

Effects of NK-4, a Cyanine Dye with Antioxidant Activities: Attenuation of Neuronal Deficits in Animal Models of Oxidative Stress-Mediated Brain Ischemia and Neurodegenerative Diseases

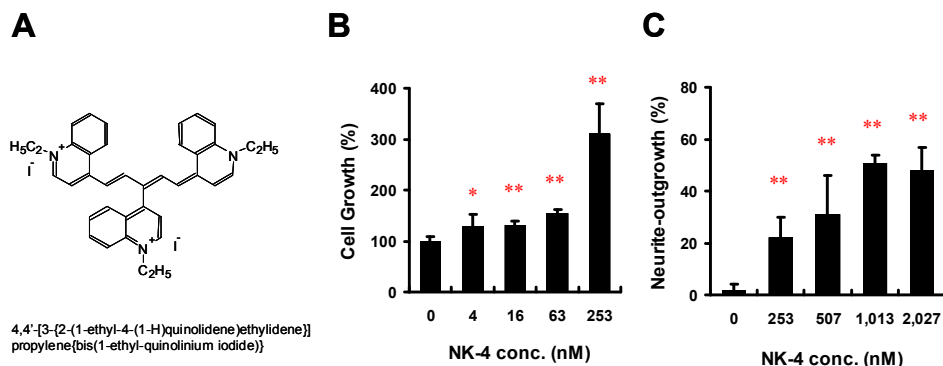
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1. Introduction

Cyanine photosensitizing dyes have been studied for over 150 years and continue to be of interest in their biology and medicine. They have been shown to possess various biological activities, including antimicrobial, antioxidant, macrophage activating, and oxidative phosphorylation uncoupling activities (Hayami, 1984; Ushio et al., 2009; Ishihara & Fujisawa, 2007; Kunikata et al., 2002; Takeguchi et al., 1985). Some dyes have been used as immunomodulators to treat allergy and rheumatoid arthritis, cancer, and to promote wound healing (Trauner et al., 1998; Motoyoshi et al., 1991). Since cumulative evidence suggests an involvement of oxidative stress and neuroinflammation as the common feature in the pathogenesis of neurodegenerative disorders (Amor et al., 2010; Wolozin & Behl, 2000a, 2000b), it would be reasonable to expect that cyanine dyes with anti-oxidative and anti-inflammatory properties could protect the central nervous system from neuroinflammatory-related brain insults.

Recently, we screened more than 250 cyanine dyes for their neurotrophin-like activity and found that NK-4 and some other related compounds are potent neurotrophic agents for the promotion of growth and differentiation of neuronal rat adrenal pheochromocytoma cell line PC12. NK-4 is a divalent cationic pentamethine trinuclear cyanine dye that contains three quinolinium rings, N-alkyl side chains, and two iodine anions (Fig. 1A). Addition of NK-4 into the culture at nanomolar concentrations significantly augmented cell growth of PC12 cells in 3-day cultures (Fig. 1B). NK-4 also promoted nerve growth factor (NGF)-primed neurite-outgrowth at micromolar and submicromolar concentrations (Fig. 1C). Since the intervention using neurotrophic or neuroprotective small molecules is thought to have potential for treating neurodegenerative disorders, we investigated the neuroprotective effects of NK-4 against neurotoxic insults *in vitro* and further evaluated its pharmacological effects using animal models of neurodegeneration, including ischemic stroke, cerebellar ataxia, and Alzheimer's disease (AD).

In this chapter, we focus on the neuroprotective effects of NK-4 against oxidative damage *in vitro* and *in vivo*. We introduce the multipotent properties of NK-4, which may act in concert to attenuate the common pathological pathways of neurodegeneration, and discuss the potential use of NK-4 for neurodegenerative disease therapy.



(A) Chemical structure of NK-4. It was synthesized in HAYASHIBARA Co., Ltd. (B) The proliferative effect of NK-4 on PC12 cells. Cell growth was assessed by AlamarBlue® assay in 3-day cultures. (C) The promotive effect of NK-4 on NGF (5 ng/ml)-primed neurite-outgrowth in PC12 cells. Neurite-outgrowth was evaluated by counting neurite-outgrowth positive cells in 3-day cultures. The cells were defined as positive when the length of the longest neurite was >2-fold longer than that of the cell body. Data are means \pm SD (n=3). *P<0.05 and **P<0.01 vs. control (no NK-4).

Fig. 1. Chemical structure and neurotrophin-like effects of NK-4.

2. In Vitro Properties of NK-4

2.1 Free radical-scavenging activity

As it has been known that some cyanine dyes show significant antioxidative property due to the extended π -electron conjugated system contained within the structure (Ishihara & Fujisawa, 2007), the free radical-scavenging capacity of NK-4 for superoxides, hydroxyl, and peroxy radicals was evaluated (Koya-Miyata et al., 2010). These radicals are produced *in vivo* in many experimental models of ischemia and reperfusion, and they are therefore generally regarded as the primary free radicals involved in oxidative stress-mediated injury. Half-maximal inhibitory concentrations (IC₅₀) of NK-4 against these radicals are shown in Table 1.

Ascorbate, a well-characterized free radical-scavenging agent, and edaravone were used as positive controls. Edaravone, also known as MCI-186, is a hydroxy radical scavenger, and has been used for acute phase stroke therapy in Japan (Abe & Kogure, 1988). NK-4 displayed a direct and powerful hydroxyl radical-scavenging activity, significantly greater than those of ascorbate and edaravone. The scavenging activity for peroxy radicals was also potent in NK-4 and to a lesser extent in ascorbate. NK-4 and ascorbate acted on superoxides to a comparable extent; however, edaravone did not act on superoxides as previously reported (Tanaka, 2002). The highly reactive hydroxyl radical oxidizes cellular lipids, proteins, and DNA, leading to cell death, and is therefore highly detrimental (Gilgun-Sherki

et al., 2002). Accordingly, the antioxidants that effectively scavenge hydroxyl radicals and other free radicals should be able to eliminate oxidative injury (Barinaga, 1996), and NK-4 could be one of such molecules.

Compound	IC ₅₀ (μM)		
	Hydroxyl Radical	Peroxy Radical	Superoxide
NK-4	7.6 ± 0.6	5.2 ± 2.5	107 ± 2.2
Ascorbate	47.6 ± 0.2	30.5 ± 1.7	130 ± 24
Edaravone	779 ± 18	181 ± 19	> 3,800

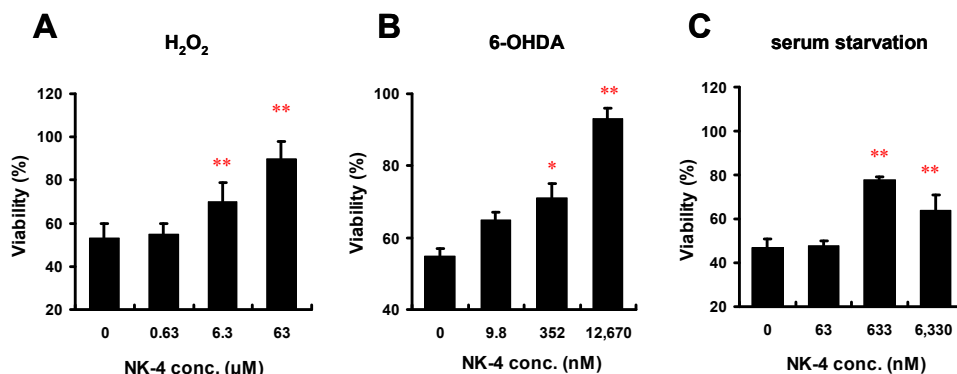
Free radical-scavenging efficacy was evaluated in a cell-free *in vitro* system using ESR. Superoxides, hydroxyl radicals, and peroxy radicals were produced and stabilized in DMPO-hypoxanthine/XOD, DMPO-DTPA/H₂O₂/FeSO₄ and DMPO-AAPH systems, respectively. The signal intensity of each radical was recorded in the presence or absence of test samples. The scavenging activity of the samples was expressed as IC₅₀ against each radical. Means ± SD (n=3). Reproduced in part with permission from Biological & Pharmaceutical Bulletin Vol.33 No.11. Copyright [2010] Pharmaceutical Society of Japan.

Table 1. Free radical-scavenging effects of NK-4 and other antioxidants.

2.2 Neuroprotective effects against various cytotoxic stresses

Neurotrophins, such as NGF or brain-derived neurotrophic factor (BDNF) regulate the growth, survival, and differentiation of central neurons (Blum & Konnerth, 2005). For example, NGF protects PC12 cells against 6-hydroxydopamine (6-OHDA)- and hydrogen peroxide (H₂O₂)-induced oxidative stress (Salinas et al., 2003; Wang et al., 2001). Since NK-4 displayed a remarkable neurotrophin-like activity, namely the promotion of cell growth and NGF-primed neurite-outgrowth in PC12 cells (Fig. 1B, 1C), we next examined whether NK-4 protects neuronal cells from oxidative or starvation stress (Ohta et al., 2011). An acute oxidative stress challenge was induced by 2-hr treatment with H₂O₂ or 24-hr treatment with 6-OHDA. These treatments cause a significant decrease in metabolic capacity, which reflects a decline in cell viability, and this is accompanied by an enhancement of apoptotic markers (Franco et al., 2010). Particularly, 6-OHDA has been widely used in experimental models of Parkinson's disease, and its neurotoxicity involves oxidative damage to catecholaminergic neurons via the generation of hydroxyl radicals, monoamine oxidase-mediated formation of H₂O₂, and mitochondrial generation of superoxide (Blum et al., 2001).

Treatment of PC12 cells with H₂O₂ decreased cell viability to approximately 50% of control. In contrast, NK-4 at concentrations of 6 μM or above significantly protected the cells from the acute oxidative damage (Fig. 2A). NK-4 also attenuated 6-OHDA-induced oxidative stress in PC12 cells at nanomolar concentrations in a dose-dependent manner (Fig. 2B). Next we tested the effect of NK-4 on trophic factor depletion-induced cell death. Extended serum depletion in PC12 cells triggers ATP shortage, which results in mitochondrial reactive oxygen species (ROS) generation and activation of apoptotic pathways (Troy et al., 2001). NK-4 at submicromolar concentrations significantly increased cellular viability of PC12 cells over 3 days of serum starvation (Fig. 2C). These results suggest that the antioxidative effects of NK-4, together with its neurotrophic properties, engender strong survival signals in PC12 cells.



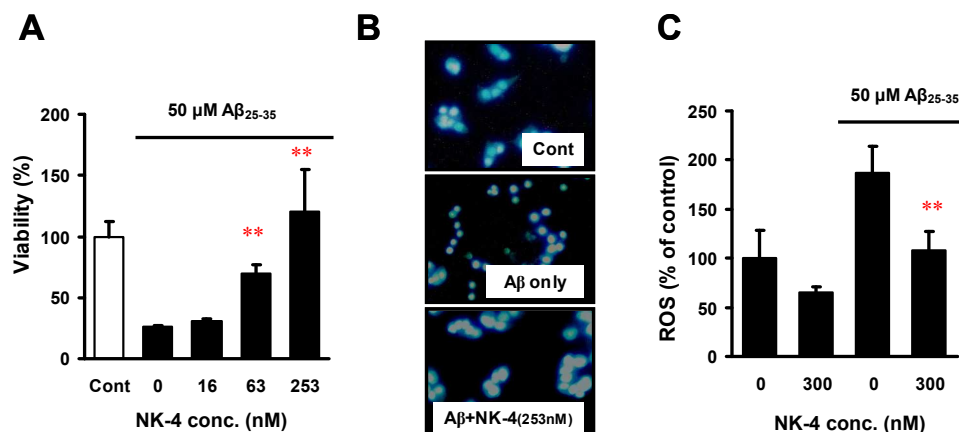
PC12 cells were seeded at a density of 2×10^4 cells/well and cultured in serum supplemented D-MEM for 24-hr at 37°C in collagen-coated microplates. (A) H_2O_2 (200 μM) or (B) 6-OHDA (100 μM) was applied in the presence or absence of indicated concentrations of NK-4 and incubated for 2-hr or 24-hr, respectively. (C) After the pre-culture, serum-containing D-MEM was replaced with serum-free medium and the cells were cultured for additional 3 days. Cell viability was assessed by the AlamarBlue® method. Results are shown as means \pm SD ($n=3$). * $P<0.05$ and ** $P<0.01$ vs. no NK-4.

Fig. 2. Protective effect of NK-4 from cytotoxic stresses in PC12 cells.

2.3 Neuroprotective effect against β -amyloid ($A\beta$) toxicity

Next, we determined whether NK-4 was effective against β -amyloid ($A\beta$)-induced neurotoxicity *in vitro* using PC12 cells (Ohta et al., 2010a). PC12 cells are reported to be highly sensitive to $A\beta$ peptide or the aggregated $A\beta_{25-35}$ fragment (Shearman et al., 1994). The viability of PC12 cells treated with 50 μM of aged $A\beta_{25-35}$ for 72-hr was about 25% compared with controls without $A\beta_{25-35}$ (Fig. 3A). NK-4 dose-dependently attenuated the cytotoxic effect of $A\beta_{25-35}$ and the effects were significant at doses over 60 nM. $A\beta$ peptides induce morphological changes associated with apoptotic cell death, such as somal shrinkage, plasma membrane blebbing, chromatin condensation, and nuclear fragmentation (Ivins et al., 1999). Nuclear staining by Hoechst 333442 dye demonstrated the typical nuclear fragmentation in $A\beta_{25-35}$ -treated PC12 cells and this was clearly inhibited by the addition of NK-4 (Fig. 3B).

Since PC12 cells undergoing $A\beta$ -mediated apoptosis produce large amounts of ROS due to deficits in mitochondrial function (Kadowaki et al., 2005), we next examined ROS generation in $A\beta_{25-35}$ -treated PC12 cells (Fig. 3C). Compared with the control, the amounts of intracellular ROS significantly increased after addition of 50 μM $A\beta_{25-35}$ in 48-hr cultures, and 300 nM of NK-4 almost totally suppressed the ROS induction. Under normal culture conditions, NK-4 also decreased basal ROS levels in normal PC12 cells; however, the effect was not statistically significant. These data suggest that NK-4 attenuates $A\beta$ -mediated cytotoxicity by reducing the unfavorable ROS accumulation in PC12 cells, probably through the restoration of mitochondrial function.



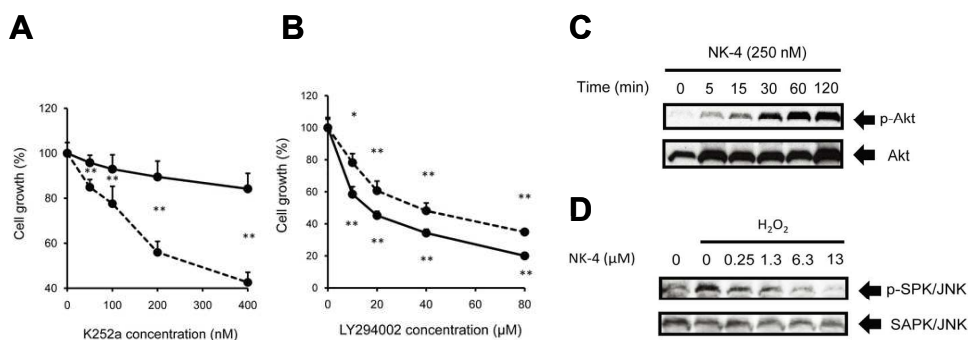
(A) A β_{25-35} was dissolved in saline and incubated at 37 °C for 4 days, which is termed an “aging” process. PC12 cells were treated with 50 μ M aged A β_{25-35} for 72-hr in the absence or presence of the indicated concentrations of NK-4. Cell viability was assessed by AlamarBlue® assay. Results are shown as means \pm SD (n=3). **P<0.01 vs. no NK-4. (B) PC12 cells were treated as (A) and stained with Hoechst 33342 dye. (C) PC12 cells were treated as (A), but for 48 hr and the amount of intracellularly produced ROS was assessed by H₂DCF-DA dye fluorescence (ex.492nm/em.527nm). Results are shown as means \pm SD (n=3). **P<0.01 vs. no NK-4.

Fig. 3. Cytoprotective effects of NK-4 on A β_{25-35} -induced cytotoxicity in PC12 cells.

2.4 Intracellular signaling

Neurotrophins act by binding to two kinds of plasma membrane receptors, the Trk receptor tyrosine kinases (Trks) and the p75 pan-neurotrophin receptor (p75^{NTR}). There are several subtypes of Trk receptor kinases characterized by their specific affinities for different neurotrophins. NGF binds preferentially to TrkA, whereas BDNF and neurotrophin-4/5 show a high affinity for TrkB (Berg et al., 1992). To address whether the effects of NK-4 are mediated by Trk activity, K252a, a non-specific inhibitor of Trks, was applied in growth assays of PC12 cells. Pretreatment with K252a dose-dependently inhibited NGF-induced cell growth, but did not inhibit growth induced by NK-4 (Fig. 4A). This suggests that NK-4 acts independently of TrkA activation in PC12 cells. A similar inhibitory profile of K252a was also found in the neurite-outgrowth of PC12 cells (data not shown).

We next examined whether NK-4 activates phosphatidylinositol 3-kinase (PI3K) and its downstream signaling effector Akt. This cascade is implicated in survival signaling mediated by NGF in serum-deprived PC12 cells, and in neurogenesis in PC12 cells (Martin et al., 2004; Kim et al., 2004). LY294002, a specific PI3K inhibitor (Vlahos et al., 1994), blocked the cell growth-promoting activity of both NK-4 and NGF (Fig. 4B). Also, as shown in figure 4C, NK-4 strongly induced phosphorylation of Akt at Ser 473 in a time-dependent manner. The dose of NK-4 required for the induction of Akt phosphorylation was consistent with that required for promotion of PC12 cell growth. These results suggest that sequential activation of PI3K and its downstream signaling effector Akt are important for NK-4-induced neurotrophic effects in PC12 cells.



(A, B) Effects of K252a or LY294002 on PC12 cell growth induced by NK-4 (bold line) or NGF (broken line). PC12 cells were preincubated in serum-free D-MEM with the indicated concentrations of K252a (A) or LY294002 (B) for 15 min. NK-4 (250 nM) or NGF (50 ng/ml) was added and the cells were further incubated for 72-hr. Cell viabilities were assessed by AlamarBlue® assay. Values are means \pm SD ($n=3$). * $P<0.05$, ** $P<0.01$ vs. control. (C) PC12 cells were treated with 250 nM NK-4 for the indicated times. Whole cell lysates were analyzed by Western blotting using an anti-phospho-Akt (Ser 473) mAb (upper) or an anti-Akt mAb (lower). (D) PC12 cells were treated with 400 μ M H_2O_2 for 2-hr in the presence of the indicated concentrations of NK-4. Phospho-SAPK/JNK (upper) and SAPK/JNK (lower) were analyzed by Western blotting using whole cell lysates.

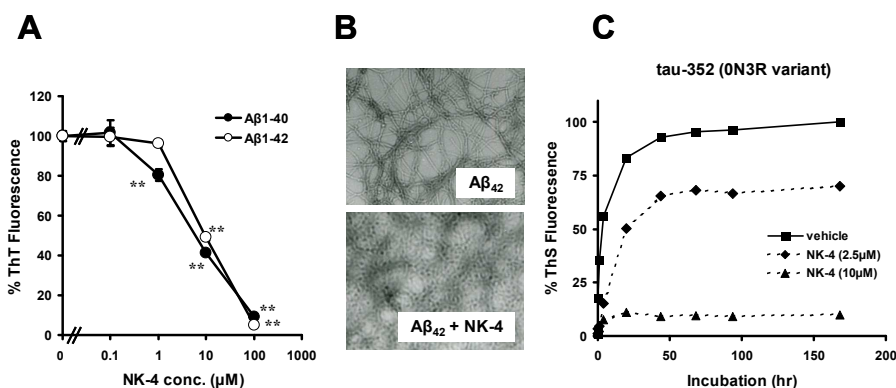
Fig. 4. NK-4 alters phosphorylation of Akt and SAPK/JNK in a Trk-independent manner.

On the other hand, we also found that the SAPK/JNK stress-induced signaling pathway was altered by NK-4. SAPK/JNK is a major cellular stress-responsive protein activated by oxidative stress. Activated SAPK/JNK translocates from the cytosol to the nucleus and regulates the activity of transcription factors such as c-Jun, ATF-2, p53, SMAD4, and Elk-1 (Kyriakis & Avruch, 2001). PC12 cells treated with cytotoxic levels of H_2O_2 displayed augmented phosphorylation of SAPK/JNK, and this was attenuated by NK-4 in a concentration-dependent manner (Fig. 4D). This observation suggests that NK-4 might attenuate H_2O_2 -induced cellular stress upstream of SAPK/JNK.

2.5 Anti-aggregative effect on A β and tau

To examine the mechanisms of NK-4-mediated protection from amyloid toxicity in neuronal cells, we evaluated the effect of NK-4 on fibrillization of A β (Fig. 5), since oligomerized or aggregated A β seems more toxic to neuronal cells (Lesne et al., 2006, Shearman et al., 1994). ThioflavinT (ThT) fluorescence is enhanced upon binding to A β fibrils in proportion to the amount of fibrils in solution (Lashuel et al., 2002). Therefore, the effect of NK-4 on A β fibril formation was evaluated using ThT (Fig. 5A). In the absence of NK-4, both A β_{1-40} and A β_{1-42} solutions displayed greatly enhanced emission at 482 nm, which is characteristic for ThT bound to amyloid fibrils. Significant decreases in ThT fluorescence were detected in the presence of NK-4 at concentrations of 10 μ M or higher against 100 μ M of A β . An equimolar concentration of NK-4 almost totally inhibited the increase in ThT fluorescence. Under electron microscopic observation, A β_{1-42} formed long and dense fibrils after 3 days of incubation (Fig. 5B, upper). In contrast, A β_{1-42} incubated with 10 μ M of NK-4 produced fewer and shorter filaments in soluble assemblies (Fig. 5B, lower).

In addition to A β deposition, intraneuronal tau aggregates in the brain is a hallmark of AD. Since the mutated tau transgenic mouse P301L exhibited a clear correlation between tau aggregates and neurodegeneration (Lewis et al., 2000), small molecule inhibition of tau aggregation is considered a potential therapeutic strategy, alongside A β anti-aggregative strategy (Pickhardt et al., 2007). Self-aggregation of tau protein occurs in the presence of polyanions such as heparin or poly(Glu), and can be quantitatively assessed by thioflavin S (ThS) fluorescence (Friedhoff et al., 1998). Therefore, we evaluated the inhibitory effect of NK-4 on tau aggregation using recombinant human tau-352 protein (0N3R variant, 15 μ M) and heparin (2.5 μ M) (Fig. 5C). As a result, NK-4 reduced ThS fluorescence in a dose-dependent manner and 10 μ M of NK-4 substantially inhibited ThS fluorescence of tau aggregation. This result, together with the inhibition of A β aggregation, strongly suggests that NK-4 is an effective inhibitor of protein aggregation *in vitro*. Probably, NK-4 associates at the interface of the β -sheet domains of proteins by its planar structure and would thus act as a “ β -sheet breaker” (Suh & Checler, 2002).



(A) Effects of NK-4 on A β fibril formation. Solutions of A β 1-40 and A β 1-42 (100 μ M each) were incubated at 37°C for 3 days in the absence or presence of NK-4 at the indicated concentrations. Thioflavin T fluorescence was measured. Values are means \pm SD (n=3). **P<0.01 vs. control. (B) Electron microscopy of A β 1-42 fibrils. The solution of 100 μ M A β 1-42 was incubated for 3 days alone (upper) or with 10 μ M of NK-4 (lower). Incubated A β was adsorbed on copper grid and negatively stained by uranyl acetate, then observed with EM at 80 keV. (C) Effects of NK-4 on tau-352 aggregation. Solutions of tau-352 (15 μ M) were incubated with 2.5 μ M of heparin at 37°C in the absence or presence of NK-4 (2.5 or 10 μ M) and thioflavin S fluorescence was measured.

Fig. 5. NK-4 inhibits A β fibril formation as well as tau aggregation.

2.6 Cholinesterase (ChE) inhibitory activity

As mentioned above, NK-4 showed remarkable neurotrophic and neuroprotective activities *in vitro*, and it might have potential to modulate A β and tau pathologies, which is implicated in AD. In relation to a possible application of NK-4 for AD, we examined whether NK-4 has an inhibitory effect on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) since the progressive deterioration of cholinergic innervation in the cerebral cortex leads to cognitive deficits in AD, and AChE inhibitors are still the primary choice for treatment of AD (Ellis, 2005).

A comparison of IC₅₀ values between NK-4 and other ChE inhibitors suggests that NK-4 is a potent and selective inhibitor of AChE (Table 2). The inhibitory effect of NK-4 against AChE was weaker than that of donepezil, the most popular drug worldwide for the treatment of dementia, but almost comparable to tacrine, and stronger than galantamine (Ohta et al., 2010a). These results suggest that NK-4 might have potential for the amelioration of disturbed cholinergic transmission in AD patients.

Compound	IC ₅₀ (nM)		
	AChE	BChE	Specificity (B/A)
NK-4	88 ± 2.5	> 6,333	> 70
Donepezil	6.7 ± 0.35	7,400 ± 130	1100
Tacrine	77 ± 1.4	69 ± 1.4	0.90
Galantamine	1,200 ± 33	18,000 ± 333	15

Inhibitory activities toward AChE and BChE by NK-4 and other ChE inhibitors are shown. IC₅₀ values of NK-4 for AChE and BChE were determined based on the Ellman method using purified enzymes of human origin. Other data are cited from a review article (Sugimoto, 2004). Values are means ± SD.

Table 2. ChE inhibitory activities and specificity of NK-4 and other ChE inhibitors.

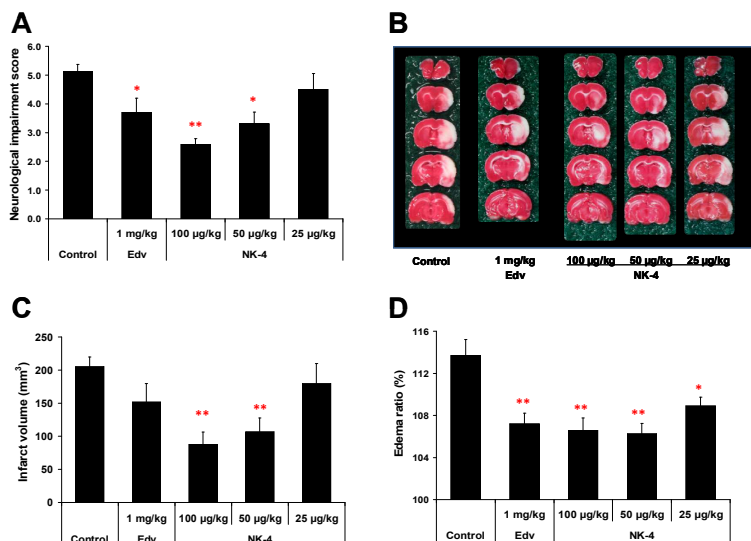
3. Anti-neurodegenerative effects of NK-4

NK-4 exhibits a neurotrophin-like activity and a potent free radical-scavenging capacity *in vitro*. Moreover, it confers significant stress tolerance to neuronal cells via activation of survival signaling pathways. Therefore, we examined whether NK-4 produces effects in animal models of neurodegenerative disorders, including models of ischemic stroke, cerebellar ataxia, and AD.

3.1 Stroke model (MCAO Rats)

Stroke is the second most common cause of death and a major cause of long-lasting disability worldwide. In relation to the massive socio-economic impact of ischemic stroke, neuroprotective agents with different modes of action and/or extended application time windows are therefore urgently needed. We evaluated the effect of NK-4 on ischemic stroke using a focal and transient ischemia model in rats. The middle cerebral artery occlusion (MCAO) method in rats is an animal model of focal brain ischemia (Bederson et al., 1986) frequently used to evaluate drug efficacy.

In the brains of MCAO rats, infarct volume increased mainly in the ipsilateral cerebral cortex and stratum (sites affected by MCAO; Fig. 6B). Administration of NK-4 substantially decreased infarct size (Fig. 6C), especially in the cortex penumbra region, a zone of incomplete cerebral ischemia. Edaravone also reduced infarct volume by approximately 25% compared to the vehicle control, but the difference was not statistically significant (Fig. 6C). Regarding the edema ratio, both NK-4 and edaravone significantly attenuated brain swelling compared to the vehicle control (Fig. 6D). Although edaravone (1,000 µg/kg) and a lower dose of NK-4 (25 µg/kg) failed to significantly reduce infarct size, they still significantly decreased brain edema.



The rat right middle cerebral artery was occluded by a silicon embolizer for 2-hr and then reperused for 24-hr. Drugs were administered intravenously twice at the indicated doses 1-hr after the occlusion and at the start of reperfusion. (A) After the 24-hr reperfusion, neurological impairment scores of rats were rated with a maximum impairment score of 6.0. (B) Representative serial brain sections (2 mm thickness) stained with 2,3,5-triphenyltetrazolium chloride (TTC) after 2-hr MCAO and 24-hr reperfusion. (C) Quantification of hemispheric lesion volumes. Infarct volumes were calculated from serial brain sections by Scion Image software. (D) Cerebral edema ratios were calculated based on the values from volumetric analysis. NK-4 groups, n=5 each; edaravone group, n=8; control group, n=8. Data are means \pm S.E.M. *P<0.05, **P<0.01 vs. vehicle control group. Reproduced in part with permission from Biological & Pharmaceutical Bulletin Vol.33 No.11. Copyright [2010] Pharmaceutical Society of Japan.

Fig. 6. Suppressive effect of NK-4 on MCAO-induced infarct formation in rats.

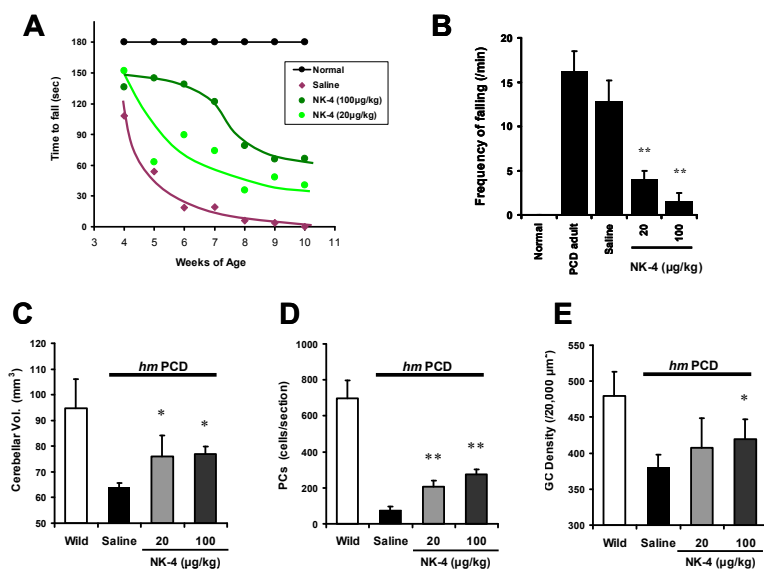
We demonstrated that the efficacy of NK-4 was superior to that of an existing drug (edaravone) in scavenging free radicals *in vitro* (Table 1) and in protecting MCAO model rats from brain injury *in vivo* (Fig. 6). Thus the antioxidant function of NK-4 may foster an overall protective environment against neuronal damage after focal brain ischemia. Since NK-4 elicits neurotrophic activity *in vitro* in PC12 cells aside from its antioxidant function, it may also enhance endogenous neurotrophic signals *in vivo* and thereby play an important role in the protection of neuronal cells from ischemic damage.

3.2 Ataxia model (PCD hamsters)

In the ischemic stroke model, intravenously administered NK-4 showed a significant neuroprotective effect (Fig. 6). This model reflects an acute oxidative insult at MCA-affected areas in the brain. We next tested the effects of NK-4 on progressive chronic neurodegeneration using a genetic ataxia model in the Syrian hamster characterized by Purkinje cell degeneration (hmPCD model). This animal model was established in HAYASHIBARA Co., Ltd. and is thought to be homologous to the well-characterized *pcd* mutant mice. Both animal models display a suppressed expression of the brain *nnal* gene,

which encodes a putative zinc carboxypeptidase originally identified by its induction in spinal motor neurons during axonal regeneration (Akita & Arai, 2009; Mullen et al., 1997; Fernandez-Gonzalez et al., 2002; Harris et al., 2000). Homozygous mutants of hmPCD show a moderate ataxia beginning at 7 weeks of age and exhibit an adult-onset degenerative loss of cerebellar Purkinje cells (PCs) followed by a slow, mild reduction in granule cell (GC) density (Akita et al., 2007).

To examine the effect of NK-4 on cerebellar ataxia in the hmPCD model, animals were administered 20 or 100 $\mu\text{g}/\text{kg}/\text{day}$ of NK-4 (intraperitoneally) for 8 weeks, starting at 3 weeks of age. Motor coordination in ataxic and non-ataxic animals was evaluated weekly with a rota-rod test. As shown in figure 7A, a low dose of NK-4 (20 $\mu\text{g}/\text{kg}$) elicited a moderate, but significant, effect in attenuating the deterioration of motor function. A high dose of NK-4 (100 $\mu\text{g}/\text{kg}$) produced a considerable improvement in their rota-rod performance that lasted for the entire test period. NK-4 could not halt or reverse the disease symptoms in hmPCD, however; it profoundly delayed the progress of disease. At the end of the study (10 weeks of age), the motor ability of NK-4-treated animals was evaluated by counting the frequency of falling. The hmPCD animals began to fall frequently in their cage



(A) Effect of NK-4 on motor performance in the rota-rod test. Animals were tested weekly for the ability to remain on the rotating rod at a constant speed (6 rpm), and the time spent on the rod was recorded. (B) Effect of NK-4 on frequency of falling in hmPCDs (10 weeks of age). Spontaneous falling of each animal was counted for 60 s. (C) Effect of NK-4 on cerebellar size in hmPCDs. The volume of the hamster cerebella at 10 weeks of age was calculated as an approximate oval sphere. (D) Effect of NK-4 on the number of PCs in hmPCDs. PCs were counted in the mid-sagittal section of the cerebellum from hmPCDs. (E) GC density in the cerebellum from hmPCDs and wild type controls. H&E stained sections of cerebellum cortex from hmPCDs and wild type controls were counted for GCs in an area of 20,000 μm^2 . Graphs show the mean \pm S.E.M. of 6 hamsters at 10 weeks of age. * $P < 0.05$, ** $P < 0.01$ vs. hmPCD control. Adapted from Ohta H *et al*, *PLoS ONE* 6(2):e17137 (Ohta et al., 2011).

Fig. 7. Effects of NK-4 on motor coordination and cerebellar degeneration in hmPCDs.

from around 7 weeks of age and the frequency of falling increased with age. Animals treated with the low and high doses of NK-4 showed a significant reduction in falling frequency compared to saline-treated controls (Fig. 7B). These observations demonstrate that NK-4 is effective in treating motor discoordination associated with cerebellar ataxia in the hmPCD model.

The mutants also showed severe cerebellar atrophy and volumetric reduction at 10 weeks of age (Fig. 7C). Animals treated with low or high doses of NK-4 had a significantly larger cerebellum volume compared with saline-treated controls. H&E staining and calbindin immunohistochemistry of cerebellar cortical sections revealed a large reduction in the number of cerebellar PCs in the brain of hmPCDs at 10 weeks of age (Fig. 7D). The PC dendrites in high dose NK-4-treated hmPCDs were significantly longer and thicker than those in surviving PCs in saline-treated hmPCDs (data not shown). In the brain of hmPCDs, the cerebellar GC density was moderately reduced compared to wild type controls. NK-4 dose-dependently attenuated cellular atrophy and prevented the reduction in GCs (Fig. 7E).

In this model, daily intraperitoneal injection of NK-4 at a dose of 20 or 100 $\mu\text{g}/\text{kg}$ for 8 consecutive weeks was effective in attenuating motor discoordination and degenerative loss of both PCs and GCs with no detectable adverse events. This suggests that NK-4 can attenuate neurodegeneration in the central nervous system via a peripheral route of administration. PCs are susceptible to ischemic damage because of their reduced capacity to isolate glutamate and reduced ability to generate energy during anoxia (Welsh et al., 2002). GCs are also vulnerable to a variety of toxins that decrease glutathione levels and this makes the cells more vulnerable to cellular damage from ROS (Fonnum & Lock, 2004). Direct scavenging of free radicals by NK-4 may protect these cells. In addition, NK-4 appears to activate survival-signaling pathways in degenerating cerebellar neurons.

3.3 Alzheimer's Disease (AD) mouse models

AD, the most common form of dementia, is characterized clinically by ongoing declines in cognitive and functional ability and emergence of behavioral and psychological symptoms. More than 35 million people living with dementia worldwide in 2010, increasing to 65.7 million by 2030 and 115.4 million by 2050 (Wimo & Prince, 2010). However, no effective disease-modifying therapy is available. A report from Alzheimer's association predicts the total costs of care for AD patients will increase five-fold per year, and the treatments which delay the onset of Alzheimer's disease or slow the progression of this condition dramatically reduce the costs of Medicare or Medicaid (Alzheimer's association, 2010). Presently, the only approved therapies for AD are ChE inhibitors and an N-methyl-D-aspartate (NMDA) receptor antagonist, and the beneficial effects of these symptomatic treatments appear limited and are not long lasting (Lanctôt et al., 2009). Thus, more evidence-based effective therapies, whether they are symptomatic treatment or disease-modifying strategies, are urgently needed.

NK-4 shows potent neuroprotective and neurotrophin-like activities *in vitro* (Fig. 1~3) and also *in vivo* (Fig. 6, 7). Furthermore, it remarkably and selectively inhibits AChE *in vitro* (Table 2). These multiple properties of NK-4 raised the possibility that it could halt or slow the progression of AD. To date, several mouse models for AD based on the amyloid hypothesis or the tau hypothesis have been developed (Bloom et al., 2005). Therefore, we tested the potential effects of NK-4 on AD using *in vivo* studies incorporating the amyloid

precursor protein (APP) transgenic mice Tg2576 (Hsiao et al., 1996) and the A β -induced amnesia model (Maurice et al., 1996).

3.3.1 Effects of NK-4 in APP-Tg (Tg2576) mice

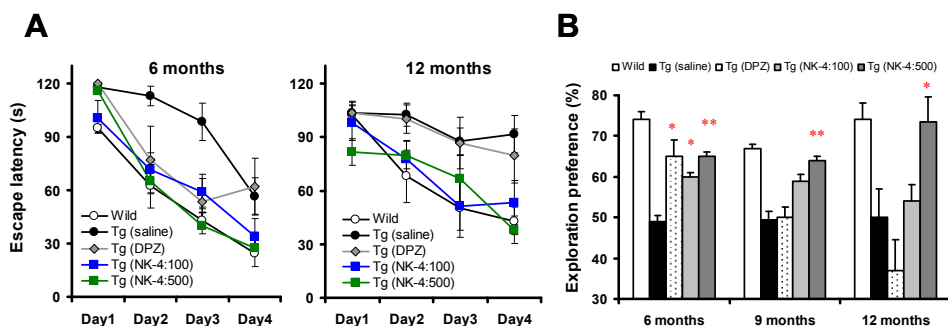
The Tg2576 mouse is the most thoroughly characterized AD mouse model and is considered to reflect human amyloid pathology most closely among mouse models. This mouse develops a considerable amount of A β deposits in the brain with age and this is accompanied by gradual declines in cognitive function (Hsiao, 2001). We examined whether chronic administration of NK-4 produced an effect on the cognitive deficits in Tg2576 mice (Ohta et al., 2010b). Tg2576 mice were administered NK-4 as doses of 100 or 500 μ g/kg/day once a day, 5 times a week for 9 months, starting at 3 months of age. Donepezil, an existing drug for dementia based on its AChE inhibitory properties (Sugimoto, 2004) was used as a control drug and was administered at a dose of 200 μ g/kg/day.

Wild type and Tg2576 mice were tested for spatial learning ability in a Morris water maze test (Morris, 1984) at ages 6 and 12 months. There were significant differences in escape latency between the wild type controls and saline-treated Tg2576 mice at 6 and 12 months of age (Fig. 8A). For the saline-treated Tg2576 group, the latency to reach the hidden platform did not shorten during the whole test period at 12 months of age, suggesting that the spatial learning ability of saline-treated Tg2576 mice became impaired with age. We found significant differences in spatial learning ability between the NK-4-treated Tg2576 mice (at both low and high doses) and saline-treated Tg2576 mice. Saline-treated Tg2576 mice consistently exhibited longer escape latencies compared to the NK-4-treated Tg2576 group at 6 and 12 months of age. Donepezil-treated mice showed a partial improvement in spatial memory deficits at 6 months of age, although the drug became less effective over time. At 12 months of age, the escape latencies of donepezil-treated and saline-treated Tg2576 mice showed no significant difference. These results suggest a long-lasting ameliorative effect of NK-4 on A β -mediated spatial memory impairment.

We also evaluated the object recognition memory of Tg2576 mice in a novel object recognition test (Nagai et al., 2003). Long-term recognition memory was tested at ages of 6, 9, and 12 months (Fig. 8B). Saline-treated Tg2576 mice displayed significantly decreased object recognition memory compared to wild type mice at the same ages. Tg2576 mice treated with low and high doses of NK-4 or donepezil spent a longer time exploring the novel object than did the saline-treated controls at 6 months of age. However, at 9 and 12 months of age, the exploration preference values of low dose NK-4 and donepezil-treated groups decreased, and the differences between these two groups and saline-treated group became insignificant. In contrast, the high dose of NK-4 group spent a significantly longer time exploring the novel object versus the saline-treated mice throughout the test period, and the exploratory preference was comparable to that of wild type controls at 12 months of age. This suggests that NK-4 administration for a longer period and at high dosing might be more effective for the improvement of object recognition memory.

At the end of experiment (12 months of age; after 9-month treatment), the effects of NK-4 on plasma and brain A β levels in Tg2576 mice were evaluated (Fig. 9). Dose-dependent increases in A β ₁₋₄₀ and A β ₁₋₄₂ were observed in plasma from the NK-4-treated mice (Fig. 9A). Although donepezil treatment also increased both plasma A β ₁₋₄₀ and A β ₁₋₄₂ levels, the effect

was lower compared to NK-4 treatments. Brain levels of A β were estimated separately in detergent-insoluble and -soluble fractions. Both detergent-insoluble and soluble A β_{1-40} levels were significantly decreased by NK-4 treatment (Fig. 9B, upper graphs). Donepezil did not affect A β concentrations in brain. Because the levels of A β_{1-42} were very low in all groups compared to those of A β_{1-40} (Fig. 9B, lower graphs), the levels of A β_{1-40} seem to reflect the total amount of A β in the brain.

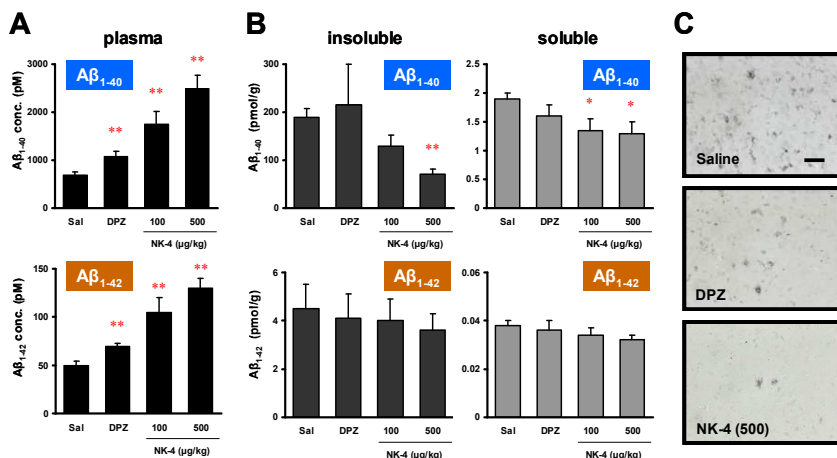


NK-4 was injected intraperitoneally to Tg2576 mice at a dose of 100 or 500 $\mu\text{g}/\text{kg}$ once a day, five times a week for 9 months, beginning at 3 months of age ($n=10$ each). Donepezil (DPZ) was administered at a dose of 200 $\mu\text{g}/\text{kg}$ ($n=5$). Control mice received 200 μl of saline (wild type, $n=10$; Tg2576, $n=10$). (A) Morris water maze test at 6 and 12 months of age. The escape latency represents the average time to find a hidden platform placed in a fixed location in a circular pool ($\phi 130$ cm). Two trials per day were conducted for 4 consecutive days with an upper cutoff time of 120 sec. (B) Novel object recognition test at 6, 9 and 12 months of age. Two objects X and Y were placed in the test box and the exploration behavior of mice was recorded. A retention session was performed 24 hr after the training session. One of the familiar objects Y was replaced by a novel object Z. Mice were allowed to explore freely for 10 min, with the time spent in exploring each of the two objects recorded. The exploratory preference was expressed as a ratio of the time spent exploring the novel object (Tz) over that spent on the two objects (Tx+Tz). Data are expressed as means \pm S.E.M. * $P<0.05$, ** $P<0.01$ vs. saline-treated Tg2576 group. Wild: non-transgenic mice. Tg(saline), saline-treated Tg2576 mice; Tg(DPZ), donepezil-treated Tg2576 mice; Tg(NK-4: 100), low-dose NK-4-treated Tg2576 mice; Tg2576 (NK-4: 500), high-dose NK-4-treated Tg2576 mice.

Fig. 8. Effect of NK-4 on recognition memory in Tg2576 mice.

We next surveyed brain A β deposition in Tg2576 mice by A β immunohistochemistry (Fig. 9C). A β -immunoreactive small diffuse plaques were abundantly present in the cortex of saline-treated Tg2576 mice (upper panel), and they were visibly reduced by high dose NK-4 treatment both in size and in quantity (bottom panel). The donepezil-treated group showed a slight decrease in A β -immunoreactivity (middle panel).

Nine months of NK-4 administration to Tg2576 mice significantly attenuated the cognitive decline as assessed by behavioral tests (Fig. 8), and decreased the levels of A β in the brain (Fig. 9B, C) while augmenting levels in the plasma (Fig. 9A). These results imply that NK-4-induced cognitive improvement was attributable to decreased A β brain levels. Although there is still no consensus as to the mechanism by which some drugs, including anti-A β antibodies, alter amyloid deposition in the brain (Levites et al., 2006), A β may be cleared across the blood-brain barrier to the blood in NK-4-treated Tg2576 mice.



(A) Plasma A β concentrations of Tg2576 mice at 12 months of age. A β levels were separately measured by ELISA for A β_{1-40} and A β_{1-42} . (B) Brain A β levels of detergent-insoluble or soluble fractions at 12 months of age. Hemi-brains from Tg2576 mice were homogenized and separated into detergent-insoluble and soluble fractions. A β_{1-40} and A β_{1-42} in individual samples were measured and are expressed as picomoles/g wet brain weight. Values are the means \pm S.E.M. Saline (Sal), n=10; Donepezil (DPZ), n=5; NK-4 (100), n=9; and NK-4 (500), n=8. *P<0.05, **P<0.01 vs. saline-treated group. (C) A β -immunohistochemistry of the cerebral cortex from Tg2576 mice at 12 months of age. Hemi-brains were fixed with 10% buffered formalin and embedded in paraffin. Coronal sections of cerebral cortex with 5- μ m thickness were immunostained with rabbit anti-A β -peptide polyclonal antibody. Bar represents 50- μ m.

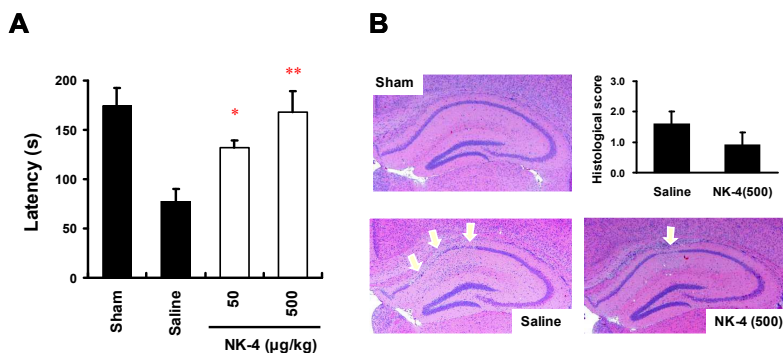
Fig. 9. Effect of NK-4 on the plasma and brain concentrations of A β in Tg2576 mice.

Oxidative mechanisms are thought to be involved in cell loss and other neuropathologies associated with AD (Zhu et al., 2001; Cutler et al., 2004). During AD pathogenesis, ROS impair mitochondrial redox activity and further increase ROS generation (Shearman et al., 1994; Hensley et al., 1994). A β induces the production of ROS and leads to apoptotic neuronal cell death that can be inhibited by antioxidants (Behl et al., 1994; Mattson & Goodman, 1995). Pathological and biochemical studies suggest that ROS induced by fibrillar A β produce neurotoxic effects (Gevasis et al., 1999). Since NK-4 is a potent scavenger of ROS (Table 1) and is an inhibitor of A β aggregation (Fig. 2), these features might work in concert to attenuate A β toxicity in AD.

3.3.2 Effects of NK-4 in A β -icv mice

To further determine whether the effect of NK-4 was directly attributable to mitigation of A β pathology, an A β -induced amnesia model was employed (Ohta et al., 2010a). In this model, ICR mice received intracerebroventricular (icv) administration of aggregated A β_{25-35} peptide to induce memory deficits due to cholinergic dysfunction (Maurice et al., 1996). Long-term recognition memory was evaluated by the step-through type passive avoidance test 12 days after A β_{25-35} injection. In this behavioral assay, daily NK-4 treatment significantly and dose-dependently improved memory deficits induced by A β_{25-35} (Fig. 10A). There was a significant difference in step-through latencies between sham operated mice

and $A\beta_{25-35}$ -injected ICR mice. Both low (50 $\mu\text{g}/\text{kg}$) and high (500 $\mu\text{g}/\text{kg}$) doses of NK-4 successfully prolonged the step-through latencies with statistically significant differences ($p < 0.05$ for the low dose and $p < 0.01$ for the high dose, respectively; Fig. 10A). Improved long-term memory retention following NK-4 administration was also confirmed using this animal model in a novel object recognition test (data not shown).



(A) Passive avoidance test of $A\beta_{25-35}$ -icv mice. Male ICR mice were injected aged $A\beta_{25-35}$ (9 nmol/mouse) into the left lateral ventricle using the following coordinates from Bregma: 0.5 mm posterior, 1.0 mm lateral, and 2.0 mm ventral. A low (50 $\mu\text{g}/\text{kg}$) or high (500 $\mu\text{g}/\text{kg}$) dose of NK-4 was administered intraperitoneally to mice for twelve consecutive days starting from the next day of $A\beta$ injection. Then, mice were tested for step-through passive avoidance during days 9-12. The step-through latency in the retention session (24-hr after the training session) was recorded. Values are means \pm S.E.M. ($n=10$). Sham, sham-operated group; saline, saline-treated $A\beta_{25-35}$ -icv group; NK-4, NK-4-treated $A\beta_{25-35}$ -icv groups. * $P < 0.05$, ** $P < 0.01$ vs. saline-treated group. (B) Histological damage scores in the hippocampal CA1 region. Hippocampal neuronal cell loss was assessed in coronal H&E sections. Scores are represented as means \pm S.E.M. ($n=10$). Arrows indicate the sites of degeneration.

Fig. 10. Effects of NK-4 on $A\beta_{25-35}$ -induced cognitive impairments in ICR mice.

These beneficial effects of NK-4 on recognition memory are in agreement with histological findings shown in figure 10B. A single icv injection of $A\beta_{25-35}$ caused significant hippocampal neuronal loss mainly in the CA1 region. The difference in histological scores was not statistically significant between the saline-treated (1.6 ± 0.41) and the high dose NK-4-treated mice (0.9 ± 0.41 , $p = 0.067$). However, NK-4 ameliorated the $A\beta_{25-35}$ -induced injury of pyramidal neurons in the hippocampus (Fig. 10B). Reportedly, associative learning in the passive avoidance test strongly depends on hippocampal function (Phillips & LeDoux, 1992). In this context, neuronal injury in the hippocampal CA1 region following $A\beta_{25-35}$ -icv injection provides a reasonable explanation for the impaired long-term memory. These results, in combination with the results from Tg2576 mice, and AChE inhibitory property of NK-4 suggest that NK-4 treatment effectively improves cognitive deficits in AD model mice by both attenuating $A\beta$ pathology and enhancing cholinergic transmission in the brain.

4. Conclusion

In this chapter, we showed that NK-4, a type of cyanine dye, exerts a wide spectrum of biochemical and biological activities implicated in neuroprotection. NK-4 significantly ameliorated neurological and cognitive deficits, as well as neurodegeneration, in four

distinct animal models (MCAO rats, ataxic hamsters, APP-transgenic mice, and A β -icv mice). Molecular mechanisms by which NK-4 acts against neurodegeneration remain unclear, although it was found that NK-4 activates the PI3K-Akt pathway independently of Trk receptors. Thus, activated Akt may be a key mediator of the beneficial effects on neural cell survival. In addition, since activated Akt is required for PI3K-mediated synaptic plasticity and memory consolidation via activation of the downstream regulator CREB (Brightwell et al., 2007), induction of Akt phosphorylation might play a critical role in NK-4-mediated memory improvement in animal models of dementia. In addition to the modification of intracellular signaling, a direct antioxidative property of NK-4 may also be involved in survival and functional maintenance of neurons.

Regarding the safety profile, there were no specific adverse events in mice that received intraperitoneal injections of NK-4 at doses of up to 500 μ g/kg/day, 5 days a week, for 9 months (3.3.1. Tg2576 study). Furthermore, NK-4 was well tolerated in rats up to 4,000 mg/kg in an acute toxicity study and 100 mg/kg in a subacute toxicity study (both via the oral route of administration) based on mortality, clinical observations, body weight, hematology, blood chemistry, organ weights, and histological examination of a complete tissue list (Ohmori et al., 1983). Furthermore, NK-4 has been used as an active ingredient of over the counter (OTC) medicine in Japan for treating allergy, and for promoting wound healing since the 1950s (Suzue, 1969). Additionally, NK-4 was not mutagenic in the standard Ames test (our unpublished data). These observations suggest that NK-4 is a safe compound that will not cause serious adverse reactions.

Application of small neurotrophic molecules that modulate neuronal survival and synaptic function is a promising and valid therapeutic approach for neurodegenerative disorders. Lines of evidence described here strongly suggest the potential utility of NK-4 as a treatment for neurodegenerative disease. The evaluations of pharmacokinetics, bioavailability, as well as the efficacy of orally administered NK-4 are ongoing. In addition, assessment of NK-4 in human neurodegenerative therapy will require further studies.

5. Acknowledgment

We would like to thank Dr. S. Arai, Ms. C. Ushio, and Dr. M. Takeuchi (HAYASHIBARA Co., Ltd.) for their helpful support.

6. References

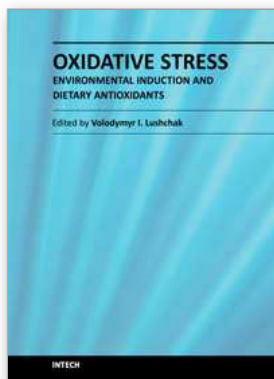
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Oxidative Stress - Environmental Induction and Dietary Antioxidants

Edited by Dr. Volodymyr Lushchak

ISBN 978-953-51-0553-4

Hard cover, 388 pages

Publisher InTech

Published online 02, May, 2012

Published in print edition May, 2012

This book focuses on the numerous applications of oxidative stress theory in effects of environmental factors on biological systems. The topics reviewed cover induction of oxidative stress by physical, chemical, and biological factors in humans, animals, plants and fungi. The physical factors include temperature, light and exercise. Chemical induction is related to metal ions and pesticides, whereas the biological one highlights host-pathogen interaction and stress effects on secretory systems. Antioxidants, represented by a large range of individual compounds and their mixtures of natural origin and those chemically synthesized to prevent or fix negative effects of reactive species are also described in the book. This volume will be a useful source of information on induction and effects of oxidative stress on living organisms for graduate and postgraduate students, researchers, physicians, and environmentalists.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Hitomi Ohta, Kenji Akita and Tsunetaka Ohta (2012). Effects of NK-4, a Cyanine Dye with Antioxidant Activities: Attenuation of Neuronal Deficits in Animal Models of Oxidative Stress-Mediated Brain Ischemia and Neurodegenerative Diseases, *Oxidative Stress - Environmental Induction and Dietary Antioxidants*, Dr. Volodymyr Lushchak (Ed.), ISBN: 978-953-51-0553-4, InTech, Available from: <http://www.intechopen.com/books/oxidative-stress-environmental-induction-and-dietary-antioxidants/effects-of-nk-4-a-cyanine-dye-with-antioxidant-activities-attenuation-of-neuronal-deficits-in-ani>

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