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J Neural Transm (2006) [Suppl] 71: 67–77

Journal of Neural Transmission
Printed in Austria

Involvement of type A monoamine oxidase in neurodegeneration: regulation of mitochondrial signaling leading to cell death or neuroprotection

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Summary In neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, apoptosis is a common type of cell death, and mitochondria emerge as the major organelle to initiate death cascade. Monoamine oxidase (MAO) in the mitochondrial outer membrane produces hydrogen peroxide by oxidation of monoamine substrates, and induces oxidative stress resulting in neuronal degeneration. On the other hand, a series of inhibitors of type B MAO (MAO-B) protect neurons from cell death. These results suggest that MAO may be involved in the cell death process initiated in mitochondria. However, the direct involvement of MAO in the apoptotic signaling has been scarcely reported. In this paper, we present our recent results on the role of MAO in activating and regulating cell death processing in mitochondria. Type A MAO (MAO-A) was found to bind an endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol, and induce apoptosis in dopaminergic SH-SY5Y cells containing only MAO-A. To examine the intervention of MAO-B in apoptotic process, human MAO-B cDNA was transfected to SH-SY5Y cells, but the sensitivity to *N*-methyl(*R*)salsolinol was not affected, even though the activity and protein of MAO-B were expressed markedly. MAO-B oxidized dopamine with production of hydrogen peroxide, whereas in control cells expressing only MAO-A, dopamine autoxidation produced superoxide and dopamine-quinone, and induced mitochondrial permeability transition and apoptosis. Rasagiline and other MAO-B inhibitors prevent the activation of apoptotic cascade and induce prosurvival genes, such as bcl-2 and glial cell line-derived neurotrophic factor, in MAO-A-containing cells. These results demonstrate a novel function of MAO-A in the induction and regulation of apoptosis. Future studies will clarify more detailed mechanism behind regulation of mitochondrial death signaling by MAO-A, and bring out new strategies to cure or ameliorate the decline of neurons in neurodegenerative disorders.

Abbreviations: *β-PEA* β-phenylethylamine, *$DiOC_6(3)$* 3,3′-dihexyloxacarbocyanide iodine, *DMEM* Dulbecco's modified Eagle's medium, *$\Delta\Psi m$* mitochondrial membrane potential, *FACS* fluorescence-augmented flow cytometry, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *GDNF* glial cell line-derived neurotrophic factor, *HE* hydroethidine, *HPLC-ECD* high-performance liquid chromatography with electrochemical detection, *5-HT* 5-hydroxytryptamine, serotonin, *MAO-A and MAO-B* type A and B monoamine oxidase, *NMRSal and NMSSal* *N*-methyl(*R*)salsolinol and *N*-methyl(*S*)salsolinol, *mPT* mitochondrial permeability transition, *PBS* phosphate-buffered saline, *PD* Parkinson's disease, *PI* propidium iodide, *RNAi* RNA interference, *ROS* reactive oxygen species, *siRNA* small interfering RNA

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In neurodegenerative disorders, including Parkinson's disease (PD) and Alzheimer's disease, selective neurons degenerate in specified brain regions in either apoptotic or necrotic process. In PD, the degeneration of dopamine neurons is observed mainly in the substantia nigra. Understanding of the intracellular mechanism of neurodegeneration has been advanced markedly and in the intrinsic pathway to apoptosis mitochondria initiate death signaling. Oxidative and nitrosactive stress, mitochondrial dysfunction, neurotoxins, excitotoxicity, accumulation of misfolded protein and reduced activity of the ubiquitin-proteasome system activate the death cascade (Götz et al., 1990; Andersen, 2004; Bossy-Wetzel et al., 2004; Naoi et al., 2005). The detailed mechanism underlying the cell death in PD has been studied using animal and cellular models, and we found that dopamine-derived *N*-methyl(*R*)-salsolinol [*N*M*R*Sal, 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] is an endogenous neurotoxin and causes cell death in dopamine neurons (Naoi et al., 2002a, b, 2004). After continuous infusion in the rat striatum, *N*M*R*Sal induces apoptotic cell death in dopamine neurons in the substantia nigra (Naoi et al., 1996). In human dopaminergic neuroblastoma SH-SY5Y cells, *N*M*R*Sal induced apoptosis by sequential activation of death cascade; decline in mitochondrial membrane potential, $\Delta\Psi m$, opening of mitochondrial permeability transition (mPT) pore, release of cytochrome c, activation of caspase 3, nuclear translocation of glyceraldehydes-3-phosphate

dehydrogenase [GAPDH, D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12], and fragmentation of nuclear DNA (Maruyama et al., 1997, 2001a, b; Akao et al., 2002a). Analyses of clinical samples from parkinsonian patients indicate that *N*M*R*Sal might be involved in the pathogenesis of PD (Maruyama et al., 1996; Naoi et al., 1998).

The mPT is an increase in the permeability of the inner mitochondrial membrane to solutes, by opening of mPT pore, a large proteinaceous pore spanning the outer and inner membrane of mitochondria (Crompton, 1999; Green and Kroemer, 2004). The mPT pore forms a functional micro-compartment with voltage-dependent anion channel in the outer membrane, adenine nucleotide translocator in the inner membrane, and hexokinase at the contact site, but the exact composition has not yet been fully clarified. The (*R*)-enantiomer of *N*-methylsalsolinol (*N*MSal), but not the (*S*)-enantiomer, induces swelling in mitochondrial matrix (Akao et al., 2002a) and $\Delta\Psi$m reduction in SH-SY5Y cells (Maruyama et al., 2001b). The enantio-selective cytotoxicity of *N*M*R*Sal suggests the occurrence of the specified binding site recognizing *N*M*R*Sal in mitochondrial membrane. *N*M*R*Sal was found to inhibit type A, but not type B, monoamine oxidase [MAO-A and MAO-B, monoamine: oxygen oxidoreducatse (deaminating), EC 1.4.3.4]. The inhibition was competitive to the substrate, and the value of the inhibitor constant, K_i, was estimated to be $59.9 \pm 5.4\,\mu M$ (mean $\pm$ SD) (Yi et al., 2006b). This suggests that MAO-A may bind *N*M*R*Sal at or near the substrate-binding site. However, it has never been reported whether MAO is involved directly in apoptotic cascade, or MAO itself is a component of mPT pore.

MAO is localized in the outer membrane of mitochondria and catalyses the oxidative deamination of neuroactive, vasoactive and xenobiotic amines generating hydrogen peroxide and aldehydes. The two MAO isoenzymes, MAO-A and MAO-B, share 70% amino acid sequence identity and are encoded by two closely linked genes in the X chromosome (Bach et al., 1988; Shih et al., 1999). These two isomers have distinct specificities for the substrates and inhibitors (Tipton et al., 2004). MAO-A has substrate preference for 5-hydroxytryptamine (5-HT, serotonin) and norepinephrine, and very high sensitivity to an irreversible inhibitor, clorgyline [*N*-methl-*N*-propargyl-3(2,4-dichlorophenoxy)-propylamine], whereas MAO-B oxidizes β-phenylethylamine (β-PEA) and benzylamine and is inhibited by low concentrations of (−)deprenyl [*N*, α-dimethyl-*N*-2-propynylbenzene-ethanlamine] and rasagiline [*N*-propargl-1(*R*)- aminoindan] (Youdim et al., 2001). In human brain MAO-A is expressed in catecholamine neurons, whereas serotonergic neurons and astrocytes contain MAO-B (Westlund et al., 1988). The studies of MAO-A and MAO-B knockout mice clearly proved that these two MAO isoenzymes have distinct functions in monoamine metabolism and play important roles in neurological and psychiatric disorders, including depression and PD (Cases et al., 1995; Lim et al., 1994; Shih et al., 1999). In human brain MAO-B levels increase 2–3 folds in an age-dependent way, resulting in increased oxidative stress, which may induce vulnerability of the brain in age-dependent neurodegenerative disorders.

A series of MAO-B inhibitors with a propargyl moiety, rasagiline and (−)deprenyl, protect neurons from cell death induced by various insults (Maruyama et al., 2001a; Youdim et al., 2005a, b). Rasagiline is now the most potent in neuro-rescue or -protective function, as shown in animal and cellular models of PD, Alzheimer's disease and brain ischemia, and the neuroprotective effect has been also suggested in clinical trials (Parkinson Study Group, 2004). The anti-apoptotic function is due to the direct stabilization of mPT pore (Maruyama et al., 2001a, 2001b) and induction of prosurvival genes, such as antiapoptotic Bcl-2 and Bcl-xL (Akao et al., 2002a, b) and glial cell line-derived neurotrophic factor (GDNF) (Maruyama et al., 2004). However, the neuroprotective function may not necessarily depend on the inhibition of MAO-B activity, as suggested by the facts that the neuroprotective potency is observed with propargylamines without MAO-inhibition (Maruyama et al., 2001c; Yi et al., 2006a), and at the concentration quite lower than those for MAO inhibition (Akao et al., 2002a; Maruyama et al., 2001a, 2004).

In this paper, the role of MAO in the apoptotic cascade was studied by use of *N*M*R*Sal in SH-SY5Y cells containing only MAO-A (wild SH), in relation to the *N*M*R*Sal binding, $\Delta\Psi$M reduction and apoptosis. To confirm the role of MAO-A in apoptotic cascade, the effects of RNA interference (RNAi) targeting MAO was examined by use of small interfering RNA (siRNA) to silence MAO-A in the cells. In addition, the involvement of MAO-B in apoptosis by *N*M*R*Sal was examined in SH-SY5Y cells transfected with cDNA of human MAO-B (MAO-B-SH). The role of MAO-A and -B in inducing anti-apoptotic genes by rasagiline, a MAO-B inhibitor, was studied by use of these SH cells, and also Caco-2 human colon adenocarcioma cells expressing only MAO-B (Wong et al., 2003). The role of MAO isoenzymes is discussed in relation to the regulation of apoptotic signaling in mitochondria, and their possible involvements in neurodegenerative disorders including PD.

Materials and methods

Materials

*NMR*Sal was synthesized according to Teitel et al. (1972). Kynuramine, 4-quinolinol, dihydroethidine (HE) and dopamine were purchased from Sigma (St. Louis, MO, USA); propidium iodide (PI), MitoTracker Orange and Green, and 3,3′-dihexyloxacarbocyanide iodine [$DiOC_6(3)$] from Molecular Probes (Eugene, OR, USA); 5-hydroxytryptamine (5-HT, serotonin) from Merck (Darmstadt, Germany). Clorgyline, a MAO-A inhibitor, and rasagiline and (−)deprenyl (selegiline), MAO-B inhibitors, were kindly donated by May and Baker (Dagenham, U. K.), TEVA (Netanya, Israel), and Dr. Knoll (Semmellweis University, Budapest, Hungary), respectively. Dulbecco's modified Eagle's medium (DMEM), β-PEA and other drugs were purchased 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 according to Desagher et al. (1999). Caco-2 cells were cultured in DMEM supplemented with 10% FCS and 1% nonessential amino acids.

RNAi of MAO-A in SH-SY5Y cells

To reduce MAO-A expression in mitochondria, siRNA targeting MAO-A mRNA (Sc-35874) was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The siRNAs were transfected into the cells to be 20–35 nM in the final concentration by use of cationic liposomes TransIT-TKO (Mirus Bio, Madison, WI, USA). The transfection efficiency was evaluated by the transfection of the cells with a duplex siRNA-FITC. Non-specific control duplex (57% GC content; Dharmacon, Lafayette, CO, USA) was used as control for non-specific effects. The effects of RNAi targeting MAO-A on the protein amount and activity of MAO and the binding of *NMR*Sal were determined at 36 h after the transfection. MAO protein was detected by Western blot analyses, using antibody recognizing both MAO-A and -B prepared according to Gargalidis-Moudanos et al. (1997). The polyclonal antisera were isolated from rabbits immunized with the peptide TNGGQ ERKFVGGSGQ, corresponding to amino acids 210–227 in MAO-A and 202–217 in MAO-B, and purified on an affinity column conjugated with the antigen peptide. Bound antibodies were detected using enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

DNA transfection of MAO-B gene in SH-SY5Y cells

To establish transfectants expressing human MAO-B, a pIRES1neo eukaryotic expression vector (Invitrogen, San Diego, CA, USA) was used (Yi et al., 2006b). pIRES1-neo-MAO-B was constructed by including the full-length human MAO-B gene in pECE vector (Lan et al., 1989) and digested with HindIII and inserted into the pIRESneo vector. SH-SY5Y cells were transfected with pIRES1neo or pIRES1neo-MAO-B by using cationic liposomes (Lipofect-AMINE). Selection was started 2 days after the transfection using the culture medium containing geneticin (GIBCO BRL). Individual clones were isolated and characterized by RT-PCR, as described previously (Akao et al., 2002a). Stable clones overexpressing MAO-B protein (MAO-B-SH) were obtained by limiting dilution and confirmed by RT-PCR.

Assay for MAO-A and MAO-B activity

MAO activity in mitochondria was measured fluorometrically by use of kynuramine as a substrate, according to Kraml (1965). Mitochondria prepared from control SH-SY5Y (wild SH) cells were used as a MAO-A sample, and those from MAO-B-SH cells were pre-treated with 1 μM clorgyline at 37°C for 20 min and used as a MAO-B sample. Protein concentration was determined according to Bradford (1976).

Assay for the binding of NMRSal to mitochondria

Mitochondria were suspended in 100 μl of 10 mM Tris-HCl buffer, pH 6.0, and incubated with 10–100 μM *NMR*Sal for 60 min at 4°C. Then, the cells were washed successively with 1.5 ml of phosphate-buffered saline (PBS) containing 1% bovine serum albumin and twice with PBS alone by centrifugation at 6000 *g* for 10 min. The cells were suspended in 200 μl of 10 mM perchloric acid solution containing 0.1 mM EDTA, mixed, centrifuged, filtered through a Millipore HV filter (pore size 0.45 μm), and applied to high-performance liquid chromatography with electrochemical detection (HLC-ECD), as reported previously (Naoi et al., 1996).

Measurement of $\Delta\Psi m$

The effects of *NMR*Sal on $\Delta\Psi m$ were quantitatively measured by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Becton Dickinson, San Jose, CA, USA), and MitoTracker Orange and Green, or 3,3′-dihexylpxacarbocyanide iodine [$DiOC_6(3)$] were used as fluorescent indicators (Yi et al., 2006a, b). The cells were cultured in 6-well poly-L-lysine-coated tissue culture flasks, washed with Cosmedium-001 without FCS, and incubated with 100–500 μM *NMR*Sal or dopamine for 3 h at 37°C. The effects of 5-HT and β-PEA were also examined by addition of 100–500 μM 5-HT and β-PEA. After stained with 100 nM MitoTracker Orange and Green for 30 min at 37°C, or 2.5 nM $DiOC_6(3)$ (Stock solution: 1 μM in ethanol) for 15 min at 37°C. Then, the cells 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. $DiO_6(3)$ fluorescence was measured with FL-1.

Assessment of apoptosis induced by NMRSal or dopamine

Apoptosis was quantitatively measured by FACS, as described previously (Yi et al., 2006a). The cells cultured in 6-well poly-L-lysine-coated culture flasks were incubated in DMEM with 100–500 μM *NMR*Sal or 100 μM dopamine at 37°C for 24 h, and treated with trypsin, gathered, and washed with PBS. The cells were stained with 75 μM PI solution in PBS containing 1% Triton X-100 at 24°C for 5 min in the dark, washed and suspended in PBS, then subjected to FACS analysis. Cells with a lower DNA content, as shown by PI staining less than G1, were defined to be apoptotic (subG1 peak) (Eckert et al., 2001).

Rasagiline-induced bcl-2 expression in the cells

Wild SH and MAO-B-SH cells and Caco-2 cells were cultured with 10 μM–10 pM rasagiline overnight and Bcl-2 contents in the cells were quantitatively determined by Western blot analysis as reported (Akao et al., 2002b).

Statistics

Experiments were repeated 3 to 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.

Results

Binding of NMRSal to mitochondrial MAO-A and the effects of siRNA for MAO-A

The binding of *NMR*Sal to mitochondria prepared from control SH-SY5Y (wild SH) cells was kinetically studied

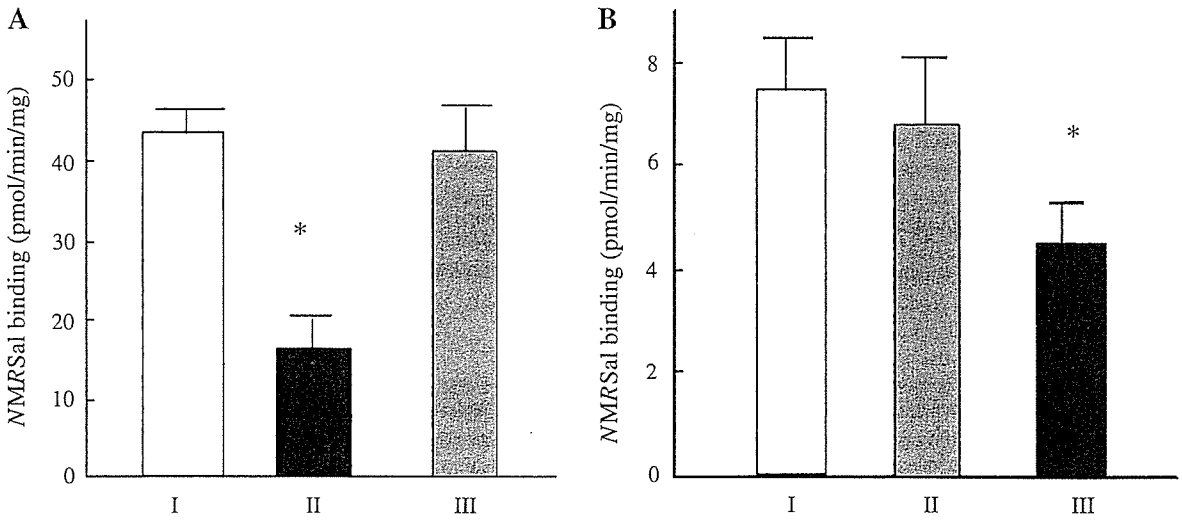

Fig. 1. The effects of MAO-inhibitors and RNAi targeting MAO on *NMR*Sal binding to mitochondria. **A** Effects of clorgyline and (−)deprenyl on the *NMR*Sal binding to mitochondria. Mitochondria were treated with 1 μM MAO inhibitors for 20 min at 37°C, then incubated with 10 μM *NMR*Sal for 1 h at 4°C. The column and bar represent the mean and SD of triplicate measurement of 2 experiments. **B** Effects of MAO-A RNAi. Crude mitochondria were prepared from wild SH (I), negative control (II) and siRNA-treated cells (III), and incubated with *NMR*Sal. *NMR*Sal binding was quantified by HPLC-ECD. The column and bar represent the mean and SD of triplicate measurements. $^{*}p < 0.05$ from control and negative control cells

and the binding kinetics followed the Michaelis-Menten equation. The values of the apparent Michaelis constant, K_m, and the maximal velocity, V_{max}, were obtained to be 80 ± 15 μM and 2.7 ± 0.5 nmol/h/mg protein, respectively. The involvement of MAO in the binding was examined by use of clorgyline and (−)deprenyl, the selective inhibitor of MAO-A and MAO-B, respectively. As shown in Fig. 1A, clorgyline reduced *NMR*Sal binding significantly, but (−)deprenyl did not affect the binding.

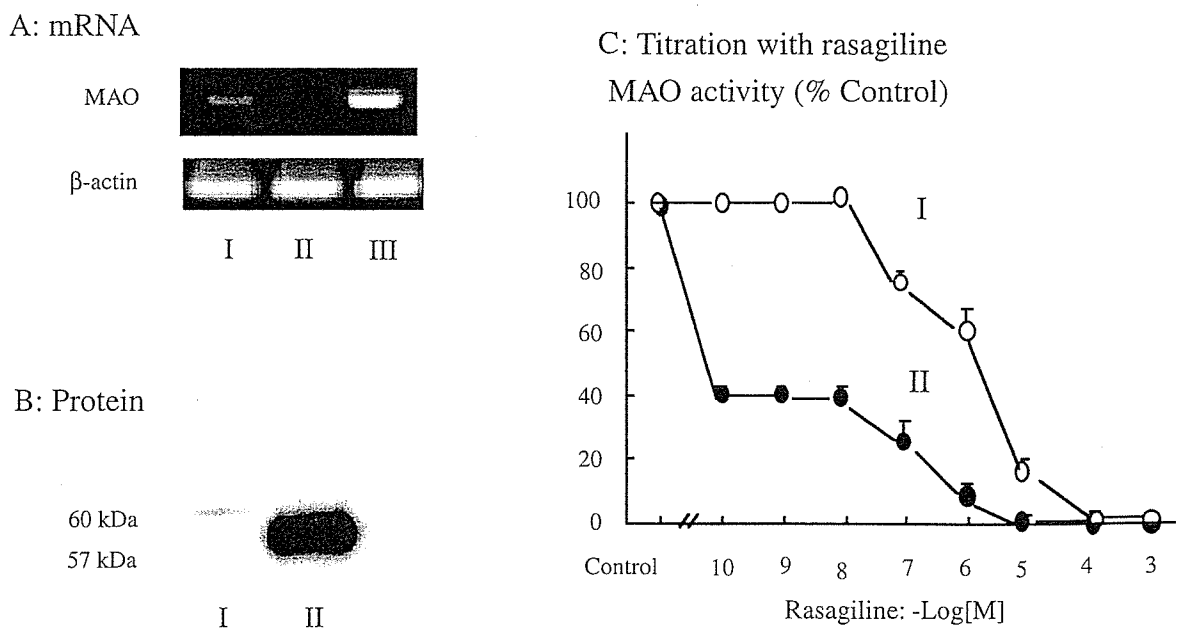

Fig. 2. Establishment of SH-SY5Y cells transfected with human MAO-B. SH-SY5Y cells were transfected with human MAO-B cDNA. **A** mRNA isolated from wild SH cells (I), cells transfected with IRES vector alone (II), and with full length MAO-B cDNA (III). β-Action was used as control. **B** Western blot analyses of MAO protein in mitochondria isolated from wild SH (I) and MAO-B-SH cells (II). MAO protein was detected with the antibody recognizing both MAO-A and -B. **C** Effects of rasagiline, a MAO-B inhibitor, on MAO activity. Mitochondria were prepared from wild SH (I) and MAO-B-SH cells (II), and MAO activities were measured with 100 μM kynuramine as a substrate, after treatment with rasagiline (0.1 nM–1 mM) at 37°C for 20 min. Each point and bar represent the mean and SD of triplicate measurements

In order to confirm whether *N*MRSal binds to MAO-A in mitochondria, MAO-A expression was inactivated using RNAi. In the siRNA-transfected cells, MAO protein with about 60 kDa was significantly reduced, whereas in non-specific siRNA-transfected cells the protein amount was almost the same as in control. The functional effects of RNAi were confirmed by reduction in MAO activity to 0.22 ± 0.02 nmol/min/mg protein in the siRNA treated-cells from 0.34 ± 0.03 nmol/min/mg protein in control. In non-specific siRNA-transfected cells, the MAO activity was the same as in control, 0.34 ± 0.01 nmol/min/mg protein. Figure 1B shows that RNAi targeting MAO-A markedly reduced *N*MRSal binding to 4.47 ± 0.88 pmol/mg protein in siRNA-treated cells from 7.46 ± 0.95 and 6.83 ± 1.40 pmol/mg protein in control and non-specific siRNA-treated cells.

Effects of transfected MAO-B on MMRSal binding

To specify the role of MAO-A and -B in the binding of *N*MRSal and the induction of apoptosis, SH-SY5Y cells transfected with human MAO-B cDNA (MAO-B-SH) were prepared from control cells expressing only MAO-A (wild SH). The expression of mRNA of MAO-B was confirmed in MAO-B-SH cells (Fig. 2A). MAO-A and MAO-B protein in wild SH and MAO-B-SH cells were detected by Western blot analyses and their apparent molecular weights were determined to be approximately 60 and 57 kDa, respectively (Fig. 2B). MAO activity in mitochondria isolated from MAO-B-SH cells increased significantly to be 22.9 ± 0.93 from 2.82 ± 0.18 nmol/min/mg protein in those from wild SH cells. The sensitivity to rasagiline, an irreversible inhibitor of MAO-B, increased by MAO-B transfection, as shown by the inhibitor concentration-ac-

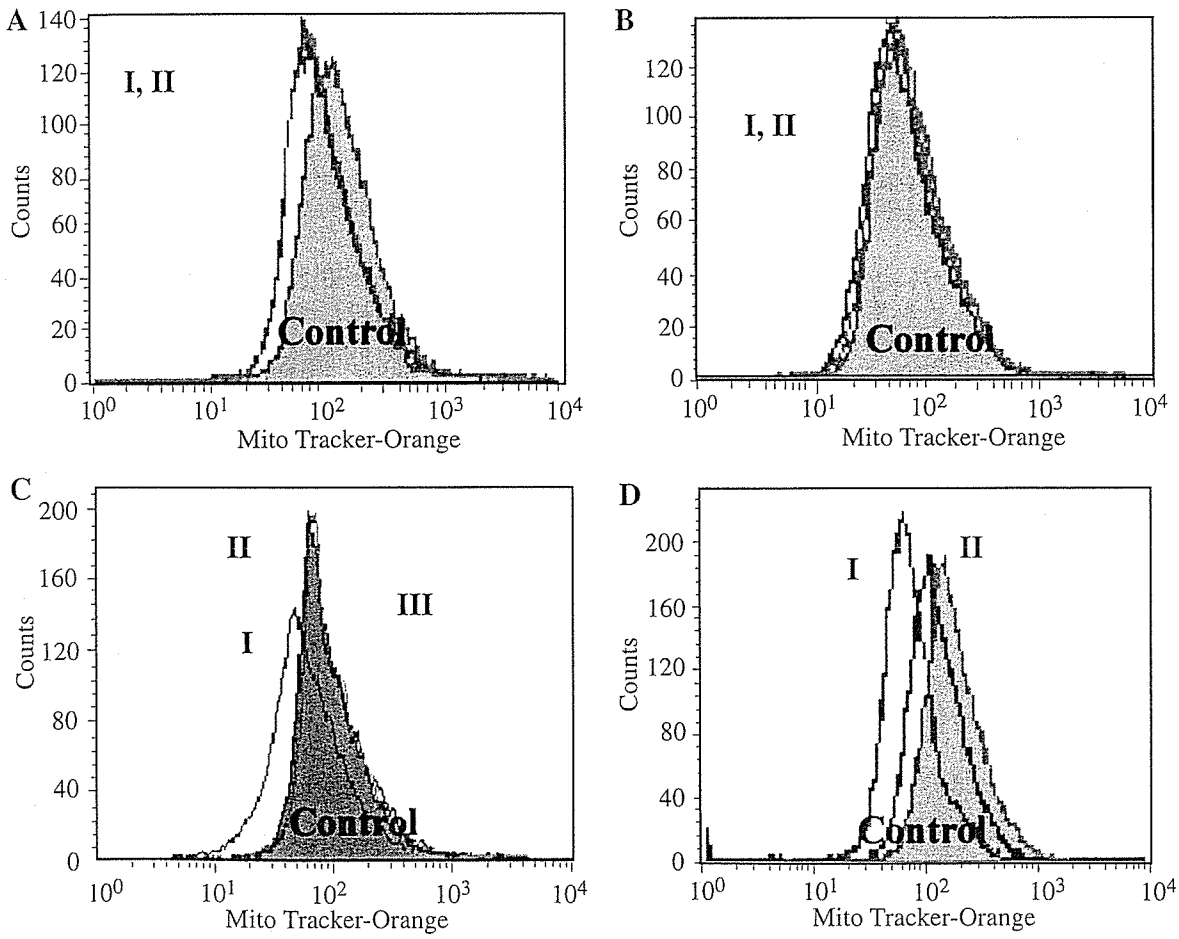

Fig. 3. *N*MRSal reduced $\Delta\Psi$m in isolated mitochondria. **A** and **B**: Mitochondria were prepared from wild SH (A) and MAO-B-SH cells (B) and incubated with 500 μM (I) and 250 μM *N*MRSal (II) at 37°C for 3 h. **C** Mitochondria isolated from wild SH cells were incubated with 100 μM *N*MRSal in the absence (I) and presence of 100 μM 5-HT (II), or treated with 5-HT alone at 37°C for 3 h. **D** Mitochondria prepared from wild SH were incubated with the anti-MAO antibody diluted by 100-folds (I) or 500-folds (II) at 37°C for 3 h. $\Delta\Psi$m was measured by FACS after stained with MitoTracker Orange and Green

tivity studies (Fig. 2C), indicating that increased MAO activity was due to transfected MAO-B.

The binding of *NMR*Sal to mitochondria prepared from wild SH and MAO-B-SH cells was examined. The binding velocity of *NMR*Sal to mitochondria isolated from wild SH and MAO-B-SH cells were 163.6 ± 52.6 and 150.1 ± 20.9 pmol/min/mg protein, respectively. The transfection of MAO-B did not increase *NMR*Sal binding, suggesting that *NMR*Sal did not bind to MAO-B, as shown also by the fact *NMR*Sal did not inhibit MAO-B activity.

NMRSal induced ΔΨm decline and apoptosis in MAO-A containing cells

Involvement of MAO-A and -B in apoptosis induced by *NMR*Sal was examined using mitochondria prepared from wild SH and MAO-B-SH cells. Figure 3A and B show that *NMR*Sal reduced $\Delta\Psi$m in mitochondria containing MAO-A, but did not affect $\Delta\Psi$m in those prepared from MAO-B-SH cells. 5-HT, a substrate of MAO-A, prevented $\Delta\Psi$m decline induced by *NMR*Sal (Fig. 3C), whereas β-PEA, a MAO-B substrate, did not. In addition, clorgyline, an irreversible inhibitor of MAO-A reduced $\Delta\Psi$m, which 5-HT prevented. On the other hand, a reversible MAO-A inhibitor moclobemide did not. Figure 3D shows that the antibody against MAO reduced $\Delta\Psi$m in a doe-dependent way.

The role of MAO-A in apoptosis was shown by competition with 5-HT. *NMR*Sal induced apoptosis in wild SH, which 5-HT prevented completely (Fig. 4A). The number of apoptotic cells after *NMR*Sal treatment was 36.9% of the total and reduced to 5.3% by addition of 5-HT, which was almost the same as in control cells or cells treated with 5-HT alone; 5.5 and 4.6%. Clorgyline also induced apoptosis in the cells at the concentration higher than 100 nM (Fig. 4B). Clorgyline (50 μM) increased the number of apoptotic cells to 28.7% of the total from 12.4% and 8.4% in control and 5-HT alone (1 mM)-treated cell, and 5-HT reduced the number of clorgyline-induced apoptotic cells to 14.7%.

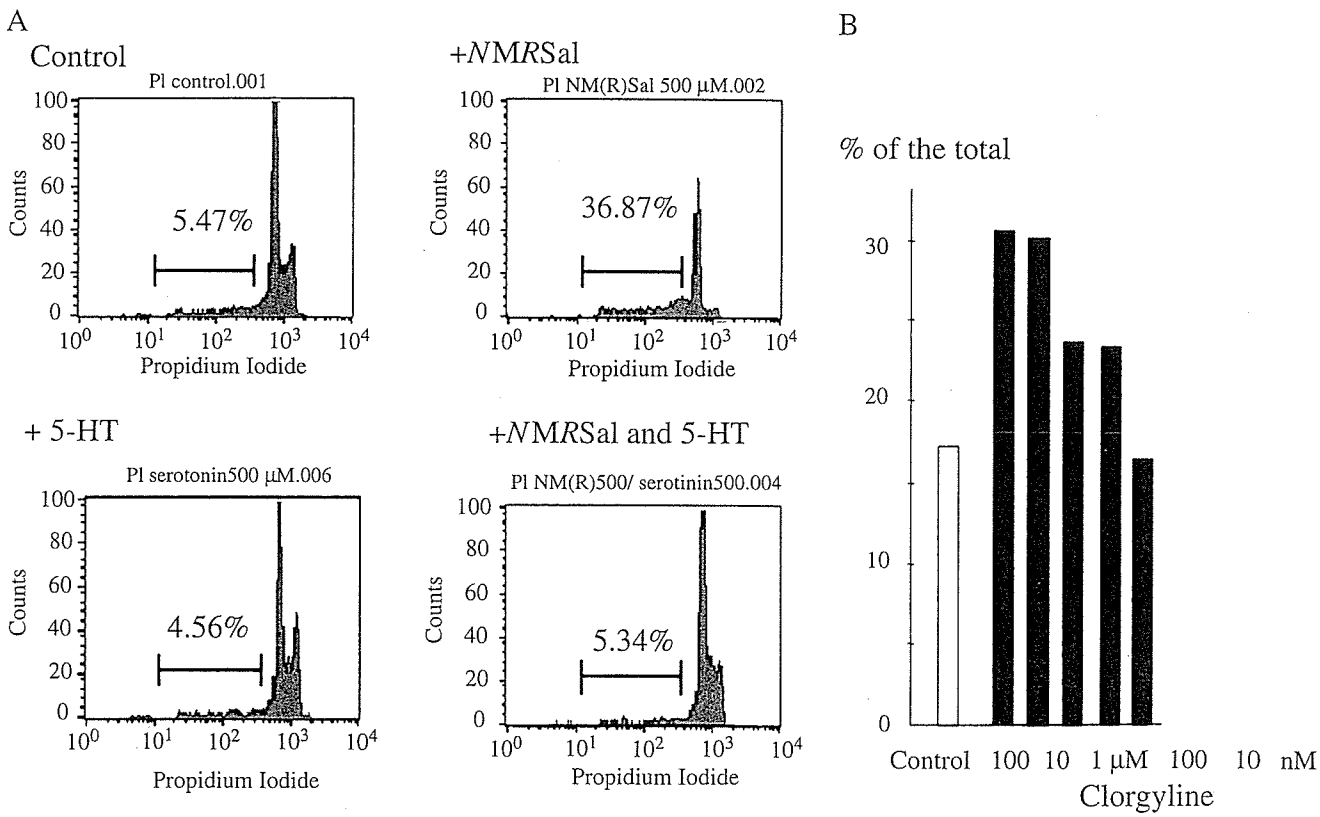

Fig. 4. Effects of 5-HT on *NMR*Sal-induced apoptosis and apoptosis by clorgyline in wild SH cells. **A** Control cells were incubated with *NMR*Sal (+*NMR*Sal) or 500 μM 5-HT (+5-HT) or *NMR*Sal and 5-HT (+*NMR*Sal and 5-HT) at 37°C overnight. Apoptotic cells were quantified by FACS after staining with PI. The cells with lower DNA content showing less PI staining than G1 were defined to be apoptotic. The number in Fig. 4A represents the number of apoptotic cells in the total (%). **B** Wild SH cells were incubated with 10 μM–10 nM clorgyline at 37°C overnight and apoptotic cells were quantified by FACS-PI method. The column represents the number of apoptotic cells as % of the total

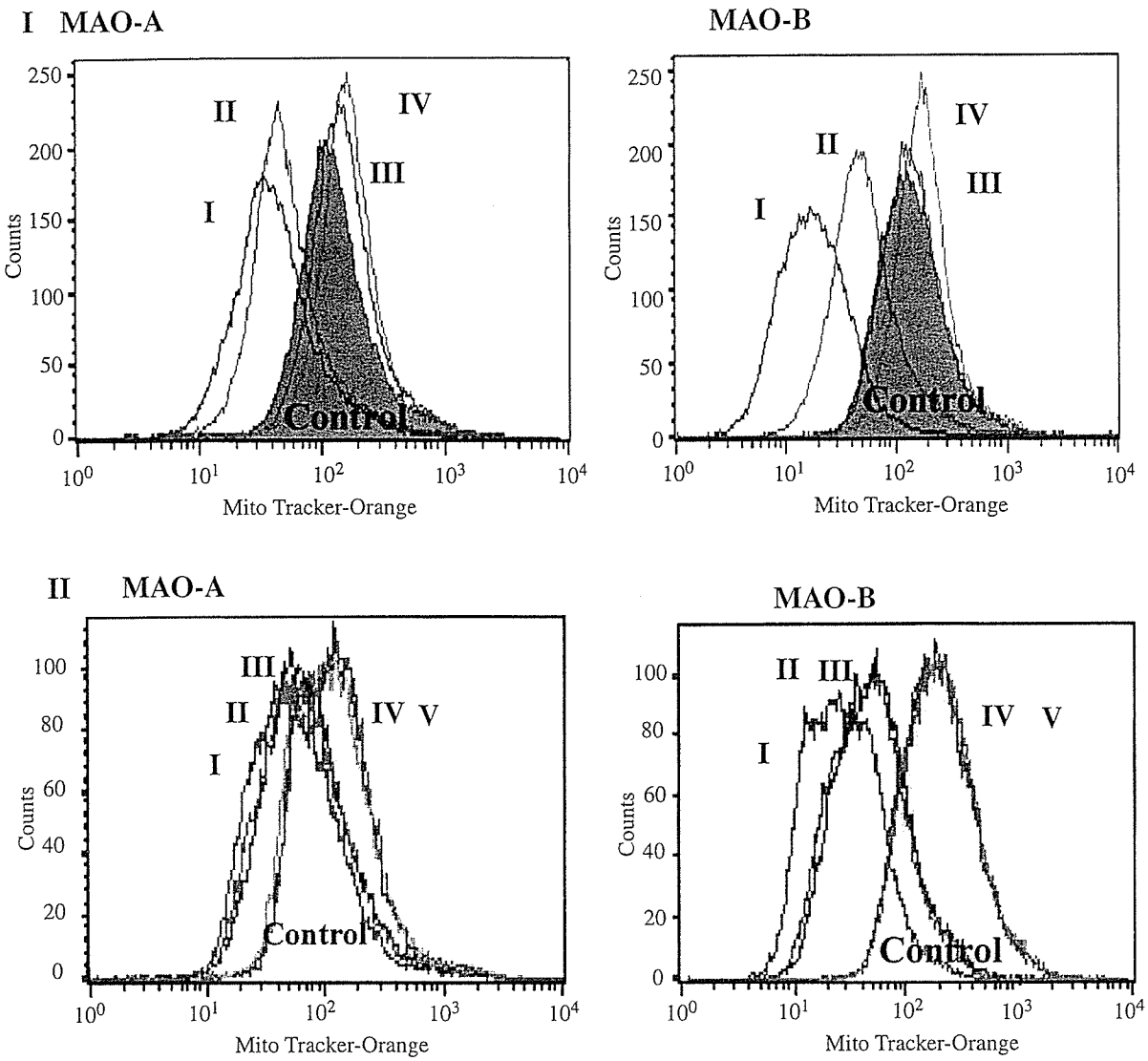

Fig. 5. $\Delta\Psi$m reduction by dopamine oxidation and effects of MAO inhibitors. Mitochondria were prepared from MAO-A only containing wild SH (**MAO-A**) and MAO-B-SH cells (**MAO-B**). **I** Mitochondria incubated at 37°C for 3 h with 100 μM dopamine (I), 100 μM L-DOPA (II), 1 μM Fe^{2+} (III) and Fe^{3+} (IV). **II** Mitochondria were treated with 100 μM dopamine in the absence (**I**) of 1 μM clorgyline (**II**) or (−)deprenyl (**III**). **IV** and **V**: Mitochondria treated with clorgyline or (−)deprenyl. $\Delta\Psi$m was measured by FACS with MitoTracker Orange

The role of MAO-A and MAO-B in the cytotoxicity of dopamine oxidation

Dopamine is oxidized either by enzymatic oxidation of MAO to 3,4-dihydrophenylactaldehyde and hydrogen peroxide, or by non-enzymatic autoxidation to dopamine-quinone and superoxide. The role of MAO-A and -B in the dopamine-induced cell death process was studied using mitochondria isolated from wild SH and MAO-B-SH cells. As shown in Fig. 5, I, dopamine and L-DOPA reduced $\Delta\Psi$m markedly in MAO-A-containing mitochondria, whereas in MAO-B-containing mitochondria dopamine reduced $\Delta\Psi$m more markedly than by L-DOPA and in MAO-A-containing mitochondria. Clorgyline and (−)deprenyl, inhibitors of MAO-A and MAO-B, did not prevent $\Delta\Psi$m decline in MAO-A-containing mitochondria, but they partially prevented the $\Delta\Psi$m decline in MAO-B-containing mitochondria (Fig. 5, II). Using FACS and fluorescent dyes, H_2DCFDA for hydrogen radical, nitric oxide and peroxynitrite (Crow, 1997) and HE for superoxide (Bindokas et al., 1996), ROS produced from dopamine oxidation was confirmed to be superoxide in MAO-A-containing mitochondria, whereas MAO-B produced hydrogen peroxide in addition to superoxide. Reduced glutathione, ascorbic acid and superoxide dismutase prevented $\Delta\Psi$m decline in

MAO-A and -B-containing-mitochondria, whereas catalase did not. Dopamine oxidation modifies SH residues in mitochondrial complex I with formation of quinoprotein and inhibits the enzymatic activity of mitochondrial oxidative phophorylation (Naoi et al., in preparation).

Involvement of MAO-A in neuroprotection by rasagiline, a MAO-B inhibitor

A series of propargylamine MAO-B inhibitors protect neuronal cells in cellular and animal models of PD and other neurodegenerative disorders. The role of MAO in the neuroprotective function by rasagiline was confirmed in wild SH cells containing only MAO-A. The antiapoptotic, neuroprotective function of rasagiline is ascribed to two mechanisms. One is the stabilization of mitochondrial homeostasis and the prevention of mPT, and the other the induction of anti-apoptotic genes, bcl-2 and GDNF, as shown in Fig. 6. Opening of mPT pore leads to $\Delta\Psi$m loss and swelling of the matrix, which was completely suppressed by rasagiline. Rasagiline prevents the cytochrome c release from mitochondria caused by rupture of the outer membrane due to the swelling, and suppresses the activation of casspase 3 (Maruyama et al., 2001a; Akao et al., 2002a) and the nuclear translocation of GAPDH (Maruyama et al., 2001b). Rasagiline increases the gene expression and protein amounts of bcl-2 (Aako et al., 2002b) and GDNF (Maruyama et al., 2004) in wild SH cells, and also the activity of catalase and superoxide dismutase in rats (Carrillo et al., 2000). The gene induction has the concen-

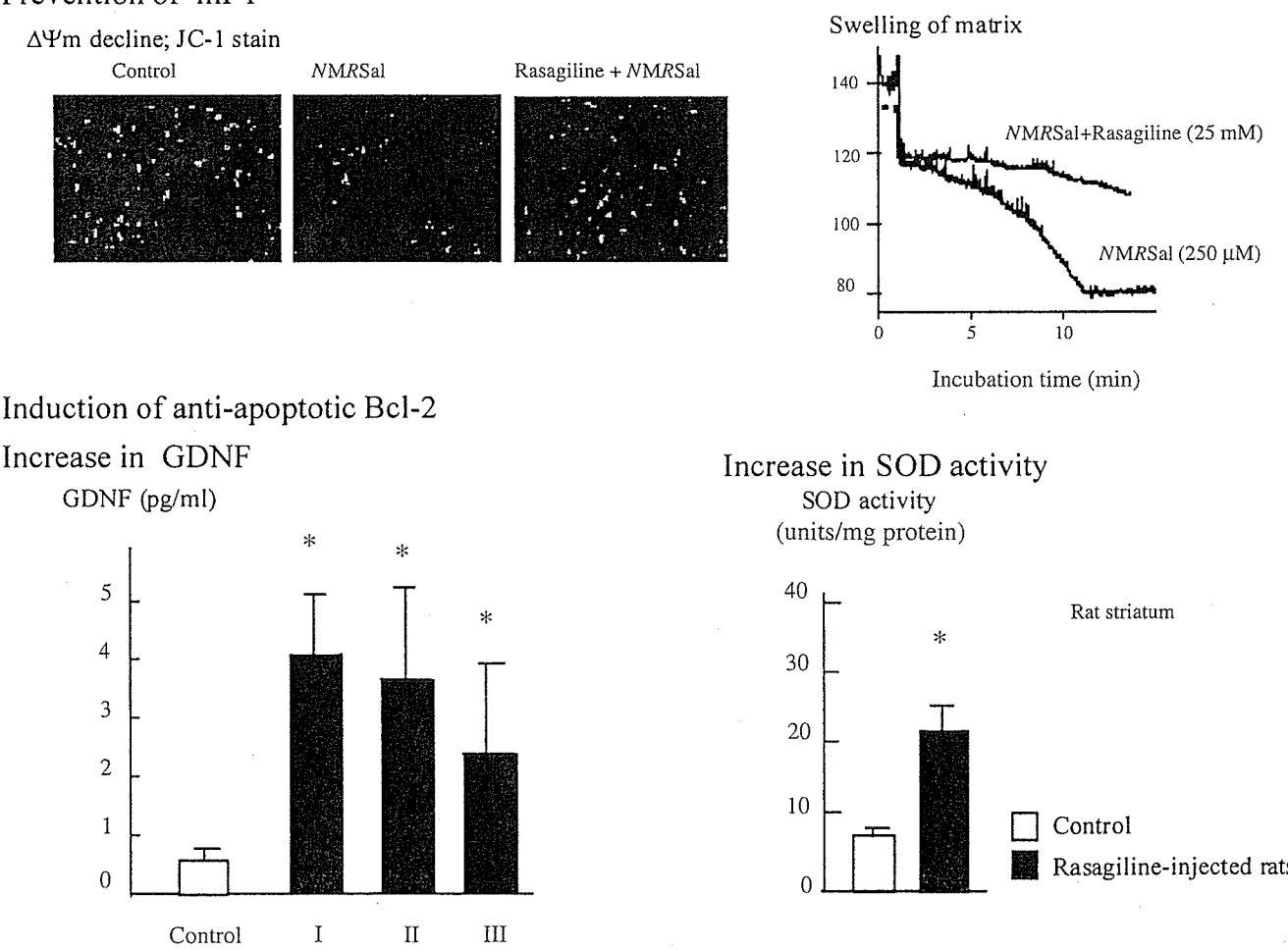

Fig. 6. Intracellular mechanism behind neuroprotective function of rasagiline. Rasagiline stabilizes mPT pore and prevents $\Delta\Psi$m collapse and swelling of mitochondrial matrix. The activation of following death cascade, release of cytochrome c, activation of caspases and nuclear translocation of GAPDH is completely suppressed. Rasagiline increases GDNF in SH-SY5Y cells in a dose-dependent way. Cells were treated with 1 μM (I), 100 (II) and 10 nM (III) rasagiline at 37°C overnight and GDNF amount was assessed by ELISA. In rat brain regions containing dopamine neurons SOD and catalase activities increases significantly after systematic administration of rasagiline for 3 weeks. $^{*}p < 0.01$

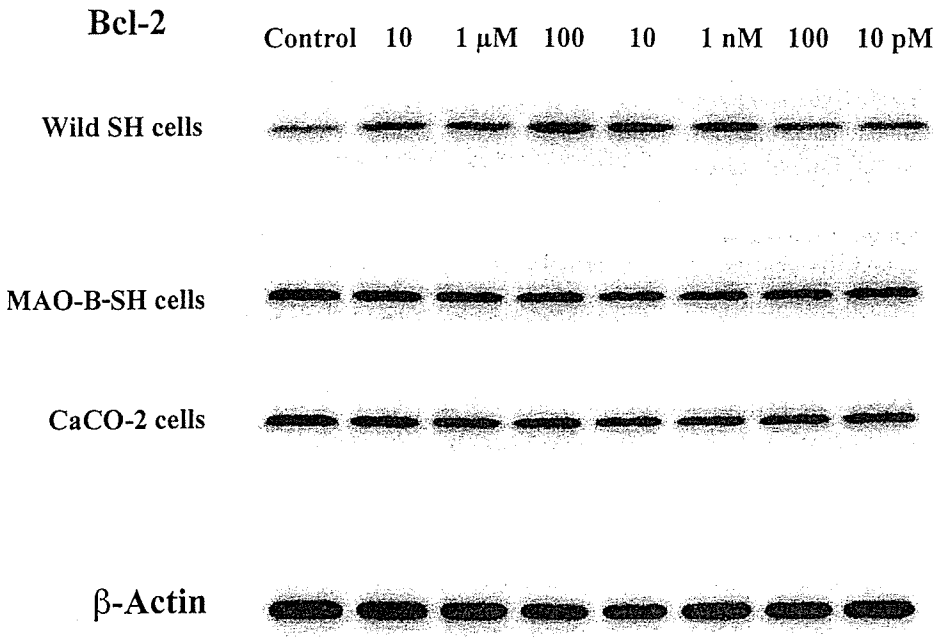

Fig. 7. Induction of Bcl-2 in wild and MAO-B-SH-SY5Y cells, and Caco-2 cells. Only MAO-A containing control and MAO-B transfected cells, and only MAO-B expressing Caco-2 cells were treated with 10 μM-10 pM rasagiline at 37°C overnight and the amount of Bcl-2 was determined by Western blot analysis. β-Action was used as a control

tration optima at two quite different ranges, 100–10 nM and 100–10 pM (Akao et al., 2002b). These concentrations were quite lower than those required for inhibition of MAO-A and -B. The IC_{50} values for inhibition of rat brain MAO activity were reported to be 412 nM and 4.4 nM for MAO-A and -B, respectively (Youdim et al., 2001).

The involvement of MAO-B in the induction of antiapoptotic genes was studied in MAO-B transfected cells and Caco-2 cells expressing only MAO-B. Even though marked expression of MAO-B was confirmed by the increased activity and protein amount, transfected MAO-B did not increase the sensitivity to rasagiline, as shown in Fig. 7. In Caco-2 cells bcl-2 was not induced by rasagiline at the concentrations of 10 μM–10 pM, suggesting that MAO-B may not be involved, or non-neuronal cells may not be responsible to rasagiline. These results suggest that MAO-A may play a major role in the antiapoptotic function of propargylamines, and that MAO-A may have a specified binding site of rasagiline other than that of the substrate and induce antiapoptotic genes. However, these results cannot exclude the possibility that MAO-B itself is involved in regulating apoptotic cascade in other types of cells. In addition, it remains to clarify how the signaling from mitochondria activates the transcription factors, such as NF-κB, which mediates the induction of Bcl-2 and GDNF by rasagiline (Maruyama et al., 2004).

Discussion

This paper reports for the first time the direct involvement of MAO-A in apoptosis. All the hitherto papers discussed the role of MAO in neuronal degeneration mainly in relation to the enzymatic oxidation of monoamines and the induction of oxidative stress (Cohen et al., 1997). In addition, the role of MAO-B in PD was augmented by the fact that MAO-B oxidizes a protoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into toxic 1-methyl-4-phenylpyridinium ion (MPP^+) (Heikkila et al., 1985). In concern to the role of MAO-A in apoptosis, higher MAO-A levels were expressed in apoptosis induced by depletion of nerve growth factor in PC12 cells, and increased ROS generation was considered to potentiate apoptosis (De Zutter and Davis, 2001). On the other hand, Malorni et al. (1998) reported that clorgyline and pargyline, inhibitors of MAO-A and MAO-A and -B, protected human melanoma M14 cells from apoptosis induced by serum withdrawal. These MAO inhibitors prevented the mPT induced by tyramine, a substrate for MAO-A and -B, in mitochondria isolated from rat liver (Marcocci et al., 2002). The protective function of MAO-A inhibitors was considered to be due to maintaining mitochondrial homeostasis by a direct effect on mPT pore in addition to inhibiting monoamine oxidation, but the detailed mechanisms were not presented in their paper.

Our results point out a novel direct involvement of MAO-A in mitochondrial apoptotic mechanism in addition to the enzymatic generation of ROS. RNAi targeting MAO-A reduced *N*MR*S*al binding to mitochondria, in almost the same degree as the reduction of MAO protein amount and enzymatic activity. Kinetic studies on the inhibition of MAO-A activity suggest *N*MR*S*al binding to the substrate binding site in MAO, as shown by competition with 5-HT, a MAO-A substrate, but not β-PEA, a MAO-B substrate. The binding of *N*MR*S*al to MAO initiates the activation of apoptotic signaling. It is supported further by the fact that overexpression of MAO-B in SH-SY5Y cells did not increase the sensitivity to cytotoxic *N*MR*S*al, and that *N*MR*S*al binding to mitochondria inhibited by clorgyline, but not (−)deprenyl. The binding of *N*MR*S*al to the active site of MAO-A may induce the conformational changes in MAO and the opening of mPT pore. The decline in $\Delta\Psi$m by anti-MAO antibody suggests the interaction of MAO with mPT pore. However, at present it requires further studies to clarify the mechanism behind the interaction of MAO with other components of mPT pore.

MAO-B is commonly considered to play a major role in the cell death of PD, since in human basal ganglia MAO-B is more abundant than MAO-A and accounts for about 80% of the total MAO activity (O'Carrol et al., 1983). MAO-B in glia cells, but not neurons, may play a major role in the enzymatic oxidation of dopamine and ROS production (Damier et al., 1996). However, in MAO-A only containing cells, superoxide and dopamine quinone produced by dopamine autoxidation induce mPT and apoptosis as well as in MAO-B overexpressed cells. These results suggest again that MAO-A may determine the cell death and survival in neurons. However, we should examine further using *in vivo* and *in vitro* models of neurodegeneration to establish the role of MAO-A and -B in regulation of death cascade and induction of antiapoptotic genes for neuroprotection by rasagiline analogues.

The results in this paper point out the direct involvement of MAO-A in apoptotic mechanism induced by a dopaminergic neurotoxin, *N*MR*S*al, and similar, but less marked, effects on $\Delta\Psi$m were observed also with MPP^+. Selective MAO-A inhibitors, *N*MR*S*al and MPP^+, might activate mitochondrial apoptotic signaling through binding to MAO-A, and induce cell death in MAO-A containing neurons. RNAi effectively reduced MAO in this cell model, suggesting that RNAi can be applied to prepare animal and cellular models by silencing MAO-A gene, and future studies by neurochemical and behavioral analyses may bring new insights on the function of MAO-A in neurodegeneratve and psychiatric disorders, such as bipolar emotional disorders.

Acknowledgements

This work was supported by a Grant-in-Aid on Scientific Research (C) and (A) (W. M.) from Japan Society for the Promotion of Science, Grant for Research on Dementia and Bone Fracture (W. M., M. N.) from the Ministry of Health, Labor and Welfare, Japan.

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JNT-0/637
Special issue
For Author's Correction Only

J Neural Transm (2007) 000: 1–11
DOI 10.1007/s00702-007-0637-4
Printed in The Netherlands

Journal of Neural Transmission

Neuroprotection by propargylamines in Parkinson's disease: intracellular mechanism underlying the anti-apoptotic function and search for clinical markers

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Summary In Parkinson's and other neurodegenerative diseases, a therapeutic strategy has been proposed to halt progressive cell death. Propargylamine derivatives, rasagiline and (−)deprenyl (selegiline), have been confirmed to protect neurons against cell death induced by various insults in cellular and animal models of neurodegenerative disorders. In this paper, the mechanism and the markers of the neuroprotection are reviewed. Propargylamines prevent the mitochondrial permeabilization, membrane potential decline, cytochrome c release, caspase activation and nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase. At the same time, rasagiline induces anti-apoptotic pro-survival proteins, Bcl-2 and glial cell-line derived neurotrophic factor, which is mediated by activated ERK-NF-κB signal pathway. DNA array studies indicate that rasagiline increases the expression of the genes coding mitochondrial energy synthesis, inhibitors of apoptosis, transcription, kinases and ubiquitin-proteasome system, sequentially in a time-dependent way. Products of cell survival-related gene induced by propargylamines may be applied as markers of neuroprotection in clinical samples.

Keywords: Apoptosis, propargylamine, rasagiline, mitochondria, permeability transition pore, GDNF, Bcl-2, nuclear transcription factor

Abbreviations

ANT	adenine nucleotide translocator
BDNF	brain-derived neurotrophic factor
BPAP	1-(benzofuran-2-yl)-2-propylaminopentane
CyP-D	cyclophilin-D
CsA	cyclosporin A
$\Delta\Psi m$	mitochondrial membrane potential
FACS	fluorescence-augmented flow cytometry
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GDNF	glial cell-line derived neurotrophic factor
R-2HMP	*N(R)*-(2-heptyl)-*N*-methyl-propargylamine
IL	interleukin
MAO-A and *MAO-B*	type A and B monoamine oxidase
MAP	mitogen-activated protein
MEM	Hanks' minimum essential medium
mPT	mitochondrial permeability transition
NM(R)Sal	*N*-methyl(*R*)salsolinol
PD	Parkinson's disease
PI	propidium iodide
TNF	tumor necrosis factor
VDAC	voltage-dependent anion channel

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disease and affects 1–2% of the aged population. PD is pathologically characterized by selective cell death of dopamine neurons in the substantia nigra pars compacta, and biochemically by depletion of dopamine neurotransmitter in the striatum. The etiology for the sporadic form of PD remains enigmatic, whereas a growing understanding of responsible genes for familiar forms of PD suggests that the processes leading to neuronal loss may be common with those in the sporadic form of PD (Eriksen et al., 2005; Vila and Przedborski, 2004). The loss of nigral dopamine neurons in PD is hypothesized as the mutations in genes detected in the familiar form sensitizes the neurons to intrinsic and extrinsic insults. Increased oxidative stress, mitochondrial dysfunction, impaired ubiquitine-proteasome system, abnormal inflammatory cytokines, and excitotoxicity are considered to cause cell death in dopaminergic neurons, in which dopamine itself should be involved by not fully clarified mechanisms. At present, available therapies for patients with PD are limited to ameliorate the symptoms. Dopamine replacement relieves the major symptoms at least for the beginning several years. However,

progressive loss of dopamine neurons results in motor fluctuation and cognitive dysfunction, hallucinations, depression and dementia. A therapy intervening the disease progress itself is now seriously required, and "neuroprotective" therapy to rescue neurons from cell death and "neurorestrorative" therapy to restore deteriorated neurons to a normal state have been proposed (Dawson and Dawson, 2002). The therapy should target intracellular death cascade, which is activated rather slowly for decades to the end point showing the clinical signs and regulated by well-conserved and -regulated cell death system (Riederer, 2004). Using cellular and animal PD models, the molecular mechanisms behind neuronal loss have been intensively studied, and several agents have been confirmed to prevent the cell death processing. In order to ameliorate the pathogenic factors, neuroprotective agents have been proposed, including antioxidants, neurotrophic factors, anti-inflammatory drugs, mitochondria supplement, inhibitors of monoamine oxidase (MAO), and drugs interfering glutamate excitotoxicity. Since signal proteins for apoptosis increase in the nigral neurons of Parkinsonian brains, anti-apoptotic agents altering apoptotic signal pathway have been gathering attention (Maruyama et al., 2002a; Mandel et al., 2003; Simpkins and Jankovic, 2003; Youdim et al., 2006). The anti-apoptotic function is confirmed in inhibitors of type B MAO (MAO-B) and caspase inhibitors, immuno-modulators, Co-Q10, NMDA receptor antagonists and neurotrophic factors in cellular and animal model systems. Recently, several clinical trials were reported to examine effects of propargylamine MAO-B inhibitors, rasagiline [*N*-propargyl-1(*R*)-aminoindan] (Youdim et al., 2001) and (−)deprenyl [selegiline, *N*, α-dimethyl-*N*-2-propynylbenzene-ethanolamine], in Parkinsonian patients, and beneficial effects were confirmed to slow the progression of the symptoms (Parkinson Study Group, 2004, 2006; Pälhagen et al., 2006). However, the final conclusion about the neuroprotective efficiency remains to be clarified (Riederer et al., 2004; Schapira and Olanow, 2004; Suchowersky et al., 2006).

Rasagiline and (−)deprenyl were applied in PD to increase dopamine availability through inhibiting the oxidative deamination by MAO (Birkmayer et al., 1977). In addition, MAO-B inhibitors inhibit the oxidation of protoxicants to toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to 1-methyl-4-phemylpyridinium ion (MPP^+), scavenge reactive oxygen species, and prevent the lipid peroxidation and the formation of toxic dopamine quinone. Later clinical observations suggest that they may protect neurons against cell loss in PD, AD and other neurodegenerative disorders. We studied the mechanism behind protection of rasagiline against apoptotic or necrotic cell death induced in human neuroblastoma SH-SY5Y cells by oxidative stress (Maruyama et al., 2002c) and neurotoxins, such as *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal] (Naoi et al., 2002a) and 6-hydroxydopamine (6-OHDA) (Maruyama et al., 2001b, 2002b). *N*M(*R*)Sal binds to type A MAO (MAO-A) in mitochondrial outer membrane, opens a mega-channel called mitochondrial permeability transition (mPT) pore, initiates rapid reduction of mitochondrial membrane potential, $\Delta\Psi m$, and swelling of mitochondria (Akao et al., 2002a; Maruyama et al., 2002a; Naoi et al., 2006; Yi et al., 2006a). Induction of mPT results in the cytochrome c release signaling subsequent apoptosis, or the loss of ATP production leading to necrosis. Bcl-2 protein family in mitochondria directly regulates the apoptotic pathway, and intracellular signaling strictly regulates the synthesis and posttranslational modification. Neuroprotective agents intervene these apoptotic processes, either by suppressing apoptogenic factors or increasing pro-survival, anti-apoptotic factors in cells.

In this paper, our recent understanding on the mechanism underlying anti-apoptotic function of propargylamines is reviewed. The effects of propargylamine derivatives were examined in relation to the regulation of mPT and the induction of pro-survival proteins, Bcl-2 and neurotrophic factors. To confirm the involvement of cell signaling, gene expression by the propargylamines was studied by cDNA array analyses. Hitherto clinical studies indicate that the more quantitative, biochemical and molecular evaluation is required to confirm the neuroprotection in Parkinsonian patients. Our recent results by use of primate suggest that gene products increased by rasagiline in the CSF and serum may be used as clinical markers to quantify the potency of putative neuroprotective drugs in clinical samples. The expected future development of neuroprotective therapy is discussed.

Materials and methods

Materials

Rasagiline and related compounds were kindly donated by Teva Pharmaceutical (Netanya, Israel). *N*-Propargylamiine and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA); JC-1, Hoechst33342, MitoTracker Orange and Green, and Rhodamine 123 from Molecular Probes (Eugene, OR, USA). Anti-Bcl-2 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA); anti-β-actin antibody from Oncogene (Boston, MA, USA); mouse monoclonal anti-GAPDH antibody from Chemicon International (Temecyla, CA, USA). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented by 5% fetal calf serum in 95% air and 5% CO_2. Bcl-2 was overexpressed in SH-SY5Y cells as reported previously (Akao et al., 2002a). Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999).

Determination of apoptosis

Apoptotic and necrotic cell death were assessed quantitatively using fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Benton Dickinson, San Jose, CA, USA) (Yi et al., 2006a). To determine apoptotic cells, the cells were stained with PI solution in phosphate-buffered saline (PBS) containing 1% Triton X-100 and subjected to FACS analysis. Cells with a lower DNA content showing less PI staining than G1were defined to be apoptotic (subG1 peak) according to Eckert et al. (2001).

Measurement of mitochondrial membrane potential, $\Delta\Psi m$

The $\Delta\Psi m$ in isolated mitochondria was quantified by FACS using MitoTracker Orange and Green. The mitochondria were treated with agents at 37°C for 3 h, and stained with 100 nM MitoTracker Orange and Green, then 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. In other experiments, mitochondria were prepared from male Donryu rat liver or transgenic mice expressing human Bcl-2 in the liver, as previously described (Shimizu et al., 1998). $\Delta\Psi m$ was assessed also by measurement of reduction in Rhodamine 123 fluorescence, which was ascribed to $\Delta\Psi m$-dependent uptake of Rhodamine 123 into the mitochondria (Narita et al., 1998).

Measurement of mRNA and protein of Bcl-2 family proteins

SH-SY5Y cells were cultured in the presence of various concentrations (10 μM–1 pM) of rasagiline for 24 h or for a various incubation time with 100 nM rasagiline. The whole cells were gathered and the total RNA was extracted by the phenol/guanidinium thiocyanate method. The cDNA was generated by reverse transcription of the total RNA, and the cDNA fragments were amplified using the PCR primers (Akao et al., 2002b). PCR products were analyzed by electrophoresis on 3% agarose gels, and β-actin cDNA was used as an internal standard.

Quantitative measurement of mRNA and protein of GDNF

SH-SY5Y cells were treated with rasagiline in 96 well plates with Hanks' minimum essential medium (MEM). The effect of sulfasalazine (100 μM), an inhibitor of IκB, was examined by adding the inhibitor 30 min before the treatment with rasagiline. The protein amount of GDNF was quantified as reported previously using the enzyme immunoassay (EIA) (Nitta et al., 2002). Samples or standard were added to GDNF antibody-coated wells, and incubated for 12–18 h at 4°C. The biotinylated secondary antibody was reacted in avidin-conjugated β-galactoside (Boehringer Mannheim) for 1 h. The enzyme activity in each well was measured by incubation with a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside. The fluorescence intensity of produced 4-methylumbelliferone was measured at 360 nm with excitation at 448 nm. The mRNA of GDNF was measured by reverse transcription-polymerase chain reaction (RT-PCR), as reported (Maruyama et al., 2004a).

Quantitation of activated NF-κB

Activation of NF-κB was determined by NF-κB binding to κB sites using NF-κB p65 transcription assay kit (Active Motif, Carlsbad, CA, USA) (Maruyama et al., 2004a). Five μg of the extract of Hela cells stimulated with TNF-α for 30 min was used as a positive control. The activation of NF-κB was expressed as % of the positive control.

cDNA array for gene expression in apoptosis

The cells were incubated with 100 nM rasagiline for 6, 12, and 24 h, and the total RNA was extracted. Using AMV reverse transcriptase, total RNA isolated from the sample and control was labeled with Cy3- or Cy5-dUTP. The levels of gene expression were quantitatively analyzed by cDNA expression array using TaKaRa IntelliGene Human Apoptosis CHIP (Takara Biomedicals, Ohtsu, Japan).

Statistics

Experiments were repeated at least 4 times and the results were expressed as mean and SD. Difference was 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.

Results

Stabilization of mitochondrial contact sites by propargylamines

A series of propargylamines, rasagiline, (−)deprenyl, aliphatic (*R*)*N*-(2-heptyl)-*N*-methylpropargylamine (*R*-2HMP) and free *N*-propargylamine, prevent the activation of apoptotic cascade and protect SH-SY5Y cells against apoptosis induced by neurotoxins, *N*M(*R*)Sal and 6-OHDA, and oxidative stress caused by dopamine oxidation and a peroxynitrite-generating agent, SIN-1 (Akao et al., 2002a; Maruyama et al., 2002a, b, c; Yi et al., 2006b). Figure 1 shows the chemical structure of examined propargylamines. An endogenous neurotoxin *N*M(*R*)Sal induces the mPT and apoptosis (Naoi et al., 2002b, 2006). As summarized in Fig. 2, these propargylamines completely suppress opening of mPT pore caused by neurotoxins and oxidative stress. Rasagiline inhibits mitochondrial swelling and $\Delta\Psi m$ reduction (Akao et al., 2002a), and prevents release of cytochrome c, caspase 3 processing and nuclear translocation of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Maruyama et al., 2002a). Rasagiline protected MAO-A-expressing SH-SY5Y cells from apoptosis and transfection-enforced expression of MAO-B did not increase the sensitivity to rasagiline, indicating that neuroprotective function does not depend on the MAO-B inhibition (Yi et al., 2006a). On the other hand, clorgyline [*N*-methyl-*N*-propargyl-3(2,4-diclorophenpxy)-propylamine] did not prevent, but induced mPT. Table 1 shows the results on the structure-activity relationship for direct stabilization of mPT among propargylamine derivatives with different hydrophobic structure, indanyl (rasagiline), phenyl (deprenyl), aliphatic (2-HMP) and benzofuranyl groups [1-(benzofuran-2-yl)-2-propylaminopentane, BPAP]. The aminoindan derivatives are the most active followed by the phenyl derivatives, and the derivatives with aliphatic and benzofuranyl structures require rather high concentrations for preventing mPT. The modification of aminoindan ring does not affect the potency to stabilize mPT pore, as shown

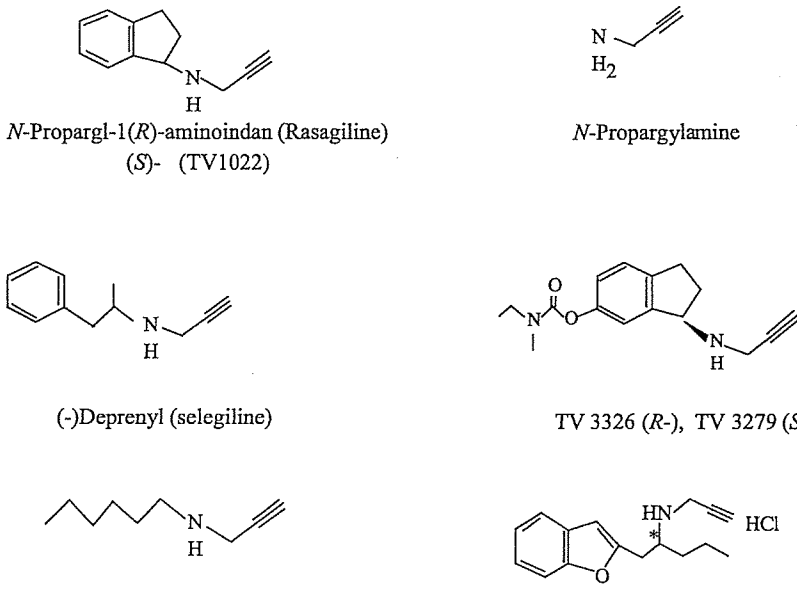

Fig. 1. Chemical structures of propargylamines with neuroprotective potency

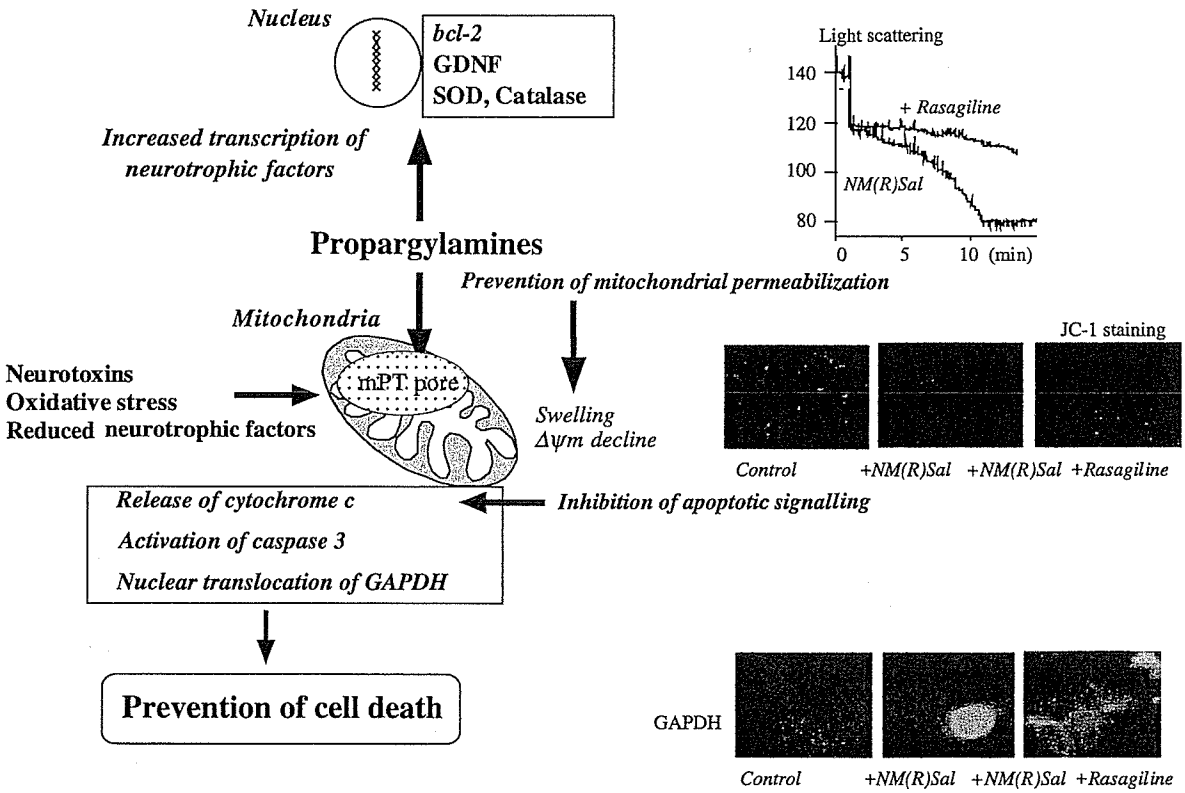

Fig. 2. Target sites of neuroprotective propargylamines in apoptosis cascade. Rasagiline and related compounds suppress mPT, as shown by prevention of mitochondrial swelling and $\Delta\Psi$m reduction. They inhibit also cytochrome c release, caspase 3 activation and nuclear GAPDH translocation. In addition, the propargylamines increase the expression of anti-apoptotic Bcl-2 family protein, neurotrophic factors (GDNF, BDNF), and antioxidant enzymes (SOD, catalase)

with TV 3326 [(*N*-propargyl)-(3*R*)-aminoindan-5-yl]-ethylmethyl carbamate and its hydroxyl metabolite, TV 3294 (Maruyama et al., 2003). In general, the *R*-enantiomers are more potent to prevent the mPT than the *S*-enantiomers (Maruyama et al., 2001a, b). The *S*-enantiomer of rasagiline, TV1022, lacks the MAO inhibiting function, but it still

Table 1. *Structure and neuroprotective characteristics of propargylamines*

Name [Structure]	Prevention of mPT	Induction of Bcl-2	Induction of GDNF
Rasagiline [*R*(+)-*N*-propyl-1aminoindan]	10 μM–1 nM	10 μM–1 nM, 10–1 pM	1 μM–100 pM
TV1022 [*S*(−)-*N*-propyl-1-aminoindan]	1 μM–100 nM	–*	–
Aminoindan	–	–	–
N-Propargylamine	1 μM–10 nM	100–1 nM	N.D.**
N-Methylpropargylamine	–	–	N.D.
Propiolaldehyde	–	–	N.D.
(−)Deprenyl	1 μM–100 nM		1 μM–10 nM
(+)Deprenyl	10 μM	–	–
Desmethyldeprenyl	10–1 nM		1 μM–10 nM
TV3326 [5-ethyl ethyl carbamate-rasagiline]	100–10 nM	–	–
TV3294 [5-hydroxyl-rasagiline]	100–10 nM	–	–
R-N-(2-Heptyl)-*N*-methylproparylamine	1 μM–100 nM	N.D.	N.D.
S-N-(2-Heptyl)-*N*-methylproparylamine	10 μM	N.D.	N.D.
R-N-(2-Heptyl)-propargylamine	1 μM–100 nM	N.D.	N.D.
R-3-(2-Heptylamine)-*N*-methylpropionic acid	–	N.D.	N.D.
R-(−)-BPAP	–	100–1 nM	1 nM***
S-(+)-BPAP	1 μM–10 nM	–	N.D.
R-(+)-*N*-(2-propynyl)-BPAP	1 μM–10 nM	100–1 nM	N.D.
S-(−)-*N*-(2-propynl)-BPAP	–	–	N.D.

* Not affective, ** not determined, *** Hirai et al. (2005).

prevents mPT, suggesting again that the anti-apoptotic function is not related to the MAO inhibition. In the case of the benzylfuranyl derivatives, the stabilization of mPT pore depends on the absolute structure of propargylamines. The compounds with dextro-rotation prevented $\Delta\Psi m$ decline by neurotoxins, whereas the corresponding enantiomer with levo-rotation did not (Maruyama et al., 2004b). The propargylamine group is essentially required for the activity as in the case with free *N*-propargylamine itself, whereas the analogues without a propargyl residue, aminoindan and *R*-3-(2-heptylamino)-propionic acid, did not prevent mPT. The methylation of the amino residue in *N*-propargylamine abolished the activity to prevent $\Delta\Psi m$ reduction (Yi et al., 2006b).

The precise mechanism leading to the permeabilization of mitochondria is still unclear, even though several models have been proposed. The mPT pore is primarily composed of adenine nucleotide translocator (ANT) in the inner membrane and voltage-dependent anion channel (VDAC) in the outer membrane, which binds to ANT at the contact sites between the inner and outer membrane. In addition, peripheral benzodiazepine receptor (PBR) and MAO in outer membrane and hexokinase at the contact site are associated with the mPT pore. Cyclophilin-D (CyP-D) binds to the matrix site of ANT and induces conformation change to form a non-specific pore leading to release of any molecules of less than 1.5 kDa, and metabolic gradients across the inner membrane are dissipated, with accumulation of Ca^{2+}. Opening of the mPT pores results in swelling of the matrix and rupture of the outer membrane, which leads to the release of apoptogenic factors (cytochrome c, apoptosis-inducing factor, Smac/DIABLO, Omi/HtrA2) resulting in activation of caspase system. Oxidative stress and other insults facilitate the mPT pore opening though cross-linking of thiol groups of cysteine residues in ANT and increases the binding of CyP-D to the ADP binding site (McStay et al., 2002). Neurotoxins, PBR ligands (PK11195, protophorphirin IX), bax and other pro-apoptotic Bcl-2 protein family, heavy metals, inorganic phosphate, fatty aids, quinines and uncouplers of mitochondrial oxidative phosphorylation system induce mPT. On the other hand, viral proteins, such as HIV viral protein R (Jacotot et al., 2001) and myxoma poxvirus protein, M11L (Everett et al., 2002), bind to the CyP-D binding site and prevent the pore formation. Another model of mPT is that Bcl-2 interacts directly with VDAC and regulates ANT activity, which was proved in a model system composed of VDAC in liposomes (Shimizu et al., 1999; Tsujimoto and Shimizu, 2000). According to this model, VDAC interacts with apoptogenic Bax and Bak, functions as "VDAC modulators", changes its conformation leading to formation of a megachannel to allow cytochrome c to pass through, whereas anti-apoptotic Bcl-xL closes the channel. In this case, the outer membrane might be intact without rupture. More recently, lipid bilayer was proposed to play an important role in mPT by interacting with ANT or other mitochondrial components (Lucken-Ardjomande and Martinou, 2005).

*N*M(*R*)Sal binds to MAO-A in the outer membrane and opens mPT pore, which CsA and bongkrekic acid antagonize through binding to CyP-D and ANT. *N*M(*R*)Sal, dopamine and its oxidation product quinone, neuromelanin, and peroxynitrite modify sulfhydryl (SH) groups in mitochondria

and induce mPT (Yi et al., in preparation). Rasagiline prevents the reduction of free SH residues in mitochondria and the mPT, regardless of the types of insults leading to mPT (toxins, PBR ligands and oxidative stress). Rasagiline is bound to MAO-B, MAO-A, or other components in mPT pore, stabilizes the contact site and prevents the conversion of ANT into a pro-apoptotic pore. The study is under way whether rasagiline can bind directly to ANT or CyP-D. In addition, propargylamines bind to several other proteins in cells. (−)Deprenyl and its analogue TCH346 [CGP3466, dibenzo(*b,f*)oxepin-10-yl-methyl-methyl-prop-2-ynyl-amin], bind to GAPDH, and prevent the *S*-nitrosylation of GAPDH, the binding to Siah and its nuclear translocation (Hara et al., 2006). Another candidate binding site is poly(ADP-ribose)-polymerase-1 (Brabeck et al., 2003). However, in apoptotic processes these putative binding sites are downstream of mPT and our results demonstrate that the binding of rasagiline to mitochondrial protein and the regulation of mPT are the primary events in preventing apoptosis.

Induction of neuroprotective Bcl-2 family proteins

It is well known that some kinds of protein, Bcl-2 family protein, anti-oxidants and neurotrophic factors, alleviate neuronal loss through suppression of oxidative stress, prevention of apoptotic signal transduction and promotion of cell survival. Rasagiline, and (−)deprenyl increase the activity of anti-oxidative enzymes, superoxide dismutase (SOD) and catalase, in the rat brain after the systemic administration (Carrillo et al., 2000, Kitani et al., 2000). (−)Deprenyl and desmethyldeprenyl increase mRNA level of SOD 1 and 2, Bcl-2 and Bcl-xL, nitric oxide synthase, c-JUN, and NAD dehydrogenase in PC12 cells (Tatton et al., 2002). Our and Youdim's group have clarified the detailed mechanism underlying the induction of anti-apoptotic proteins by rasagiline analogues.

The family of Bcl-2-related proteins constitutes one of biologically most relevant regulatory gene products against apoptosis through controlling mitochondrial permeabilization (Kroemer, 1997). Bcl-2 family proteins are subdivided into three groups on the basis of the pro- and anti-apoptotic function and the Bcl-2-homology (BH) domains (BH1 to BH4). Anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) have 4 BH domains, whereas pro-apoptotic multidomain protein (Bax, Bak, Bok/mtd) lacks BH4. BH3 only proteins (Bid, Bim/Bod, Bad, Bmf) are also pro-apoptotic and link specific apoptotic stimuli to mPT. Bcl-2 is mainly localized in the mitochondrial inner membrane, and the family proteins form homo- or hetero-dimers between anti- and pro-apoptotic members and determine cellular sensitivity to apoptotic stimuli by titrating one another's function. Anti-apoptotic Bcl-2 family proteins prevent apoptosis either by inhibiting pro-apoptotic Bcl-2 members directly, controlling endoplasmic reticulum and mitochondrial homeostasis, or defending against oxidative stress. On the other hand, pro-apoptotic Bcl-2 family proteins induce mPT and trigger the release of mitochondrial apoptogenic factors into the cytosol, as discussed above.

Overexpression of Bcl-2 protects various neuron paradigms *in vivo* and *in vitro* from death induced by neurotoxins and other insults. Bcl-2-overexpression in SH-SY5Y cells prevented apoptosis induced by *N*M(*R*)Sal, which is relevant with the results that $\Delta\Psi$m decline induced by *N*M(*R*)Sal was suppressed in mitochondria prepared from Bcl-2 overexpressed mouse liver (Akao et al., 2002a; Maruyama et al., 2002a). These results suggest that rasagiline may induce Bcl-2 protein, in addition to the direct stabilization of the mPT pore. We found that rasagiline increases the mRNA and protein levels of *bcl-2* and *bcl-xL* in SH-SY5Y cells, as shown in Fig. 3 (Akao et al., 2002b). Rasagiline showed a reverse-bell shape curve of the concentration-activity relationship and the increase of Bcl-2 was detected at 10 μM–10 nM, and also at 10–1 pM. Bcl-2 protein level increased from 6 to 24 h of the treatment. Rasagiline induced mRNA levels of anti-apoptotic *bcl-2* and *bcl-xL*, but not those of pro-apoptotic *bax* and *mcl-l*. Other MAO-A and -B inhibitors, clorgyline and pargyline, did not affect the mRNA level at the concentrations examined.

The results of structure-activity relationship of propargylamine derivatives to Bcl-2 induction are summarized in Table 1. Rasagiline and *N*-propargylamine increased Bcl-2 mRNA and protein, whereas aminoindan and *N*-methylpropargylamine did not (Maruyama et al., 2002b; Yi et al., 2006b). The structure required for Bcl-2 induction is the propragylamine group, as in the case for preventing mPT. Also among BPAP derivatives, *R*(−)-*N*-propynyl compound, FDFS-1180, induced Bcl-2, more than FDFS-11169 without proynyl group (Maruyama et al., 2004b). For Bcl-2 induction, *R*-propargylamines are more potent than the *S*-enantiomers.

Induction of neurotrophic factors by propargylamines

Neurotrophic factors, including nerve growth factor, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor, prevent cell death in specified type neurons. GDNF is a member of the transforming growth factor-β superfamily and effectively protects dopaminergic neurons against

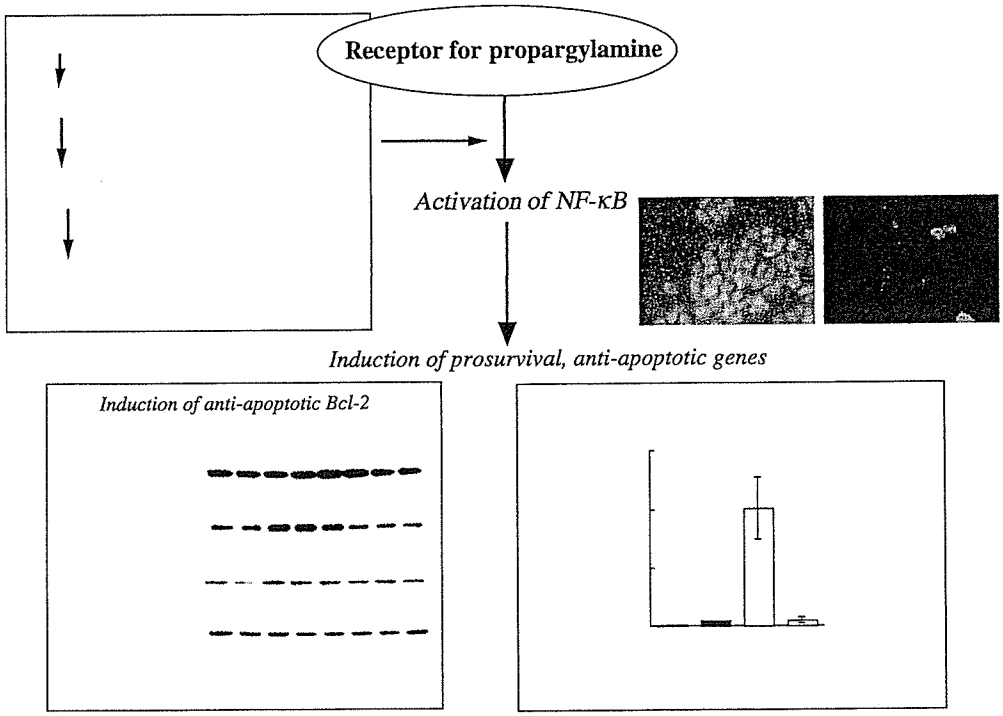

Fig. 3. Rasagiline increases anti-apoptotic Bcl-2 family and GDNF, a dopamine neuron-specific neurotrophic factor, through activation of ERK-NF-κB pathway. Anti-apoptotic propargylamines bind to the putative receptor on the membrane and activate the MEK1/2-ERK1/ERK2 pathway. The activated phosphorylated forms of ERK1/2 were detected after 30 min incubation with 100 μM rasagiline. After 3 h treatment with rasagiline, NF-κB was activated and p65 subunit was translocated into nuclei, as shown by staining using anti-p65 antibody for GAPDH and Hoechst 33342 for nuclei. The involvement of NF-κB in the induction of GDNF and Bcl-2 was also confirmed by use of an inhibitor of IκB kinase, sulfasalazine, which inhibited the increase of GDNF protein in SH-SY5Y cells treated with 100 nM rasagiline. The structure required for the Bcl-2 induction is a propargylamine structure, since aminoindan without a propargyl residue did not increase Bcl-2 levels

cell death in various animal PD models prepared with 6-hydroxydopamine and MPTP. Since GDNF and other neurotrophic factors cannot penetrate into the brain though the blood-brain barrier, several trials have been reported, delivering GDNF in the substantia nigra by direct administration, gene therapy, and cell implant (Bauer et al., 2000; Gill et al., 2003).

As shown in Fig. 3, rasagiline increases GDNF in SH-SY5Y cells. GDNF mRNA was virtually not detectable in SH-SY5Y cells, but after the treatment with 100 nM rasagiline for 3 h considerable amount of GDNF mRNA was detected. GDNF protein level in the control cells was less than 1 pg/ml and increased to be more than 100 pg/ml after rasagiline treatment. Induction of neurotrophic factors, GDNF, BDNF, NGF and neurotrophin-3 (NT-3), by propargylamines was examined in SH-SY5Y cells. Depending on the type of propargylamines, different neurotrophic factors were induced; rasagiline induced GDNF, and (−)deprenyl BDNF (Maruyama et al., in preparation). This result suggests that a specified propargylamine compound can induce a definite neurotrophic factor beneficial for selective type of neurons.

Signal transduction and gene expression by rasagiline for neuroprotection

These results on Bcl-2 and GDNF induction suggest that rasagiline may activate intracellular signals for induction of genes coding these anti-apoptotic proteins. NF-κB is the common transcription factor to induce anti-apoptotic *bcl-2*, neurotrophic GDNF and anti-oxidative SOD, all of which were increased by rasagiline (Carrillo et al., 2000; Akao et al., 2002b; Maruyama et al., 2004a). NF-κB consists of 2 subunits of 65 kDa (p65: RelA) and 50 kDa (p50) or 52 kDa (p52), and is sequestered in the cytoplasm as an inactive complex with NF-κB inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, dissociated from the complex and degraded by the ubiquitin-proteasome system. This reaction allows translocation of free, active NF-κB complex into nuclei, where it binds to specific DNA motifs in the promoter/enhancer regions of target genes and activates transcription, as shown by the p65 binding assay. The translocation of activated p65 subunit into nuclei by rasagiline was confirmed by Western blot analysis of the subcelluar fractions and also by immunohistochemical

observation using the p65 antibody and Hoechst 33342 for nuclear staining (Fig. 3) (Maruyama et al., 2004a). The involvement of phosphorylation of inhibitory IκB subunit on the activation of NF-κB, was demonstrated by use of sulfasalazine, an inhibitor of by IκB kinase (Fig. 3). Sulfasalazine inhibited also the increase of mRNA of *bcl-2* and *bcl-xL* as in the case with GDNF, suggesting the involvement of NF-κB transcription factor in the induction of neuroprotective proteins in common.

Rasagiline and related propargylamines protect cellular and animal models of neurodegenerative disorders, including PD, AD and ischemia (Mandel et al., 2003, 2005). By screening the signal factors activated rasagiline, we found that extracellular-regulated kinase-1/2 (ERK1/ERK2) was activated as an upper signal of NF-*k*B activation (Maruyama et al., 2004a) (Fig. 3). After treatment with 100 nM rasagiline, phosphorylated ERK1/ERK2 was increased in a time-dependent way, which PD98059, an inhibitor of mitogen-activated protein (MAP) kinase/ERK kinase-1 (MEK 1/2), inhibited. CF10923x and Calphosin, inhibitors for protein kinase C (PKC), suppressed the increase of Bcl-2 and activated NF-κB by rasagiline, suggesting the involvement of the pathway through activation of PKC, Ras/Raf and MEK 1/2 in the induction of these proteins. Youdim and his group reported detailed data concerning the activation PKC system by rasagiline, which up-regulates MAP kinase/ERK cascades (Youdim et al., 2003■; Mandel et al., 2005; Weinreb et al., 2004). Recently, in mice treated with MPTP rasagiline was reported to activate signal pathway from neurotrophic factor responsive-tyrosine kinase receptor to phosphatidylinositol 3 kinase protein (Sagi et al., 2006). However, as shown later in DNA array studies, kinases may be activated not only primarily by rasagiline itself, but also secondarily by the following death processes. At present, it requires further studies to identify the initial signal to induce anti-apoptotic genes.

To screen the gene induction by rasagiline, we examine the time-dependent expression of genes by rasagiline. SH-SY5Y cells were treated with 100 nM rasagiline for 6, 12 and 24 h and mRNA was extracted and reverse-transcribed with biotylated dUTP (Roche Diagnostics) and gene-specific primer mixture reported as the manufacture's instruction (Takara Bio Co., Otsu, Japan). The relative expression level of a given mRNA was assessed by normalizing to a housekeeping gene, β-actin, and comparing to the control values obtained by the cells without treatment of rasagiline (Table 2). Rasagiline increased 108, 57 and 82 genes (>1.5 compared to control) and reduces 37, 54 and 104 genes (<0.5) after 6, 12 and 24 h treatment, respectively. Rasagiline affected genes with different cellular function in a time-dependent way. After 6 h treatment, mRNA of *bcl-2*, and genes related to NF-κB related transcription factors, cytokines and the receptors [interleukin (IL) receptors], mitochondrial ATP synthesis (cytochrome c oxidase, NADH-coenzyme Q reductase, ATP synthase, aconitase) and the ubiquitin-proteasome system were increased. In addition, genes of mPT pore components (ANT, VDAC and MAO-A) were also increased. On the other hand, genes coding growth factor (BDNF, transforming growth factor), cytokines and receptors [tumor necrosis factors (TNF), IL, fibroblast growth factor] were reduced. At 12 h of the treatment, most marked increase was observed in MAP-KK and cytokine receptors. In addition, rasagiline increased mRNA for ANT, VDAC and mitochondrial proteins (complex I–IV, mitochondrial transcription factor A). On the other hand, kinases associated with death signal (MAP kinase activating death domain, MAPKKK 4, TNF receptor associated factor 5, death-associated protein kinase-1), growth factors (NGF), and cytokines decreased. It is interesting that mRNA of *bcl-2*, MAO-B and also transcription factors were reduced significantly at this point. Rasagiline treatment for 24 h enhanced significantly the genes for *bcl-2*, apoptosis inhibitors (apoptosis inhibitors 1, 2 and 4, neuronal apoptosis inhibitory protein) and cell signals, including kinases (MAPK, MAPKK, cyclin-dependent kinase), cytokines and the receptors, and the transcription factors. It may be hypothesized that rasagiline sequentially increases ATP-dependent activation of kinases and transcription factors, the ubiquitine-proteasome system, which degrades the cleaved phosphorylated inhibitors of kinases and transcription factor, increases cytokines and the receptors, and finally induces pro-survival genes.

Table 2. *Gene induction in SH-SY5Y cells by rasagiline*

Rasagiline (100 nM) treatment for		
6 h	12 h	24 h
Increased genes	Increased	Increased genes
ATP-synthesis-related mitochondrial mPT pore related	Kinases	Bcl-2
Cytokine receptors	Cytokine and IL receptors	Apoptosis inhibitors
NF-κB related transcription factors	Mitochondrial complex I–IV	TNF and receptors
Ubiquitin-proteasome system	mPT pore related	Growth factors
Reduced genes	Reduced genes	
IL and TNF	Bcl-2	
Cytokin-related transcription factors	Kinases	
Growth factors	IL and TNF	
	Transcription factors	
	Growth factors	