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*Short Communication*

# Protective Effect of T-588 on Toxic Damage by Serum Deprivation and Amyloid-$\beta$ Protein in Cultured Neurons

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**Abstract.** The present study examines the effect of the cognition enhancer (1*R*)-1-benzo[*b*]thiophen-5-yl-2-[2-(diethylamino)ethoxy]ethan-1-ol hydrochloride (T-588) on neuronal injury induced by serum deprivation or amyloid-$\beta$ protein (A$\beta$). T-588 protected partially against neuronal injury induced by serum deprivation or A$\beta$ in cultured cortical neurons. T-588 did not affect the phosphorylation of extracellular signal-regulated kinase (ERK) in cortical neurons and SH-SY5Y cells. These results suggest that T-588 has a protective effect in neuronal injury models and the effect is not mediated by an ERK signal pathway.

***Keywords***: T-588, neuronal death, extracellular signal-regulated kinase

(1*R*)-1-Benzo[*b*]thiophen-5-yl-2-[2-(diethylamino) ethoxy]ethan-1-ol hydrochloride (T-588) has been selected for development as a therapeutic agent for reversing the dementia associated with Alzheimer's disease and cerebrovascular disease. This compound has an anti-hypoxic effect in mice (1) and ameliorates memory and learning impairments in animal models including cerebral embolization, basal forebrain lesion, and transient forebrain ischemia (2). However, the molecular mechanisms underlying these effects are not known. We have reported that T-588 protects astrocytes against hydrogen peroxide ($H_2O_2$)-induced injury via activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (3). However, whether T-588 stimulates ERK phosphorylation in neurons in addition to in astrocytes is not known. In this paper, we examine the effect of T-588 on neuronal injury induced by amyloid-$\beta$ protein (A$\beta$) or serum deprivation and the possible involvement of the ERK signal pathway.

Drugs were obtained from the following sources: fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), nerve growth factor (NGF), A$\beta$ fragment 25 – 35 (A$\beta_{25-35}$) (Sigma, St. Louis, MO, USA); mouse anti-microtubule-associated protein-2 antibody (Chemicon International, Inc., Temecula, CA, USA); fluorescein-conjugated goat anti-mouse IgG antibody (Organon Teknika N.V.-Cappel Product, West Chester, PA, USA); brain-derived neurotrophic factor (BDNF), Dulbecco's modified eagle medium, horse serum (Gibco BRL, Rockville, MD, USA); phospho-p44/42 MAPK antibody, p44/42 MAPK antibody (New England Biolabs, Beverly, MA, USA); T-588 was a gift from Toyama Chemical (Toyama). All other chemicals used were of the highest purity commercially available. A$\beta_{25-35}$ was dissolved in water and incubated at 37°C for 24 h before use.

Primary culture of cortical neurons was prepared as described previously (4, 5). Briefly, cerebral cortices were removed from 18-day-old embryonic rat fetuses and dissociated using papain/DNase I. Neurons were seeded at a density of $1 \times 10^6$ cells on 24-well plastic tissue culture plates and cultured in Dulbecco's modified Eagle medium containing 5% fetal bovine serum, 5%

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horse serum, 2 mM sodium pyruvate, 30 nM selenate, and antibiotics. Cells were placed in a humidified atmosphere of 95% air and 5% $CO_2$ for 24 h, and then the cells were treated with 10 $\mu$M 5-fluoro-2'-deoxyuridine for 45 h. The medium was changed every 3 days. After 7 – 8 days, the cells were used for colorimetric assay and collected for Western blot analysis. More than 90% of the cells were neurons, as confirmed by phase-contrast microscopy and positive immunostaining with anti-microtubule-associated protein-2 antibody. The SH-SY5Y cell line was a gift from Dr. Wolfgang Sadee (University of California, San Francisco). The cells were cultured in RPMI1640 medium containing 10% fetal bovine serum and antibiotics (6) and were plated at $5 \times 10^4$ cells per well in 48-well plastic tissue culture plates.

The colorimetric MTT reduction assay was performed as previously described (3). This method assesses mitochondrial activity by measuring the ability of viable cells to reduce MTT to a colored formazan. In general, each culture well was incubated in culture medium containing 0.05 mg/ml MTT for 2 h in 5% $CO_2$ at 37°C. The cells were lysed thoroughly with lysis buffer (20% sodium dodecylsulfate (SDS), 50% *N*,*N*-dimethylformamide) and the absorbance at 570 nm was measured. MTT reduction activity is expressed as a percentage of the control.

The neuronal cells were washed with phosphate-buffered saline and harvested. The cells were solubilized in sample buffer (3% SDS, 62.5 mM Tris-HCl (pH 6.8), and 10% glycerol). The protein concentration in the sample was determined by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). The sample was mixed with 125 mM Tris-HCl (pH 6.5) containing 0.25% (w/v) bromophenol blue, 0.05% (v/v) 2-mercaptoethanol, 25% glycerol and 5% SDS; boiled for 5 min; and then loaded (equal amount of protein /lane) on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane and immunoblotting was carried out as reported previously, using phospho-p44/42 MAPK antibody and horseradish peroxidase-conjugated anti-rabbit antibody (3). Protein bands were detected using an enhanced chemiluminescence system.

Data were analyzed by one-way ANOVA followed by the Dunnett test (Fig. 1A) and Student's *t*-test (Fig. 1B). Statistical analyses were performed with a software package (StatView 5.0; SAS Institute, Inc., Cary, NC, USA). *P* values of 5% or less were considered statistically significant.

Previous studies showed that serum deprivation and A$\beta_{25-35}$ cause apoptosis in cultured cortical neurons (7, 8). Figure 1 shows the effect of T-588 on neuronal injury induced by serum deprivation or A$\beta_{25-35}$. Serum deprivation for 96 h or treatment with 10 $\mu$M A$\beta_{25-35}$ for 48 h decreased MTT reduction activity in cultured cortical neurons. The decrease in MTT reduction activity was due to cell death, especially apoptosis, since the number of microtubule-associated protein 2-immunopossitive cells was decreased and Hoechst 33342 staining showed

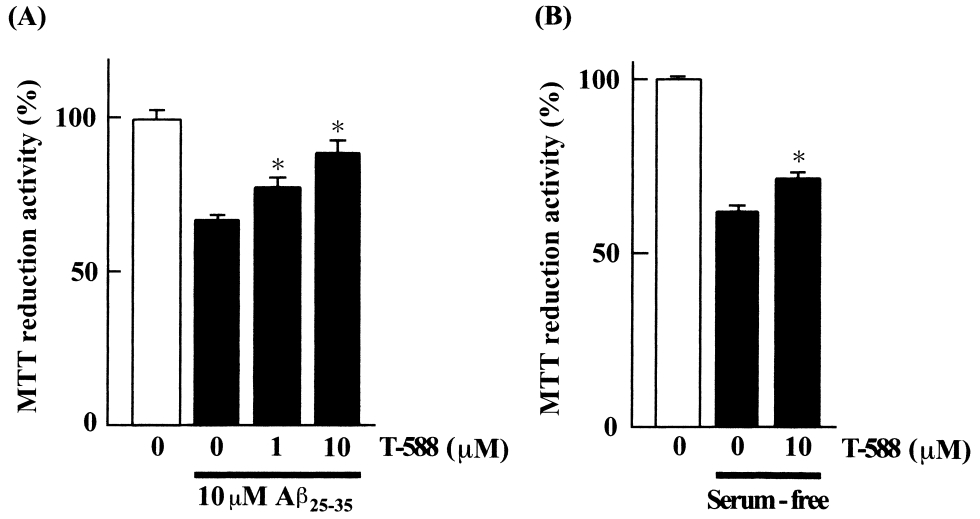


**Fig. 1.** Effect of T-588 on neuronal injury induced by serum deprivation or A$\beta_{25-35}$ in cultured rat cortical neurons. A: Effect of T-588 on A$\beta$-induced neuronal injury. The indicated concentrations of T-588 were added 24 h before A$\beta_{25-35}$ (10 $\mu$M) exposure and then incubated for 48 h. B: Effect of T-588 on serum deprivation-induced neuronal injury. T-588 was added 24 h before serum deprivation and then incubated for 96 h. Results are means ± S.E.M of 9 – 15 wells and were obtained from 3 to 5 separate experiments. *$P$<0.05, compared with neuronal injury group without T-588.

nuclear condensation (data now shown). BDNF at 50 ng/ml showed a significant protection against A$\beta_{25-35}$-induced toxic damage in cultured neurons (data not shown). T-588 at 1 – 10 $\mu$M protected cultured neurons against A$\beta_{25-35}$-induced toxic damage in a dose-dependent way (Fig. 1A). T-588 at 10 $\mu$M also protected the neurons against serum deprivation-induced damage (Fig. 1B). The effective concentrations are similar to those in the previous study in astrocytes (3). It should be noted that T-588 at 10 $\mu$M appeared to be more protective against A$\beta_{25-35}$-induced damage than that induced by serum deprivation. This suggests the difference in mechanism between neuronal injury models induced by A$\beta_{25-35}$ and serum deprivation. T-588 at 10 $\mu$M did not affect the levels of phospho-ERK in cultured neurons and SH-SY5Y cells (Fig. 2).

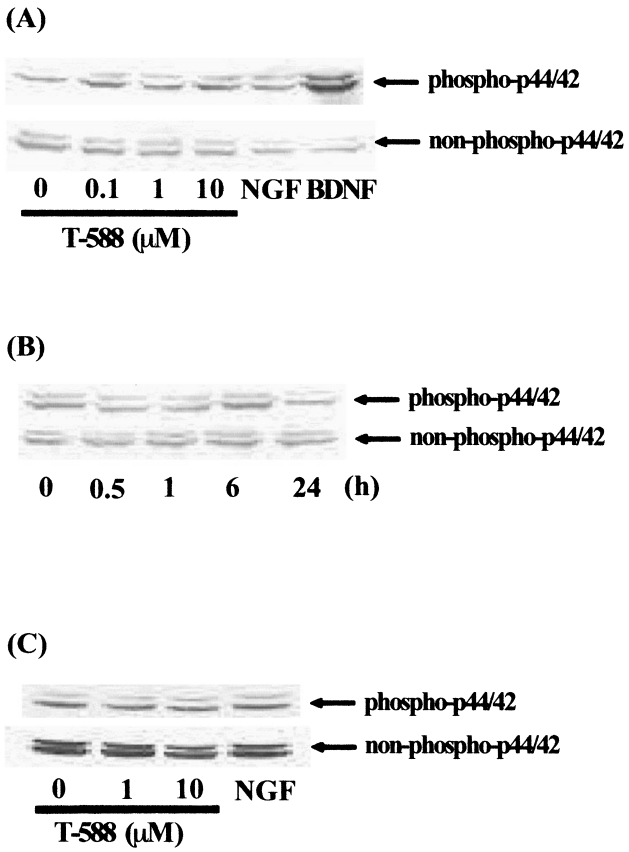


**Fig. 2.** Effect of T-588 on ERK phosphorylation in cultured rat cortical neurons or neuroblastoma SH-SY5Y cells. A typical experiment is shown. A: The rat cortical neurons were treated with T-588 at different concentrations (lane 1, none; lane 2, 0.1 $\mu$M; lane 3, 1 $\mu$M; lane 4, 10 $\mu$M) for 10 min; and the cell extract (20 $\mu$g) was used for immunoblotting using phospho-ERK (upper) and non-phospho-ERK (lower) antibodies. Lane 5 shows the effect of 50 ng/ml NGF on phospho-ERK as a negative control, and lane 6 shows the effect of 50 ng/ml BDNF on phospho-ERK as a positive control. B: The rat cortical neurons were treated with 10 $\mu$M T-588 for different times (lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; lane 4, 6 h; lane 5, 24 h); and the cell extract (20 $\mu$g) was used for immunoblotting using phospho-ERK (upper) and non-phospho-ERK (lower) antibodies. C: The neuroblastoma SH-SY5Y cells were treated with T-588 at different concentrations (lane 1, none; lane 2, 1 $\mu$M; lane 3, 10 $\mu$M) for 10 min; and the cell extract (20 $\mu$g) was used for immunoblotting using phospho-ERK (upper) and non-phospho-ERK (lower) antibodies. Lane 4 shows the effect of 50 ng/ml NGF on phospho-ERK as a negative control.

Since an ERK signal pathway is proposed to play a role in cell survival (9 – 12), the ERK signal pathway may be a target for drugs to ameliorate ischemia/reperfusion injury. We have previously demonstrated that T-588 protected against $H_2O_2$-induced cell injury via stimulation of ERK phosphorylation in cultured astrocytes (3). Furthermore, we found that the neuroprotective agent CV-2619 inhibited the $H_2O_2$-induced astrocyte injury via the ERK signal pathway (13). These observations suggest that the ERK signal pathway may be a target for drugs to protect against cell toxicity. In this study, we examined whether the ERK signal pathway is involved in the effect of T-588 in cultured neurons. We found that T-588, like BDNF, which was reported to stimulate MAPK (8), had a neuroprotective effect in cultured neurons. However, we failed to observe any stimulatory effect of T-588 on ERK phosphorylation in the cells. In addition, T-588 did not affect ERK phosphorylation in the human neuroblastoma SH-SY5Y cells. The SH-SY5Y cells were used as a pure neuronal preparation, since cultured cortical cells used here contained not only neurons but also other cells such as astrocytes and microglia. These results suggest that T-588 has a protective effect in neuronal injury models and its effect is not mediated by an ERK signal pathway. It is not known why the ERK signal pathway differs in the response to T588 between neurons and astrocytes, although it is likely that T-588 affects indirectly ERK phosphorylation in astrocytes. Nevertheless, the previous (3) and present findings imply that T-588 has a neuroprotective effect via the interaction not only with astrocytes but also with neurons.

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