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N-Propargylamine protects SH-SY5Y cells from apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol, through stabilization of mitochondrial membrane and induction of anti-apoptotic Bcl-2

H. Yi[1], **W. Maruyama**[2], **Y. Akao**[1], **T. Takahashi**[3], **K. Iwasa**[4], **M. B. H. Youdim**[5], and **M. Naoi**[1]

[1] Department of Neurosciences, Gifu International Institute of Biotechnology, Kakamigahara, Gifu,
[2] Department of Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi,
[3] Aichi-Konan College, Konan, Aichi, and
[4] Kobe Pharmaceutical University, Kobe, Japan
[5] Technion-Israel Institute of Technology, Faculty of Medicine, Eve Topf and NPF Centers, Haifa, Israel

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Summary. Propargylamine derivatives, rasagiline and (–)deprenyl, are anti-Parkinson agents and protect neurons from cell death as shown by *in vivo* and *in vitro* experiments. The studies on the chemical structure-activity relationship proved that the propargyl moiety is essentially required for the neuroprotective function. In this paper, neuroprotective activity of free *N*-propargylamine was studied using SH-SY5Y cells expressing only type A monoamine oxidase (MAO) against apoptosis induced by an endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol. *N*-Propargylamine prevented apoptosis, whereas *N*-methylpropargylamine and propiolaldehyde did not. *N*-Propargylamine stabilized mitochondrial membrane potential and induced anti-apoptotic Bcl-2 at 1 μM–10 nM. *N*-Propargylamine inhibited MAO-A in competition to substrate with the apparent K_i value of 28 μM, which was significantly higher than the concentration required for neuroprotection. It indicates that MAO inhibition is not prerequisite for the protective function of *N*-propargylamine. The anti-apoptotic function of *N*-propargylamine is discussed in terms of neuroprotection by propargylamines in neurodegenerative diseases, including Parkinson's disease.

Keywords: Propargylamine, neuroprotection, apoptosis, mitochondrial membrane potential, Bcl-2, monoamine oxidase inhibitor.

Abbreviations

DMEM Dulbecco's modified Eagle's medium; *ERK* extracellular signal-regulated kinase; *FACS* fluorescence-augmented flow cytometry; *FCS* fetal calf serum; *2-HMP* *N*-(2-heptyl)-*N*-methylpropargylamine; *MAO*

monoamine oxidase; *MAO-A* and *MAO-B* type A and B MAO; *MAPK* mitogen-activated protein kinase; *NM(R)Sal N*-methyl(*R*)salsolinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline]; *PBS* phosphate-buffered saline; *PD* Parkinson's disease; *PI* propidium iodide; *PKC* protein kinase C; *PT* permeability transition.

Neuroprotection to delay or halt progressive degeneration of specified neurons is now proposed as a causal therapeutic strategy for Parkinson's disease (PD), Alzheimer's disease, and amyotrophic lateral sclerosis. In these disorders, apoptosis has been suggested to contribute to neuronal death, but this remains to be accepted in general. The well-regulated and slowly advancing process of apoptosis is proposed as a target of neuroprotection (Naoi and Maruyama, 2001; Vila and Przedborski, 2003). Various insults, including oxidative stress, metabolic compromise, excitotoxicity and endogenous and exogenous neurotoxins, are known to induce apoptosis in neurons. Apoptotic signaling is a multi-step pathway initiated by opening a mitochondrial mega-channel, called permeability transition (PT) pore, followed by decline in membrane potential ($\Delta\Psi$m), release of cytochrome c and other apoptosis-inducing factors, activation of caspases and final fragmentation of nuclear DNA. Mitochondrial PT and following apoptotic cascade are regulated by Bcl-2 protein family, and Bcl-2 and Bcl-xL prevent, and BAX and BAD promote cell death (Tsujimoto and Shimizu, 2000).

Recently, various candidates of neuroprotective agents have been proposed and some are now subjected for clinical trials (Ravina et al., 2003). A series of propargylamine derivatives, (–)deprenyl [selegiline, *N*-(phenylisopropyl)-*N*-methyl-propargylamine] (Riederer and Lachenmayer, 2003) and rasagiline [*N*-propargyl-1(*R*)-aminoindan] (Parkinson Study Group, 2002), were reported to have beneficial symptomatic effects in patients with PD. More recently clinical controlled study with rasagiline in Parkinsonian subjects was successfully carried out pointing to its possible action to modify or slow down the disease progress (Parkinson Study Group, 2004). The patients treated with rasagiline for 12 months showed less functional decline than those whose treatment was delayed for 6 months. *In vivo* and *in vitro* experiments confirmed that (–)deprenyl (Tatton et al., 1994; Maruyama and Naoi, 1999), rasagiline (Finberg et al., 1998; Maruyama et al., 2000, 2001a, 2002; Youdim et al., 2001b), and *N*-(2-heptyl)-*N*-methylpropargylamine [2-HMP] (Yu et al., 1994; Maruyama et al., 2001b) protect neuronal cells against apoptosis as induced by various insults. Significant insights into the anti-apoptotic function of propargylamine derivatives have been documented, and mitochondria emerge as a key organelle playing a regulatory role in apoptosis. Our previous studies have shown that the neuroprotection by propargylamine derivatives, specially rasagiline, is attributed to (1) the stabilization of $\Delta\Psi$m and prevention of PT (Maruyama et al., 2001a, b; Naoi et al., 2002a), (2) the induction of anti-apoptotic Bcl-2 family regulating PT (Akao et al., 2002a, b), (3) the release of glial cell line-derived neurotrophic factor (GDNF), a neurotrophic factor selective to dopamine neurons (Maruyama et al., 2004), and (4) activation of anti-oxidant enzymes, such as superoxide dismutase and catalase (Carrillo et al., 2000).

Most of the neuroprotective propargylamines, such as (–)deprenyl (Knoll et al., 1978) and rasagiline (Youdim et al., 2001a), are inhibitors of type B monoamine oxidase [monoamine, oxygen oxidoreductase (deaminating); EC 1.4.3.4; MAO-B], suggesting the involvement of MAO in the neuroprotective potency. (–)Deprenyl and 2-HMP (Yu et al., 1992) possess a methyl group at the nitrogen position of the propargylamine moiety and this gives them the potent MAO inhibitory activity (Yu et al., 1993). However, the desmethyl

metabolites of (–)deprenyl and 2-HMP, and (*S*)-enantiomer of rasagiline do not inhibit MAO-B, but protect neurons (Mytilineou et al., 1997; Maruyama et al., 2001c). These results suggest that the neuroprotective function of propargylamines may not depend on the MAO-inhibitory activity. On the other hand, the structure activity relationship of rasagiline analogues has indicated that *N*-propargylamine itself may stabilize $\Delta\Psi m$ and prevent apoptotic process (Maruyama et al., 2003). Indeed, free *N*-propargylamine was reported to protect PC12 cells from cell death induced by serum withdrawal (Weinreb et al., 2004) similar to rasagiline.

This paper describes the effects of *N*-propargylamine on apoptosis induced by a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, *N*M(*R*)Sal] (Maruyama et al., 1996, 2001b; Naoi et al., 2002b) in human dopaminergic neuroblastoma SH-SY5Y cells. Cell death induced by *N*M(*R*)Sal in SH-SY5Y cells has been confirmed to be apoptotic, not necrotic (Naoi et al., 2003), suggesting that this model may be adequate to examine the anti-apoptotic potency of *N*-propargylamine. The regulation of apoptotic signaling, namely stabilization of mitochondrial PT and expression of anti-apoptotic *bcl-2*, and effects on MAO activity by *N*-propargylamine were examined in SH-SY5Y cells, where only MAO-A activity is expressed. In addition, the structure-activity relationship in concern to anti-apoptotic function and MAO inhibiting potency were examined among *N*-propargylamine-related compounds, *N*-methylpropargylamine and propiolaldehyde.

Materials and methods

Materials

*N*M(*R*)Sal was prepared according to Teitel et al. (1972). Aminoindan was kindly supplied by TEVA Pharmaceutical Industries (Netanya, Israel). *N*-Propargylamine, kynuramine and 4-quinolinol were purchased from Sigma (St. Louis, MO, USA); *N*-methylpropargylamine from Aldrich (Milwaukee, WI, USA) and propiolaldehyde from Wako (Osaka, Japan); propidium iodide (PI), Mito-Tracker Orange and Green from Molecular Probes (Eugene, OR, USA), Dulbecco's modified Eagle's medium (DMEM) and other drugs from Nacalai tesque (Kyoto, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum (FCS) in an atmosphere of 95% air–5% CO_2. Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999).

Inhibition of MAO-A activity by N-propargylamine

MAO activity in mitochondria prepared from SH-SY5Y cells was measured fluorometrically according to the method reported by Kraml (1965) by use of kynuramine as a substrate. Kinetics for MAO activities and the inhibition by *N*-propargylamine were studied with 8 different concentrations of kynuramine. The values of the apparent Michaelis constant, K_m, and the apparent inhibitor constant, K_i, were calculated from the double-reciprocal plot of the reaction velocity against the substrate concentration. Protein concentration was determined according to Bradford (1976).

To determine the reversibility of the inhibition by *N*-propargylamine, MAO samples were incubated with 100 μM *N*-propargylamine for 30 min at 37°C, and then dialyzed against 10 mM sodium phosphate buffer, pH 7.4, at 4°C overnight. The MAO activities were quantified before and after the dialysis.

Assessment of apoptosis induced by NM(R)Sal

Apoptosis was quantitatively measured by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Becton Dickinson, San Jose, CA, USA). The cells cultured in a 6-well poly-L-lysine-coated culture flask were incubated in DMEM with or without 1 μM–1 nM *N*-propargylamine at 37°C for 30 min, then with 250 μM *N*M(*R*)Sal for 24 hr. The cells were treated with trypsin, gathered, washed with phosphate-buffered saline (PBS). To determine apoptotic cells, the cells were stained with 75 μM PI solution in PBS containing 1% Triton X-100 at room temperature for 5 min in the dark, washed and suspended in PBS, then subjected to FACS analysis. The fluorescence intensity at 560–640 nm (FL-2 channel) was detected for PI with excitation at 488 nm. To differentiate singlet from doublet cells, FL-2 (PI)-A (Area) and FL-2 (PI)-W (Width) parameters were used. Cells with a lower DNA content showing less PI staining than G1

were defined to be apoptotic cells (subG1 peak) (Eckert et al., 2001).

Measurement of ΔΨm in mitochondria isolated from SH-SY5Y cells

ΔΨm decline in isolated mitochondria by *N*M(*R*)Sal was quantitatively measured by FACS using MitoTracker Orange and Green. The mitochondria were suspended in DMEM and incubated with 10 μM *N*-propargylamine for 30 min at 37°C, then with 250 μM *N*M(*R*)Sal for 3 h. After stained with 100 nM MitoTracker Orange and Green for 30 min at 37°C, the mitochondria were washed and suspended with PBS and subjected to FACS. The laser emission at 560–640 nm (FL-2) and at shorter than 560 nm (FL-1) with excitation at 488 nm were used for the detection of MitoTracker Orange and Green fluorescence, respectively.

Determination of Bcl-2 protein levels in the cells treated with N-propargylamine

SH-SY5Y cells treated with 1 μM–1 pM *N*-propargylamine analogues for 24 h, and the cells were gathered, washed with PBS and suspended in RIPA buffer [10 mM Tris-HCl buffer, pH 7.5, containing 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl and 1 mM EDTA 2Na]. The lyzed protein (5 μg) was separated by SDS-PAGE using 10–20% gradient polyacrylamide gel (Bio-Rad Lab., Hercules, CA, USA) and electroblotted onto PVDF membranes (Du Pont, Boston, MA, USA). After blockage with 5% nonfat milk in PBS containing 0.1% Tween 20, the membrane was incubated overnight at 4°C with anti-human Bcl-2 (100) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-tubulin

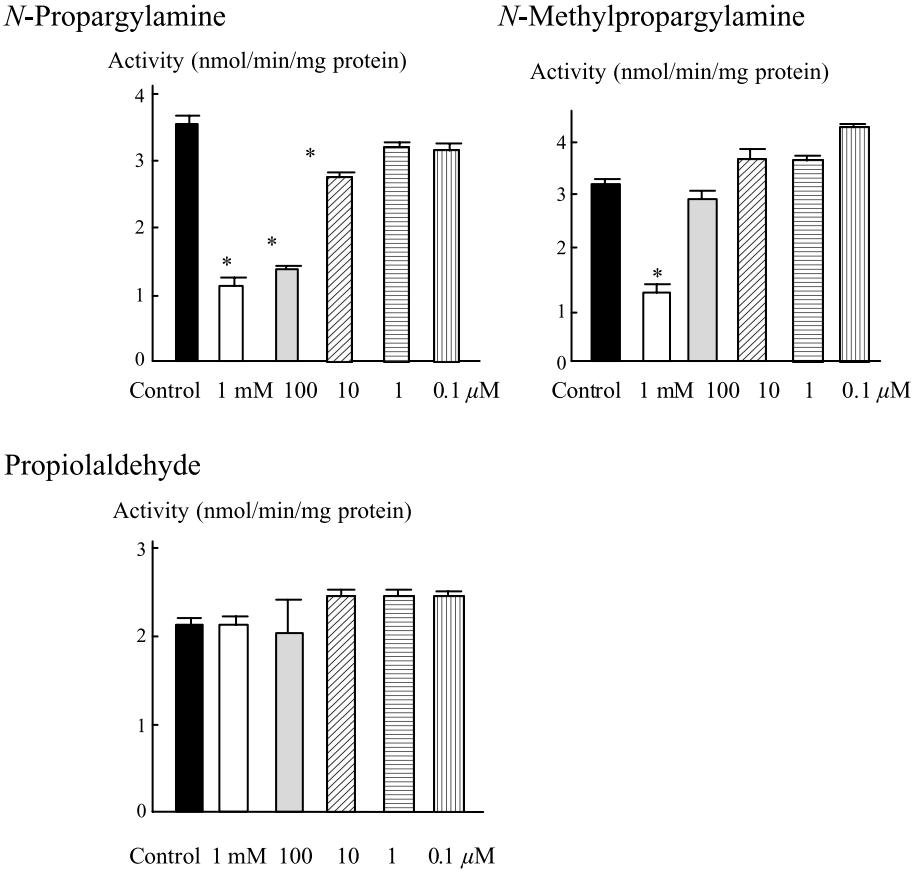


Fig. 1. The effects of *N*-propargylamine analogues on MAO-A activity. Mitochondria prepared from SH-SY5Y cells were used as MAO samples and incubated with 1 mM–0.1 μM *N*-propargylamine analogues and 100 μM kynuramine as a substrate at 37°C for 60 min, and the amounts of produced 4-hydroxyquinol were measured fluorometrically. The column and bar represent the mean and SD of 2 triplicate experiments. $P < 0.01$ from control

antibody as control (Sigma). The membranes were incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA) at room temperature. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

Measurement of bcl-2 mRNA level in the cells treated with N-propargylamine

SH-SY5Y cells were cultured in the presence of various concentrations (1 μM–1 pM) of *N*-propargylamine for 24 h, and mRNA levels of *bcl-2* were quantitatively assessed by RT-PCR method (Akao et al., 2002a, b). The cells were gathered and washed with PBS, and the total RNA was extracted by the phenol/guanidinium thiocyanate method. cDNA was generated by reverse transcription of 2 μg of the total RNA, and the cDNA fragments were amplified using the PCR primers. The linearity of the amount of PCR product to the time of PCR amplification under the used conditions was confirmed by the real-time PCR method. PCR products were analyzed by electrophoresis on 3% agarose gels, and β-*actin* mRNA was used as an internal standard.

Statistics

Experiments were repeated at least 4 times in triplicate, and the results were expressed as the mean and SD. Differences were statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's F-test. A p value less than 0.05 was considered to be statistically significant.

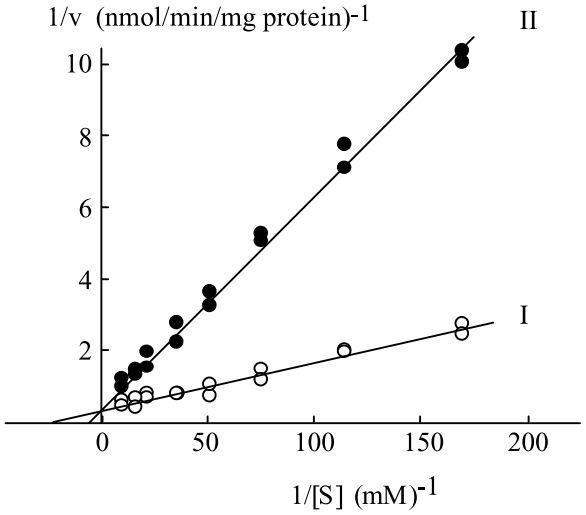


Fig. 2. The effects of *N*-propargylamine on the enzymatic activity of MAO-A. Mitochondria were prepared from SH-SY5Y cells, and MAO activity was measured fluorometrically with 8 different substrate concentrations in the absence (**I**) or presence of 100 μM *N*-propargylamine (**II**). The double reciprocal plots of the reaction velocity against the substrate concentration were used to calculate apparent K_m, K_i and V_{max} values, according the Lineweaver and Burk

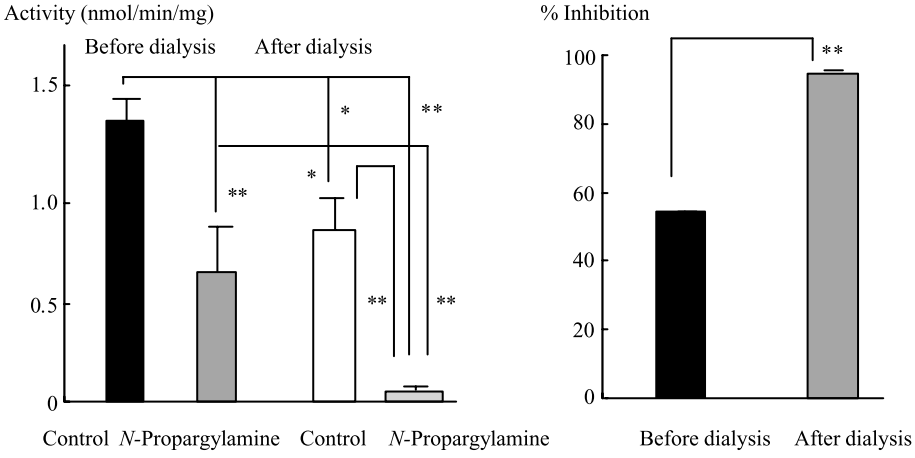
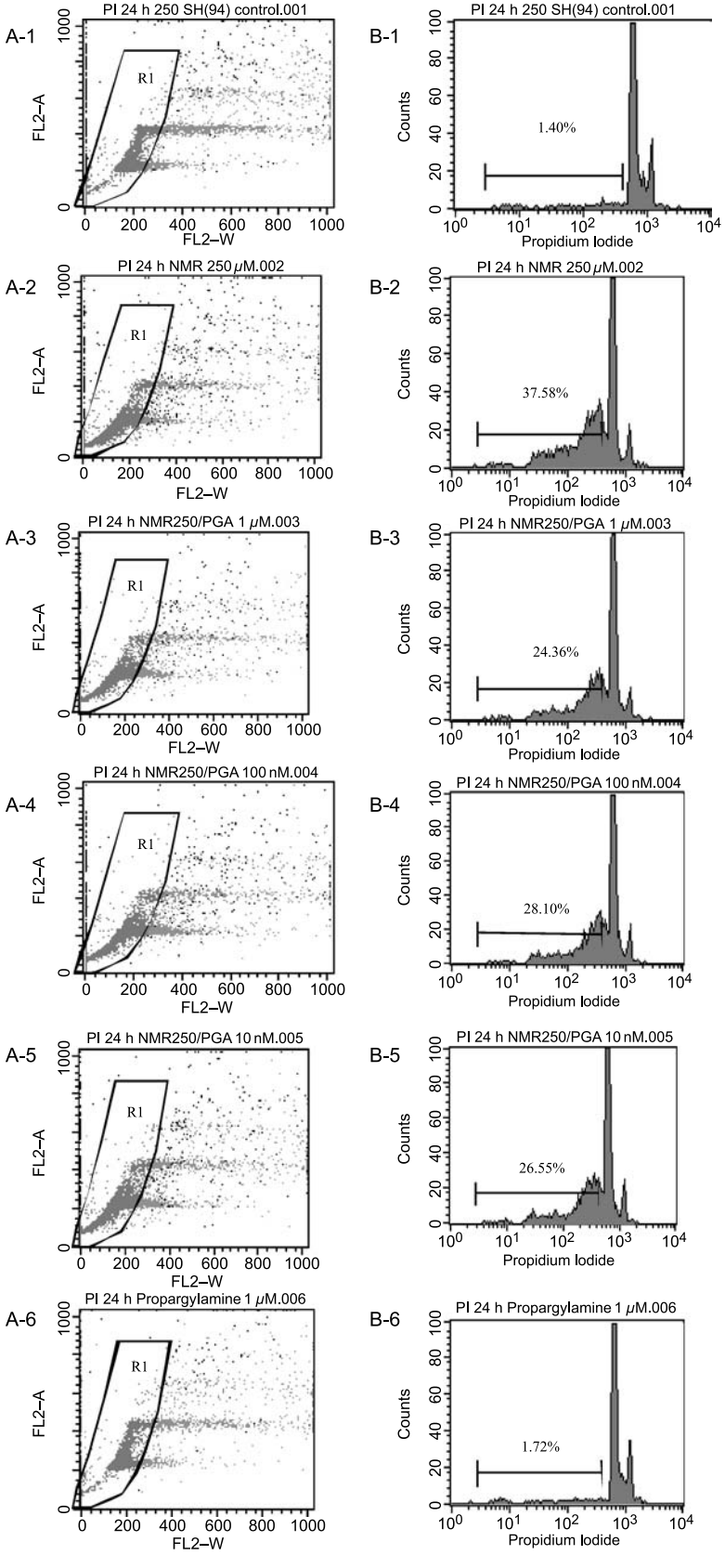


Fig. 3. Reversibility of MAO inhibition by *N*-propargylamine. MAO samples were treated with 100 μM *N*-propargylamine for 30 min at 37°C, then dialyzed against 10 mM sodium phosphate buffer, pH 7.4, at 4°C overnight. The MAO activity was measure with 100 μM kynuramine. The inhibition of MAO activity by *N*-propargylamine before and after dialysis was expressed as % inhibition by comparison of the reduced activity with the activity of control. $^{*}p < 0.05$; $^{**}p < 0.01$


A-1
PI 24 h 250 SH(94) control.001
R1
FL2–A
FL2–W
B-1
PI 24 h 250 SH(94) control.001
1.40%
Counts
Propidium Iodide
A-2
PI 24 h NMR 250 μM.002
R1
FL2–A
FL2–W
B-2
PI 24 h NMR 250 μM.002
37.58%
Counts
Propidium Iodide
A-3
PI 24 h NMR250/PGA 1 μM.003
R1
FL2–A
FL2–W
B-3
PI 24 h NMR250/PGA 1 μM.003
24.36%
Counts
Propidium Iodide
A-4
PI 24 h NMR250/PGA 100 nM.004
R1
FL2–A
FL2–W
B-4
PI 24 h NMR250/PGA 100 nM.004
28.10%
Counts
Propidium Iodide
A-5
PI 24 h NMR250/PGA 10 nM.005
R1
FL2–A
FL2–W
B-5
PI 24 h NMR250/PGA 10 nM.005
26.55%
Counts
Propidium Iodide
A-6
PI 24 h Propargylamine 1 μM.006
R1
FL2–A
FL2–W
B-6
PI 24 h Propargylamine 1 μM.006
1.72%
Counts
Propidium Iodide

Results

Inhibition of MAO-A by N-propargylamine

Inhibition of MAO-A activity in mitochondria by *N*-propargylamine is shown in Fig. 1. *N*-Propargylamine reduced MAO activity significantly until at 10 μM, whereas *N*-methylpropargylamine inhibited the activity only at 1 mM, and propiolaldehyde did not even at 1 mM. Kinetic analyses indicate that *N*-propargylamine inhibited MAO-A in competition to the substrate (Fig. 2). The apparent K_i value of *N*-propargylamine was estimated to be 28.0 μM, whereas the values of the apparent K_m and maximal velocity, V_{max}, were 45.5 μM and 2.87 nmol/min/mg protein, respectively. The dialysis experiments showed that the inhibition of MAO by *N*-propargylamine was irreversible (Fig. 3), which is similar to those reported often for (−)deprenyl, rasagiline and other propargylamine derivatives. The activity of MAO-A treated with or without *N*-propargylamine was reduced further by the dialysis procedure, which may be due to the marked un-stability of MAO-A activity.

Anti-apoptotic function of N-propargylamine

Treatment of SH-SY5Y cells with 250 μM *N*M(*R*)Sal resulted in apoptosis of 37.6 ± 3.9% of cells, whereas necrotic cells were virtually not detected. Apoptotic cells were almost negligible in the control or *N*-propargylamine alone-treated cells. Figure 4 shows typical FACS profiles of the cells treated with *N*M(*R*)Sal with or without 1 μM–1 nM *N*-propargylamine. *N*-Propargylamine reduced the number of apoptotic cells to 63–70% of that of *N*M(*R*)Sal-treated cells. The potency of anti-apoptotic function was compared with that of *N*-methylpropargylamine, propiolaldehyde, and rasagiline in a similar manner. Rasagiline was found to be most potent to prevent apoptosis followed by *N*-propargylamine (Fig. 5), but *N*-methylpropargylamine and propiolaldehyde were virtually not effective.

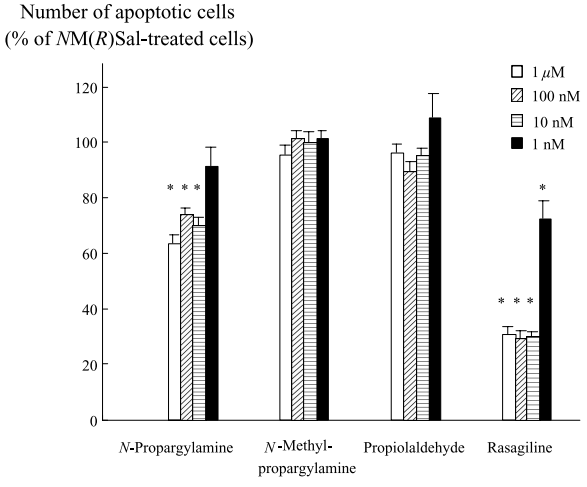


Fig. 5. The anti-apoptotic potency of *N*-propargylamine analogues and rasagiline. SH-SY5Y cells were treated with 1 μM, 100–1 nM propargylamine derivatives, and then with 250 μM *N*M(*R*)Sal overnight. The number of apoptotic cells was determined by FACS as sub G1 peak (Fig. 4) using PI as an indicator. The number of apoptotic cells was expressed as % of apoptotic cells after treated with 250 μM *N*M(*R*)Sal alone. The column and bar represent the mean and SD of three independent experiments measured in triplicate. $^{*}p < 0.01$ from cells treated with *N*M(*R*)Sal alone

Fig. 4. The effects of *N*-propargylamine against apoptosis induced by *N*M(*R*)Sal. SH-SY5Y cells were treated with 1 μM–10 nM *N*-propargylamine at 37°C for 30 min, then with 250 μM *N*M(*R*)Sal for 24 h. Apoptotic cells were quantitatively determined by FACS, after staining the cells with PI. Three-color flow cytometry was used to analyze the frequency of PI positive cells. **A** The gate to differentiate singlet from doublet cells was determined by FL-2 (PI)-A (Area) and FL-2 (PI)-W (Width) characteristics of PI-stained cells. **B** The frequency of PI-positive cells. The cells in subG1 peak were assessed to be apoptotic, and the number of apoptotic cells was represented as percent of the total, as shown in **B**. #1; control SH-SY5Y cells. #2; cells treated with 250 μM *N*M(*R*)Sal. #3, 4, and 5; cells pretreated with 1 μM, 100 nM, 10 nM *N*-propargylamine, then 250 μM *N*M(*R*)Sal. #6; cells treated with 1 μM *N*-propargylamine

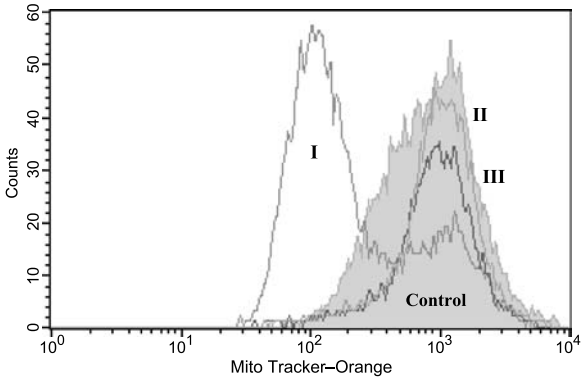


Fig. 6. The effects of *N*-propargylamine on ΔΨm decline induced by *N*M(*R*)Sal in mitochondria prepared from SH-SY5Y cells. Mitochondria were treated for 20 min without (I) or with 1 μM *N*-propargylamine (II), and then with 250 μM *N*M(*R*)Sal for 3 h at 37°C. III: Cells treated with 1 μM *N*-propargylamine alone. Mitochondria were gated by staining with MitoTracker Green, and the ΔΨm was quantitatively measured using MitoTracker Orange fluorescence

Stabilization of ΔΨm by N-propargylamine

*N*M(*R*)Sal at 250 μM induced ΔΨm decline in mitochondria isolated from SH-SY5Y cells, as shown by FACS MitoTracker Orange fluorescence representing ΔΨm (Fig. 6). Pre-

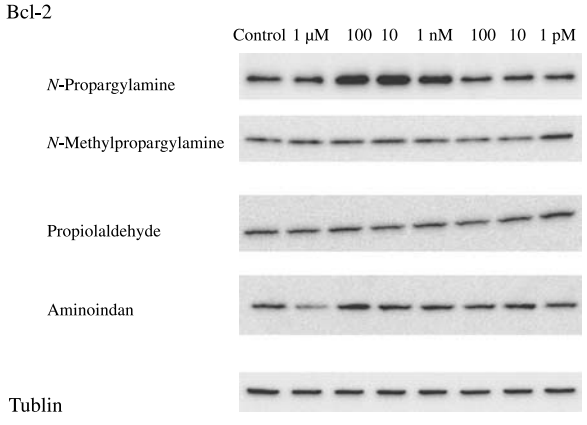


Fig. 7. The effect of *N*-propargylamine analogues and aminoindan on protein levels of Bcl-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μM-1 pM *N*-propargylamine analogues or aminoindan for 24 h, and Bcl-2 protein was quantified by Western blot analysis. Tublin in the cells was used as control

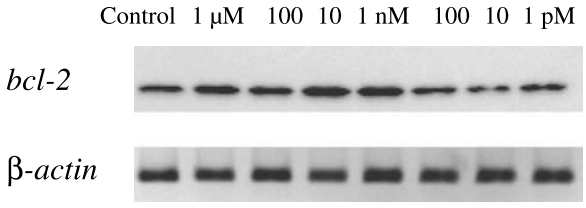


Fig. 8. The effect of *N*-propargylamine on mRNA levels of *bcl*-2 in SH-SY5Y cells. The cells were cultured in the presence of 1 μM-1 pM *N*-propargylamine for 24 h, and mRNA levels were measured by RT-PCR method, as written in Materials and methods. β-*Actin* mRNA was used as control

treatment of the cells with 1 μM *N*-propargylamine markedly prevented the decline in ΔΨm, and the protective effect was confirmed at until 10 nM of *N*-propargylamine. On the other hand, *N*-methylpropargylamine and propiolaldehyde did not prevent the decline in ΔΨm (data, not shown).

Induction of anti-apoptotic Bcl-2 by N-propargylamine

Bcl-2 is known to prevent apoptosis and promote survival, through regulating mitochondrial permeability transition. Bcl-2 protein levels in SH-SY5Y cells treated with *N*-propargylamine were estimated by Western blot analysis and increased levels of Bcl-2 protein were observed at the concentrations of *N*-propargylamine employed (Fig. 7). On the other hand, neither *N*-methylpropargylamine nor propiolaldehyde affected Bcl-2 protein levels. Aminoindan, a hydrophobic part of rasagiline, did not increase Bcl-2. The effects of *N*-propargylamine on the mRNA level of *bcl-2* were examined by RT-PCR method. As shown in Fig. 8, *N*-propargylamine significantly increased mRNA level of *bcl-2* at 1 μM–1 nM.

Discussion

N-Propargylamine, *N*-methylpropargylamine and propiolaldehyde are metabolites of a relatively non-selective MAO inhibitor, pargyline [*N*-methyl-*N*-propargylbenzylamine]

(Shirota et al., 1979; DeMaster et al., 1981), even though such metabolites have never been reported for rasagiline at present. The results in this paper clearly demonstrate neuroprotective activity of *N*-propargylamine, whereas other two metabolites did not prevent apoptosis. The results may be relevant with the fact that the propargylamine moiety plays a key role in anti-apoptotic function of rasagiline and other propargylamine derivatives (Maruyama et al., 2003; Yogev-Falach et al., 2003). Rasagiline, the (*R*)-enantiomer of *N*-propargyl-1-aminoindan, has higher anti-apoptotic activity than its (*S*)-enantiomer, TV1022 (Maruyama et al., 2001c), and only the (*R*)-enantiomer of *N*-2HMP shows anti-apoptosis activity (Maruyama et al., 2001a). These results suggest that the stereo-chemical structure of the propargylamine moiety plays a decisive role in the neuroprotective function of complex propargylamines. The dependence of anti-apoptotic activity on the stereo-chemical configuration of propargylamine residue may explain the relative weak anti-apoptotic potency of free *N*-propargylamine as reported in this paper.

In addition, our previous results suggest that there may be a binding site in the outer membrane of mitochondria, which distinguishes the enantiomeric structure of propargylamines and activates the anti-apoptotic and pro-survival cascade (Maruyama et al., 2001b). The binding protein in mitochondria remains to be elucidated, but MAO may be one of the candidates, since rasagline (Youdim et al., 2001a) and (−)deprenyl (Magyar et al., 1998) are potent irreversible inhibitors of MAO-B, as a consequence of the formation of *N*(5)-flavocyanine adduct with the FAD moiety (Nagy and Salach, 1981). *N*-Propargylamine itself irreversibly inhibits MAO-A activity in a competitive way to substrate, as reported in this paper. However, it remains to clarify whether it binds with the FAD moiety covalently, as in the case with phenolic or indane propargylamine derivatives. The parallelism between the MAO-A inhibition and anti-apoptotic function was confirmed with *N*-propargylamine and related compounds, suggesting that MAO-A may be involved in the neuroprotective function of propargylamines. Indeed, our previous work has proved the anti-apoptotic function of rasagiline in SH-SY5Y cells, where only MAO-A is expressed. These results suggest that MAO-A may be involved in neuroprotection and MAO-B inhibition is not required for the anti-apoptotic function of propargylamine derivatives, as described in Introduction. However, the possibility that propargylamines bind MAO at the site other than the substrate-binding site cannot be excluded. In addition, it remains to be clarified how *N*-propargylamine and rasagiline interact PT pore components, such as voltage-dependent anion channel, adenine nucleotide translocator or peripheral benzodiazepine receptors, and stabilize $\Delta\Psi$m.

Rasagiline increases the expression of anti-apoptotic genes, including *bcl*-2 and *bcl-xL*, but not Bax and Bad (Akao et al., 2002a, b), and GDNF (Maruyama et al., 2004). The induction of pro-survival genes by rasagiline is mediated by nuclear NF-κB transcription factor and extracellular signal-regulated protein kinase (ERK) cascade (Maruyama et al., 2004). At the same time, the involvement of mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-kinase (Yogev-Falach et al., 2002, 2003; Bar-Am et al., 2004; Weinreb et al., 2004) was also suggested by the observation that a PKC inhibitor, GF109203X and an ERK inhibitor, PD98059, prevent the neuroprotective activity of *N*-propargylamine and rasagiline (Bar Am et al., 2004; Weinreb et al., 2004). At present, the intracellular mechanisms how *N*-propargylamine and complex propargylamines activate the intracellular signaling and the transcription factors remains to be enigmatic.

These results in this paper point out that free *N*-propargylamine itself shows the anti-apoptotic activity, in a similar way as

rasagiline and other propargylamines (see Youdim 2003, for a review). If *N*-propargylamine is identified as a metabolite of rasagiline in humans, it may be involved, at least partially, in neuroprotective function of rasagiline, as shown by recent clinical trial (Parkinson Study Group, 2004). Further studies on the metabolites of rasagiline and other propargylamines in humans will clarify the pharmacodynamics of these neuroprotective and anti-apoptotic agents in PD and other neurodegenerative disorders.

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Authors' address: Dr. M. Naoi, Department of Neurosciences, Gifu International Institute of Biotechnology, 1-1 Naka-fudogaoka, Kakamigahara, Gifu 504-0838, Japan, e-mail: mnaoi@giib.or.jp