another 24 h in the continuous presence of T-817MA. For the “Normal” group, the preparations were maintained in the medium with neither T-817MA nor H₂O₂. Neuronal cell viability was quantified by measuring the MAP2-immunoreactivity as described above.
Measurement of the intracellular reduced glutathione (GSH) content in primary cortical neuron culture. T-817MA was added to the primary culture of purified neuron at concentrations of 0.01, 0.1 and 1 µM. Twenty four hours following T-817MA application, H₂O₂ was added at a concentration of 30 µM. The cells were then incubated for another 4 h in the continuous presence of T-817MA. The intracellular GSH content in the cultured neurons was measured in accordance with a previously reported method (Hiraku et al., 2002) with some modifications. In brief, the cells were washed once with ice-cold PBS, followed by addition of 100 mM perchloric acid, and then harvested. The harvested cells were homogenized for 5 sec with a microhomogenizer (SEIKO Instruments, Chiba, Japan), and then centrifuged at 10,000 g for 10 min at 4°C. The GSH content in the supernatant was measured using an HPLC system consisting of an L-7100 pump (Hitachi Ltd., Tokyo, Japan) and ECD-300 electrochemical detector (Eicom Corporation, Kyoto, Japan) equipped with an Symmetry® C18 column (i.d. 4.6 mm × 25 cm: Waters Corporation, Milford, MA), a WE-AU gold electrode (Eicom) and a 50-mm GS-50 gasket (Eicom). The mobile phase consisted of 4.4 mM phosphate buffer (pH 2.5), 88 mg/l 1-octanesulfonic acid, 4.4 mg/l EDTA-2Na and 12 % methanol. The measurement was carried out at room temperature at a flow rate of 0.8 ml/min. The voltage of the gold electrode was set at +600 mV against the Ag/AgCl reference.
electrode (RE-100, Eicom). Authentic GSH (Sigma-Aldrich) was simultaneously measured as an external standard under these conditions. Neuronal cell viability was also quantified by measuring the MAP2-immunoreactivity as described above.

**Evaluation of Neurite Outgrowth Promoting Effect**

**Hippocampal slice culture.** Organotypic hippocampal slice culture was prepared in accordance with the previously reported method (Stoppini et al., 1991) with some modifications. Hippocampal slices were prepared from 7-day-old rat pups. The dorsal hippocampus was isolated and cut into transverse slices of 350-µm thickness with a tissue chopper (Mickle Laboratory Engineering, Guilford, UK). The slices were placed onto dishes pre-coated with poly-L-lysine (3 slices in each dish) and cultured in interface configuration with DMEM containing 12.5 mM HEPES, 1% (v/v) B-27 Supplement (Invitrogen Corp.) 2 mM glutamine and 25 µg/ml gentamicin sulfate. T-817MA was added at concentrations of 0 (Control), 0.01, 0.1 and 1 µM at the initiation of the slice culture. The culture was then incubated at 37°C in the 5% CO₂/95% air for 8 days. During culture period half the volume of the medium in each dish was changed every 2 or 3 days.
Reaggregation culture of cortical neurons. Reaggregation culture of the cortical neurons was carried out referring to a previously reported method (Gao et al., 1992). The cortical neurons were harvested from rat embryos (E18). Dissociated cortical cells were suspended in DMEM containing 10% FBS, 2 mM glutamine and 25 mg/ml gentamicin sulfate, and seeded onto 100-mm non-coated dishes at a density of 50,000 cells/mm² and then cultured for 4 days. As a result of this procedure, cortical neurons formed reaggregates and floated in the medium. The suspended reaggregates of neurons were collected and seeded onto 6-well (for evaluating neurite length) or 24-well (for measuring contents of MAP2 and GAP-43) plates pre-coated with poly-L-lysine. The reaggregates of cells were cultured for 3 days. T-817MA was then added at concentrations of 0 (Control), 0.01, 0.1 and 1 µM and the reaggregates of cells were cultured further for 4 days. All the cultures were incubated in the atmosphere of humidified 5% CO₂/95% air at 37°C.

Measurement of neurite outgrowth. Following treatment of T-817MA, cultured slices or cortical reaggregation culture were fixed with methanol. The neurites, being generated from slices or cell reaggregates were subsequently immunostained (Schreyer et al., 1997) with a goat polyclonal anti-GAP-43 antibody using VECTASTAIN® ABC-PO Goat IgG Kit. Neurite length was defined as the distance from the edge of a
slice or a reaggregate to the neurite tip. Neurite outgrowth was evaluated by measuring the length of the longest neurite in each slice or reaggregate under a microscope using a micrometer. The outgrowth was measured in 3 slices or 3 reaggregates in each dish, and their mean value was regarded as the representative value of the dish. All measurements were performed in a blinded manner. GAP-43 content was quantified by EIA (Schreyer et al., 1997) using VECTASTAIN® ABC-PO kit with goat polyclonal anti-GAP-43 antibody.

**Statistical Analysis**

The results are represented as mean with S.E.M. Statistical significance of the differences between the two groups was analyzed by an analysis of variance (F-test) followed by Student’s t-test. Statistical significance of the T-817MA-treatment groups from the control group was evaluated by Dunnett test. These analyses were performed by using SAS release 8.2 (SAS Institute Japan Ltd., Tokyo, Japan). $P<0.05$ (two-tails) was considered to be significant.

**Results**

**Neuroprotective effect of T-817MA**
**Aβ(1–42)-induced neuronal death.** The protective effect of T-817MA on cortical neurons against Aβ(1–42)-induced neuronal death was investigated in the neuron/glia coculture. Typical microscope images of MAP2 immunocytochemistry are shown in Fig. 2-A, B and C. Many neurons that formed clusters were stained on the glial monolayer in the absence of Aβ(1–42) (Normal). On the other hand, few neurons were detected with 10 µM Aβ(1–42) treatment (Control). T-817MA treatment preserved the cortical neurons in the presence of Aβ(1–42). In order to evaluate the number of survived neurons, MAP2-immunoreactivity, which is known to correlate the number of neurons (Brooke et al., 1999), was measured. The exposure of Aβ(1–42) significantly reduced MAP2 immunoreactivity. T-817MA significantly prevented this reduction at 0.1 and 1 µM (Fig. 2-D). A peptide Aβ(42–1) did not reduce MAP2 immunoreactivity on the neuron/glia coculture (data not shown), which suggested that the neuronal damage was specifically induced by Aβ(1–42).

**H₂O₂-induced neuronal death.** Previous studies indicated that oxidative stress is supposed to contribute to Aβ induced neuronal damages (Butterfield et al., 2002; Markesbery et al., 2001). Based on this, the effect of T-817MA on H₂O₂-induced neuronal damage was investigated in the neuron/glia coculture. The 100 µM H₂O₂ treatment greatly reduced the number of survived neurons in the culture (Fig. 3,
Control). When T-817MA was pretreated for 24 h, T-817MA significantly prevented this neuronal damage at 0.1 and 1 µM (Fig. 3-A). On the other hand, a brief (5 min) pre-treatment with T-817MA followed by its continuous presence with H₂O₂ did not rescue the H₂O₂ treated neurons from death (Fig. 3-B).

**T-817MA attenuated H₂O₂-induced reduction of intracellular GSH contents.** A similar protective effect of T-817MA was observed in the primary cortical neuron culture. H₂O₂ exposure at 30 µM for 24 h induced neuronal death (Fig. 4-A, Control). Twenty four hours of pre-treatment followed by the continuous presence of T-817MA prevented this oxidative stress-induced neuronal death at 0.1 and 1 µM (Fig. 4-A). In order to investigate the effect of T-817MA on this oxidative stress, intracellular GSH content was measured as an index of the intracellular oxidative condition in rat cortical neurons under H₂O₂ exposure. In this experiment, primary cultures of cortical neurons were exposed to 30 µM H₂O₂ for 4 h. Viability of neurons was not altered at 4 h following 30 µM H₂O₂ exposure (Fig. 4-B). On the other hand, GSH content was significantly reduced by this stress (Fig. 4-C). The effect of T-817MA was examined with pre-treatment for 24 h, then continuous presence for 4 h with 30 µM H₂O₂ exposure. T-817MA almost completely prevented such GSH reduction at 0.1 and 1 µM.
(Fig. 4-C). T-817MA alone did not exert significant effect on the intracellular GSH content in the Normal medium (data not shown).

**T-817MA promoted neurite outgrowth**

We further investigated the neurite outgrowth promoting action of T-817MA by using two types of cultured neurons, namely the hippocampal slice culture and the cortical reaggregation culture. Hippocampal slices with 1 µM T-817MA treatment generated more and much longer neurites than Control slices (Fig. 5-A, B). T-817MA significantly increased the neurite length at 0.1 and 1 µM (Fig. 5C).

Neurite outgrowth promotion of T-817MA was similarly observed in the cortical reaggregation culture. Typical photomicrographs revealed that T-817MA treatment induced reaggregates to generate longer neurites than Control slices (Fig. 6-A, B). T-817MA significantly promoted neurite outgrowth at 0.1 and 1 µM (Fig. 6-C). In addition to the neurite length, we also quantified the immunoreactivity of neurites for GAP-43, which is specifically located in axons. GAP-43 immunoreactivity was significantly increased by T-817MA (Fig. 6-D). The effect of IGF-1, which has potential to promote neurite outgrowth in vitro (Kim et al., 1997), was evaluated in this cortical reaggregation culture as a reference experiment. IGF-1 promoted neurite outgrowth:
GAP-43 immunoreactivity (A 490 nm) was 0.236 with 100 ng/ml IGF-1, which was higher than the Control value (A 490nm: 0.158). This result indicated that the cortical reaggregation culture was useful for assessing neurite outgrowth promotion.

Discussion

AD is a neurodegenerative condition characterized by progressive neuronal loss, which may be a consequence of the neurotoxic properties of the Aβ (Naslund et al., 2000; Selkoe, 1991). The current treatment with acetylcholinesterase inhibitors focuses on the activation of the remaining functional capacities (Tariot and Federoff, 2003). Although drugs focusing on neuroprotection have been actively developed recently, causal therapy for such neurodegenerative diseases is unavailable.

T-817MA was screened as a candidate therapeutic agent for AD. The present data indicates that T-817MA exerts a neuroprotective effect and promotes neurite outgrowth in rat primary cultured neurons. Considering these neurotrophic properties, T-817MA would modify or prevent pathological deterioration in AD.

In the present study, we demonstrated that T-817MA exerted neuroprotective effect against both Aβ(1–42)-induced and H$_2$O$_2$-induced neurotoxicity in the cortical
neuron/glia coculture. These results indicate that T-817MA exerts protective effect on an in vitro model of neuropathology in AD. Aβ neurotoxicity is supposed to be associated with oxidative stress (Butterfield et al., 2002; Markesbery et al., 2001) and the reduction of endogenous antioxidant processes (Olivieri et al., 2001). Therefore, in order to understand the neuroprotective effect of T-817MA we focused our interest on oxidative stress-induced cell death.

T-817MA exerted its neuroprotecting effect when the cells were pretreated with T-817MA for 24 h prior to the H₂O₂ exposure. Unlike antioxidants (Fuson et al., 1999), T-817MA was unable to protect neurons when it was applied just prior to oxidative stress exposure. Based on this result, it is supposed that T-817MA might exert its neuroprotective effect through the modulation of endogenous anti-oxidative mechanisms, rather than scavenging the reactive oxygen species. In the current study, we employed a neuron/glia coculture because Aβ application failed to induce clear neurotoxicity in the primary culture of the enriched cortical neurons. Previous studies indicated that coexistence of glial cells enhanced Aβ-induced neurotoxicity by modifying the redox status in the neuron/glia coculture (Abramov et al., 2004; Abramov et al., 2003; Qin et al., 2002). In order to demonstrate that T-817MA interacts with neurons themselves, we also investigated H₂O₂-induced neuronal damage in a primary
cortical neuron culture. In this culture set, \( \text{H}_2\text{O}_2 \) (30 \( \mu \text{M} \) for 24 h) induced neuronal damage similar to that observed in the neuron/glia coculture. The neuroprotective effect of T-817MA against \( \text{H}_2\text{O}_2 \)-induced damage was also observed in the cortical neuron culture, thereby indicating that T-817MA might act on neuronal cells themselves. In this cortical neuron culture, brief exposure (4 h) of \( \text{H}_2\text{O}_2 \) did not significantly affect neuronal viability; meanwhile, this treatment reduced the GSH content in the neurons. GSH is an important intracellular antioxidant that protects the neurons against a variety of reactive oxygen species (Schulz et al., 2000). Decrease in GSH was supposed to contribute possibly to signaling events occurring during apoptotic neuronal death (Kane et al., 1993). Disturbance of GSH homeostasis may either lead to or result from oxidative stress in neurodegenerative disorders, and the treatments that inhibit GSH degradation may result in slowing the disease progression (Schulz et al., 2000). In our current experiment, the reduction of endogenous antioxidant processes might precede the neuronal damage. In this brief oxidative stress condition, T-817MA attenuated the preceding reduction of the intracellular GSH content, although T-817MA had no effect in the normal condition (data not shown). Based on these results we assumed that T-817MA maintained the intracellular GSH content and resulted in preventing cell death under the oxidative stress condition. In addition, pre-treatment of T-817MA was
necessary for neuroprotection in H_2O_2-induced neuronal death in the neuron/glia coculture. These results indicate that T-817MA promotes endogenous antioxidant processes to protect the neurons from H_2O_2 stress. According to the hypothesis that Aβ(1–42)-induced neuronal damage in the neuron/glia coculture was mediated by reactive oxygen species generated from astrocytes (Abramov et al., 2004), promotion of the antioxidant processes in neurons might also contribute to the neuroprotective effect of T-817MA against Aβ(1–42)-induced neuronal damage in the neuron/glia coculture, although effects of T-817MA on glia cells cannot be excluded. There are many evidences suggesting that oxidative stress might be responsible for dysfunction or death of neuronal cells in AD (Butterfield et al., 2002; Markesbery et al., 2001, Mattson et al., 2001). Oxidative stress in particular has been shown to be one of the earliest changes in disease pathogenesis in AD (Nunomura et al., 2001). On these presuppositions the antioxidative effect of T-817MA which may be able to slow down the disease progression in the early stage of AD, would be beneficial for AD treatment.

In the present study, T-817MA significantly increased neurite outgrowth. In order to evaluate neurite outgrowth we utilized an anti-GAP-43 antibody. GAP-43 is expressed in an axon and a growth cone at high levels during periods of axon elongation (Goslin et al., 1998; Meiri et al., 1986; Schereyer et al., 1997) and was reported to be a useful tool
for visualizing the sprouting of neuronal axons (McKinney et al., 1997). In the quantitative analysis with the cortical reaggregation culture, T-817MA also significantly increased GAP-43 immunoreactivity, reflecting an increase in the neurite length. Based on these results, T-817MA is considered to promote the effect of axonal outgrowth in CNS neurons.

In AD, Aβ deposition is considered to cause disruption of the neural network including progressive synaptic degeneration and neuronal loss, which consequently results in cognitive dysfunction and behavioral abnormalities (Stepanichev et al., 2004). Hence reconstructing the damaged neural network is a possible therapeutic target of the disease. The neurite projection has the potential to form a target-oriented and active synapse, and subsequently could reproduce functional connections (Li et al., 1994; McKinney et al., 1999; Stoppini et al., 1993). Therefore promoting neurite outgrowth is supposed to be essential for reconstructing the damaged neural network in AD and various other neurodegenerative diseases. It is reported that some substances, that possess neurite outgrowth promoting effect in vitro are useful in treatment of AD (Gillespie, 2003; O’Neill et al., 2004; Tohda et al., 2004).

In the present in vitro investigations T-817MA was demonstrated to have both a neuroprotective effect and a neurite outgrowth promoting effect at the same
concentration range. Although, the subcellular mechanisms underlying these pharmacological effects are not known, this set of features is similar to that of neurotrophins. These features are supposed to be important for the maintenance of the nervous system and for regulating certain aspects of neuronal survival. Activation of neurotrophic signaling pathways can protect neurons in animal and cell culture models of neurodegenerative diseases such as AD. Neurotrophic factors may protect neurons against age-related degeneration by modulating neurodegenerative cascades and stimulating survival-promoting mechanisms (Mattson et al., 2001). For example, brain derived neurotrophic factor stimulates the production of various factors, such as antioxidant enzymes and anti-apoptotic protein for protection against oxidative insult relevant to the pathogenesis of AD and other neurodegenerative diseases (Mattson et al., 2001). IGF-1 has a well described neuroprotective effect against excitotoxic, metabolic and oxidative insults in various experimental models for AD, and it promotes neurogenesis and synaptic formation throughout the brain and IGF-1 is actively transported across the blood-brain barrier (Heck et al., 1999; Gasparini and Xu, 2003; Mattson et al., 2001; Wei et al., 2002). Therefore IGF-1 has been indicated as a potential therapeutic target of AD (Gasparini and Xu, 2003). In spite of these benefits, in general, the therapeutic application of neurotrophic factors themselves to neurodegenerative
diseases is strictly limited because of their poor stability and poor CNS penetration of many of the neurotrophic factors. Considering these limitations, a neurotrophic factor-like small chemical molecule, such as T-817MA having good CNS penetration (brain level of T-817MA was about 10 times higher than blood level after oral administration: unpublished in house data), might be more favorable for therapeutic use from the viewpoint of drug delivery.

In conclusion, T-817MA exerts a neuroprotective effect and promotes neurite outgrowth in rat primary cultured neurons, indicating that this compound might have a potential for disease modification and to be useful for patients with neurodegenerative diseases, such as AD.
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Figure Legends

**Fig. 1.** Chemical structure of T-817MA

**Fig. 2.** Effect of T-817MA on Aβ(1–42)-induced neuronal death. Aβ(1–42)-induced neuronal death and the neuroprotective effect of T-817MA were assessed using rat neuron/glia coculture. Images of MAP2 immunocytochemistry showed typical responses: Neurons with vehicle treatment (Normal: A), 10 µM Aβ(1–42) treatment for 48 h (Control: B) and co-treatment of 1 µM T-817MA with 10 µM Aβ(1–42) treatment for 48 h (C). Calibration bar indicates 200 µm. D: Neuronal survival was estimated as MAP2-immunoreactivity (IR) using enzyme immunoassay. Results are expressed as absorbance at 490 nm. Columns and bars indicate the mean with S.E.M. (n=6). **P<0.01 vs. Control (Dunnett test) and #P<0.01 vs. Normal (Student’s t-test).**

**Fig. 3.** Effect of T-817MA on H₂O₂-induced neuronal death. H₂O₂ (100 µM for 24 h)-induced neuronal death and the neuroprotective effect of T-817MA were assessed with using rat neuron/glia coculture. T-817MA was pretreated for 24 h (A) or for 5 min (B) and was continuously existed with H₂O₂. Neuronal survival was estimated as MAP2-IR using enzyme immunoassay. Results are expressed as absorbance at 490 nm.
Columns and bars indicate the mean with S.E.M. (n=6). *P<0.05, **P<0.01 vs. Control (Dunnett test) and #P<0.05, ##P<0.01 vs. Normal (Student’s t-test).

**Fig. 4.** Effect of T-817MA on H₂O₂-induced neuronal death and GSH reduction.

Primary cultures of cortical neurons were pre-treated with several concentrations of T-817MA for 24 h, then exposed to 30 µM H₂O₂ for 24 h (A) or 4 h (B) in the continuous presence of T-817MA. Neuronal survival was estimated as MAP2-IR using enzyme immunoassay. Graph C shows GSH contents in the culture assessed with 4-hour H₂O₂ treatment, the same condition as B. Results are expressed as absorbance at 490 nm (A and B) or GSH contents (ng/well). Columns and bars indicate the mean with S.E.M. (n=6). *P<0.05, **P<0.01 vs. Control (Dunnett test) and #P<0.05, ##P<0.01 vs. Normal (Student’s t-test).

**Fig. 5.** Effect of T-817MA on neurite outgrowth in rat hippocampal slice cultures.

Typical images of neurite response with GAP-43 immunostained neurites are shown in Control (A) and T-817MA 1 µM for 8 days (B). Calibration bar indicates 500 µm. C: The neurite outgrowth was quantified by measuring the distance from the edge of the
slice to the tip of the longest neurite (µm). Data are shown as mean with S.E.M. (n=10).

**P<0.01 vs. Control (Dunnett test).

**Fig. 6.** Effect of T-817MA on neurite outgrowth in rat cortical reaggregation cultures.

Typical images of neurite response with GAP-43 immunostained neurites are shown in Control (A) and T-817MA 1 µM for 4 days (B). Calibration bar indicates 500 µm. C: The neurite outgrowth was quantified by measuring the distance from the edge of the aggregate to the tip of the longest neurite (µm). Quantitative analysis was performed by GAP-43 IR (D) using enzyme immunoassay and its results are expressed as absorbance at 490 nm. Data were shown as mean with S.E.M. (C: n=8, D: n=4). **P<0.01 vs. Control (Dunnett test).
Figure 2

Neuronal Survival (A490nm)

0.00 0.200 0.300 0.400 0.500

Normal

Control

Aβ(1-42), 10 µM

T-817MA, µM

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Figure 3

**A**

- Neuronal Survival (A490nm)
- T-817MA, µM (24 h)
- H₂O₂, 100 µM

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**B**

- Neuronal Survival (A490nm)
- T-817MA, µM (5 min)
- H₂O₂, 100 µM

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Figure 4

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H2O2 30 µM, 4h

T-817MA, µM

Normal Control

0.01 0.1 1

GSH contents (ng/well)

20 40 60 80 100 120 140

A

B

C

H2O2 30 µM, 24h

Normal Control

0.01 0.1 1

Neuronal Survival (A490nm)

0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80

* ** #

# *

** #

0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80

# **

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Figure 5

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Figure 6

Neurite length (µm)

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Control 0.1 1

T-817MA, µM

GAP-43 IR (A490nm)

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