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The anti-parkinson drug, rasagiline, prevents apoptotic DNA damage induced by peroxynitrite in human dopaminergic neuroblastoma SH-SY5Y cells

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Summary. Clinical trials for treatment of Parkinson's disease suggest that (−)deprenyl (selegiline), an inhibitor of type B monoamine oxidase, may slow the disease progression. However, the mechanism underlying protection of nigral dopamine neurons by selegiline remains an enigma. Recently, rasagiline, (R)(+)-N-propargyl-1-aminoindan, was reported to be neuroprotective by in vivo and in vitro experiments, which is another selective irreversible inhibitor of type B monoamine oxidase and not metabolized into amphetamine-like derivatives as in the case of selegiline. In this paper, the mechanism of the neuroprotection was examined using human dopaminergic neuroblastoma SH-SY5Y cells against apoptosis induced by peroxynitrite generated from SIN-1. After treatment with SIN-1, the apoptotic DNA damage in the cells was quantified by a single cell gel electrophoresis (comet) assay and by staining with Hoechst 33342. Change in mitochondrial membrane potential, $\Delta\psi m$, was measured by use of a fluorescent indicator, JC-1. Rasagiline reduced apoptosis with much more potency than selegiline, and the protection required 20 min pre-incubation before SIN-1 treatment. The protection by rasagiline was proved to be due to stabilization of mitochondrial membrane potential against the collapse induced by SIN-1, whereas rasagiline did not scavenge peroxynitrite directly. The studies on structure-activity relationship showed that a propargylamine group and a hydrophobic group with an adequate intermediate space were required for the protection. These results suggest that rasagiline may protect declining neurons through its anti-apoptotic activity in neurodegenerative diseases.

Keywords: Rasagiline, apoptosis, dopamine neuron, peroxynitrite, mitochondrial membrane potential, Parkinson's disease.

Introduction

The antiparkinson drug (−)deprenyl (selegiline) (Birkmayer et al., 1975, 1977) is an irreversible inhibitor of type B monoamine oxidase (MAO-B) (Knoll and Magyar, 1972) was reported to protect dopamine neurons in Parkinson's disease (PD) (Parkinson Study Group, 1989). However, further studies failed to show evidence for neurorprotection by (−)deprenyl and tocopherol-antioxidative therapy of Parkinsonism (Parkinson Study Group, 1993). The results were confounded by the symptomatic action of (−)deprenyl. Nevertheless several cell culture (PC12 cells) and in vivo studies have shown it to have neuroprotective properties. Pre-treatment with selegiline prevented parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) due to MAO-B inhibition (Heikkila et al., 1984), and protected cultured dopamine neurons from neurotoxicity of 1-methyl-4-phenylpyridinium ion (MPP^+) (Koutsilieri et al., 1996; Mytilineou and Cohen, 1985). Recently we found that selegiline protected human dopaminergic neuroblastoma SH-SY5Y cells from apoptosis induced by an endogenous dopaminergic neurotoxin, 1(R), 2(N)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline [N-methyl(R) salsolinol] (Maruyama et al., 1997a,b; Naoi et al., 1998).

On the other hand, selegiline is metabolized into amphetamine and methamphetamine [seed review, (Mahmood, 1997)], which may account for lack of neuroprotection in Parkinson subjects (Tatton, 1993). However, these metabolites cause adverse side-effects in patients treated with selegiline (Engberg et al., 1991). Among compounds structurally related to selegiline, N-propargyl-1-aminoindans (AGN-1133 and AGN-1135) are potent irreversible selective inhibitors of MAO-B, which are not metabolized into amphetamine derivatives (Finberg et al., 1981a,b; Kalir et al., 1981; Sabbagh and Youdim, 1978; Youdim et al., 2001a) and prevent MPTP neurotoxicity (Heikkila et al., 1985). The (R)enantiomer of AGN-1135, N-propargyl-1(R)-aminoindan (rasagiline), is a more selective and potent MAO-B inhibitor than the (S)enantiomer (Lamensdorf et al., 1996; Sterling et al., 1998; Youdim et al., 1995a,b, 2001a). Recent controlled (double blind) multi center phase 3 studies have shown it to have relatively potent symptomatic activity in patients with early Parkinsonism (Parkinson Study Group, 2000). Rasagiline and its S-enantiomer (TVP-1022) have recently received attention because of their possible neuroprotective-antiapoptotic properties (Youdim et al., 2001a,b, 1999), and is now under investigation as an adjuvant to L-dopa drug for Parkinson's disease (Parkinson Study Group, 2000; Oren, 1999). Rasagiline increased the survival of dopaminergic cells in a primary culture of rat fetal mesencephalic cells (Lamensdorf et al., 1996), and where as both rasagiline and TVP-1022 protected dopamine neurons and partially neuronally differentiated PC12 cells from cell death induced by serum- and NGF-withdrawal (Youdim et al., 2001a,b, 1999). The neuroprotective activities of rasagiline and TVP-1022 were further indicated by in vivo experiments, where rasagiline reversed or corrected parkinsonism induced by a neurotoxin, α-methyl-p-tyrosine (Speiser et al., 1997) and both drugs exhibited potent neuroprotection against head injury in mouse (Huang et al., 1999).

In Parkinson's disease dopamine neurons in the substantia nigra were reported to be denervated by apoptotic death process (Anglade et al., 1997). However, the role of apoptosis in Parkinson's disease is controversial since this view is not shared by others (Jellinger, 1999; Stoessl, 1999). Reactive oxygen or nitrogen species, such as hydroxyl radicals, nitric oxide and peroxynitrite, which initiate oxidative stress, have been proposed to induce apoptosis in dopaminergic neurons, and peroxynitrite is now considered to play a major role in the neuronal degeneration (Lipton et al., 1993). Recently we found that nitric oxide and SIN-1, a peroxynitrite donor, induced apoptosis in SH-SY5Y cells (Maruyama et al., 2000b).

In this paper, we studied the mechanism of neuroprotection by rasagiline and TVP-1022 against DNA damage induced by peroxynitrite. The DNA damage was quantitatively assayed by a single cell gel electrophoresis (comet) assay (Ostling and Johanson, 1984), and the structure-activity relationship was studied among rasagiline-related compounds. As the initial step in apoptosis, the changes in mitochondrial membrane potential, $\Delta\psi m$, and the effects of rasagiline were studied using a fluorescent indicator, JC-1. The results are discussed in relation to the possible protection or rescue of deteriorating neurons by rasagiline in neurodegenerative diseases.

Materials and methods

Chemicals

Rasagiline, the structurally related compounds and a metabolite, aminoindan, were prepared as reported previously (Youdim, 1995) and kindly donated by Teva Pharmaceutical Co. (Netanya, Israel). Clorgyline was kindly donated by May and Baker (Dagenham, U. K.), and selegiline by Dr. J. Knoll, Semmelweiss University of Medicine (Budapest, Hungary). Pargyline and ethidium bromide were purchased from Sigma (St. Louis, MO); SIN-1(N-morpholinosydnonimine) from Dojindo (Kumamoto, Japan); JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazoylcarbocyanine iodide), Hoechst 33342 and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) from Molecular Probes (Eugene, OR); agarose (low melting-temperature), 2′,7′-dichlorofluorescein, and other reagents from Nacalai Tesque (Kyoto, Japan).

Single cell gel electrophoresis (comet) assay

SH-SY5Y cells were cultured with a tissue culture medium, Cosmedium-001 (CosmoBio, Tokyo, Japan) supplemented by 5% Nakashibetsu precolostrum newborn calf serum (Mitsubishi Kasei, Tokyo, Japan) in atmosphere of 95% air-5% CO_2. The cells were dissociated with trypsin, washed with Cosmedium supplemented with the serum, and gathered by centrifugation. The cells were suspended in 500 μl of the Krebs-Ringer solution and incubated with various concentrations of rasagiline derivatives for 20 min, then with 10 μM of a peroxynitrite-generating agent, SIN-1 for further 3 hr. The cells (5×10^4 cells) were washed, suspended, mixed with 1% low-melting agarose, and subjected to the comet assay. The comet assay was performed as reported previously (Maruyama et al., 1997b; Ostling and Johanson, 1984). Two hundred comet images were randomly selected, and the tail length (length of DNA tail from the trailing edge of nucleus) and the comet length (nucleus plus migrated DNA tail) were measured as reported previously (Maruyama et al., 1997a,b). The effect of timing of rasagiline treatment on the DNA damage was also examined by addition of rasagiline simultaneously, 20 min before or after the incubation with SIN-1. The cells were treated to quantify the DNA damage as described above.

Staining with Hoechst 33342

To confirm apoptosis by morphological observation, the cells were cultured in a 6-well D-polylysine-coated flask overnight, them washed twice with the minimum essential medium (MEM). The cells were pre-incubated with 1 μM rasagiline for 30 min, then cultured with 100 μM SIN-1 for 18 hr. To the culture medium, Hoechst 33342 solution was added to be 20 μg/ml and the cells were incubated for 30 min. The cells were observed by fluorescence microscopy using an Olympus microscope (Tokyo, Japan) with an epi-illuminator. After then, the cells were harvested, centrifuged, and fixed with 4% paraformaldehyde and observed on a slide glass to count the number of cells with condensed and fragmented nuclei. One hundred cells were examined for 4 independent experiments.

Measurement of mitochondrial membrane potential ($\Delta\psi m$)

The cells cultured in a 6-well D-polylysine-coated flask were washed twice with MEM and incubated with JC-1 (4 μg/ml) in MEM for 30 min, then washed twice with MEM. The cells were pre-treated with 1 μM of rasagiline for 30 min, and then SIN-1 was added in the medium to be 100 μM at the final concentration. The fluorescence was observed with an Olympus BX60 inverted fluorescence microscope. JC-1 fluorescence was monitored either as green fluorescent monomer at depolarized membrane potentials or as red fluorescent J-aggregated at hyperpolarized membrane. The fluorescent intensity was quantified by computer-assisted image analysis system using NIH imaging software. Four fields containing about 200 cells were analyzed and the change of the intensity of the red fluorescence/cell or the relative intensity of red to green fluorescence was determined as the indicator of $\Delta\psi m$ in the cells treated with SIN-1 with or without pre-treatment of rasagiline.

Quantitative analysis of peroxynitrite with H_2DCFDA

Effect of rasagiline on the amount of peroxynitrite generated from SIN-1 was quantitatively examined by using H_2DCFDA as a fluorescent probe (Crow, 1997). To 100 μM SIN-1 solution in 20 mM sodium phosphate buffer, pH 7.4, 50 μM H_2DCFDA was added in the absence and presence of 100 to 0.1 μM rasagiline. The fluorescence of 2′,7′-dichlorofluorescein (2′,7′-DCF), an oxidation product of H_2DCFDA, was measured at 520 nm with excitation at 504 nm in a Shimadzu fluorospectrometer, RF-5000 (Kyoto, Japan). The increase in 2′,7′-DCF after 30 min incubation at 37°C was quantified by comparison of the standard.

Statistics

The comet length and the tail length of 200 cells were measured in each experiment. To evaluate DNA damage, the cells with the comet tail longer than 3 μm were classified as positive for DNA damage. The number of DNA-damaged cells, determined by either the comet assay or Hoechst 33342 staining was expressed as percentages of the total. Statistical significance was assessed by analysis of variance (ANOVA). All differences with $p < 0.05$ were considered to be statistically significant.

Results

Protection of the cells from peroxynitrite-induced DNA damage by rasagiline derivatives

Figure 1 shows the chemical structure of rasagiline and related compounds examined in this paper; the (R)- and (S)-enantiomers of deprenyl and N-

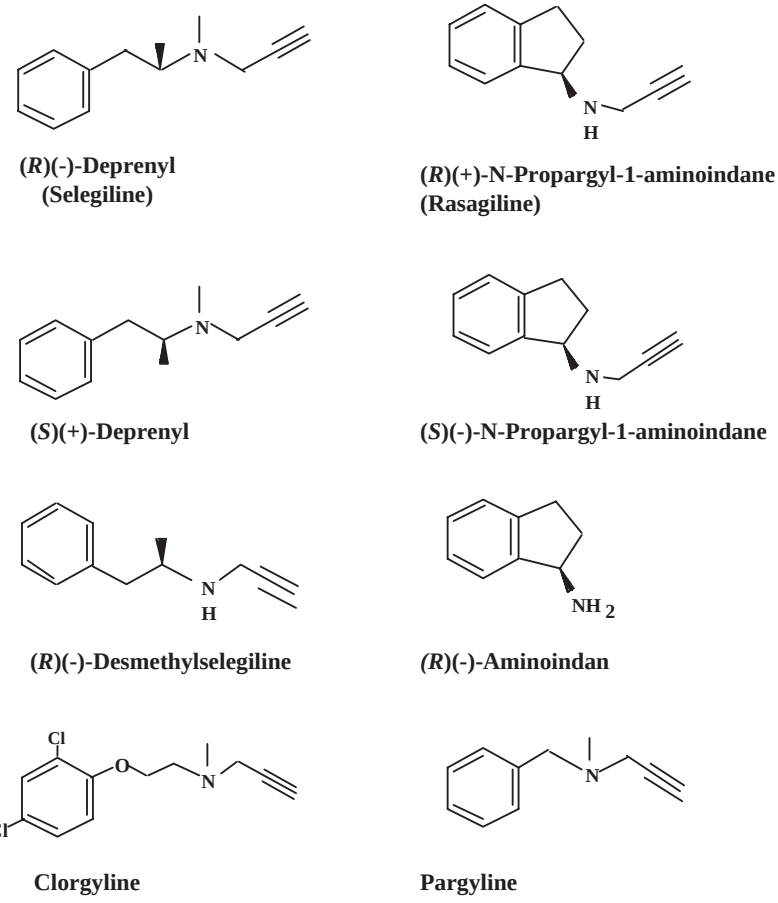

Fig. 1. Chemical structure of rasagiline and structurally related compounds

propargyl-1-aminoindan, a desmethyl derivative of selegiline, and aminoindan, a metabolite of rasagiline. To compare the neuroprotective activity among inhibitors of MAO, clorgyline, a selective MAO-A inhibitor, and pargyline, an inhibitor of both MAO-A and -B, were also examined. These compounds, except (R)-aminoindan, have a propargylamine group.

As reported previously (Maruyama et al., 2000b; Naoi et al., 1998), SIN-1 induced DNA damage in a dose-dependent way, as shown by formation of comet image with fragmented DNA. The DNA damage was confirmed to be apoptotic by staining with Hoechst 33342. After incubation with 100 μM SIN-1 for 18 hr, the cells showed typical morphological features of apoptosis; nuclear condensation and fragmentation (Fig. 2). On the other hand, the cells incubated with 1 μM rasagiline before SIN-1-treatment showed intact cell features. After incubation with SIN-1 about half of the cells showed typical apoptotic nuclei (48.9 ± 10.4%: mean ± SD of 4 independent experiments), whereas nuclear damage was detected in 3.6 ± 1.3% of control cells. The pretreatment with rasagiline protected the cells completely from DNA damage, and the number of apoptotic cells reduced to 4.0 ± 2.4%, at almost the same

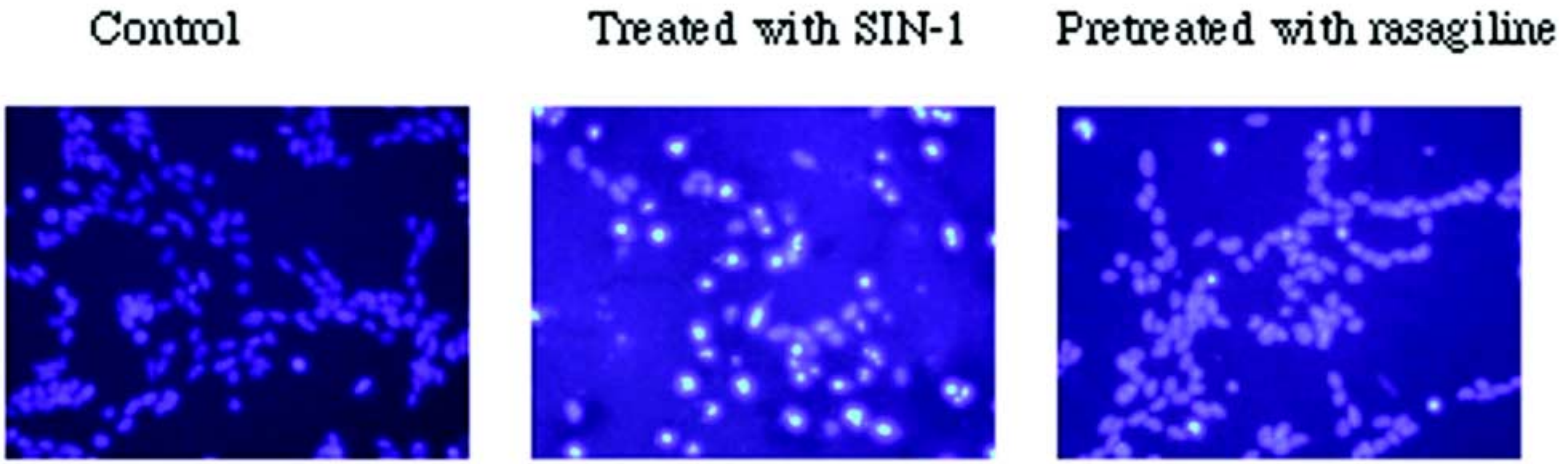
Control
Treated with SIN-1
Pretreated with rasagiline

Fig. 2

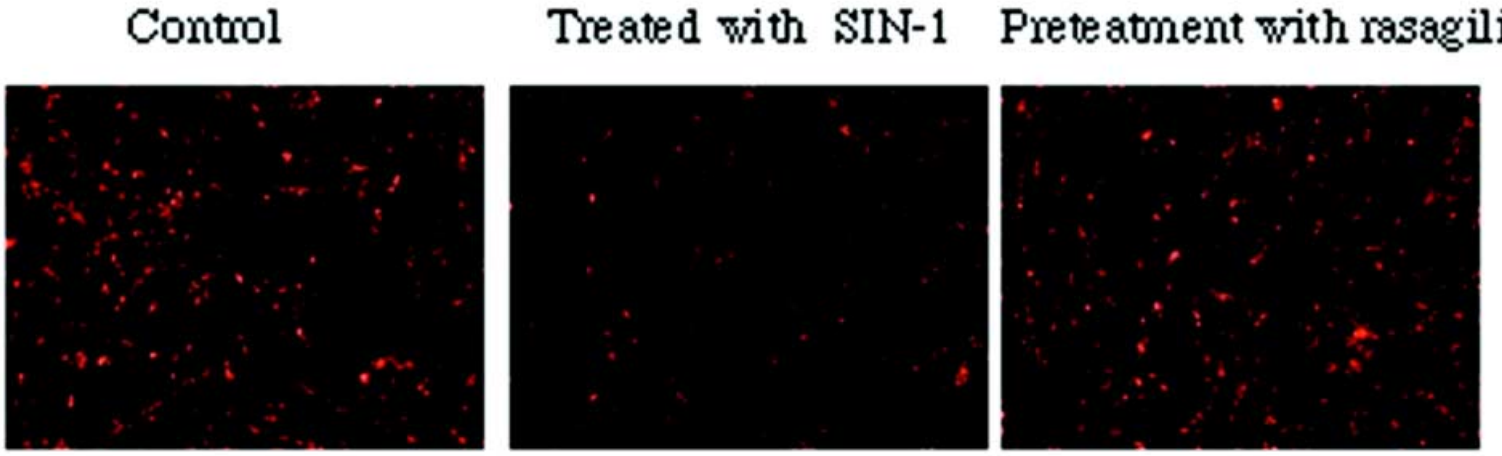
Control
Treated with SIN-1
Pretreatment with rasagiline

Fig. 4

level as in control. The number of DNA-damaged cells in the cells treated with SIN-1 alone was significantly larger than that in control and the cells pre-treated with rasagiline; $p < 0.001$ and 0.002, respectively.

The induction of DNA damage was studied among rasagiline and structurally related compounds quantitatively by the comet assay. As summarized in Table 1, tail length of control cells was less than 2.5 μm, which was clearly shorter than that in the cells treated with SIN-1 alone, longer than 6.5 μm. Rasagiline and selegiline reduced DNA damage in a dose-dependent way. At 1 μM rasagiline and selegiline completely protected the cells from DNA damage. The number of DNA-damaged cells was almost the same as in the control, 2–8 % of the total cells, whereas in SIN-1 treated cells, it increased to 35%. Rasagiline reduced DNA damage even at 10 nM, whereas selegiline did not protect the cells at the concentration lower than 100 nM. (S)Enantiomer of N-propargyl-1-aminoindan and deprenyl were slightly less potent than the

Table 1. Effects of rasagiline and TVP-1022, (R)- and (S)-enantiomer of N-propargyl-1-aminoindan and deprenyl on the DNA damage induced by a peroxynitrite-donor, SIN-1

	Tail length (μm)	
Control	2.22 ± 5.54	
Treated with SIN-1	6.61 ± 9.95	
Pretreatment with		
Rasagiline	(S)(−)-N-Propargyl-1-aminoindan	(TVP-1022)
1 μM	1.49 ± 4.55*	2.74 ± 5.34*
100 nM	2.88 ± 7.51*	2.80 ± 6.94*
10 nM	2.72 ± 6.90*	4.04 ± 8.05
Selegiline	(+)Deprenyl	
1 μM	1.72 ± 5.09*	0.32 ± 2.40*
100 nM	1.05 ± 3.70*	3.62 ± 3.46
10 nM	4.28 ± 9.89	3.46 ± 6.89

Each value represents the mean ± SD of three independent experiments with 200 cells. The cells were incubated with propargylamines 20 min before the addition of 10 μM SIN-1, and the DNA damage was quantified by the comet assay. *Statistically different from the cells treated with SIN-1 alone, by Sheffe's F test ($p < 0.05$)

Fig. 2. Effect of rasagiline-pretreatment on apoptosis induced by SIN-1. After 30-min incubation with 1 μM rasagiline, SH-SY5Y cells were treated with 100 μM SIN-1 for 18 hr, and then the cells were stained with Hoechst 33342. In the cells treated with SIN-1, fragmentation and condensation of nuclei were detected in many cells (treated with SIN-1), whereas the pre-treatment with rasagiline prevented apoptosis completely (pre-treated with rasagiline)

Fig. 4. The change in mitochondrial membrane potential after SIN-1 incubation and the effect of pre-treatment with rasagiline. SH-SY5Y cells were incubated with JC-1 and then incubated with 100 μM SIN-1, with or without pre-treatment with 1 μM rasagiline. The red fluorescence representing J-agglutinates in the intact membrane was reduced in the cells incubated with SIN-1 (treated with SIN-1) in a time-dependent way, whereas in control and cells pre-treated with rasagiline (pretreated with rasagiline) the fluorescence intensity did not change for 60 min

Table 2. Effects of (−)desmethylselegiline and (−)aminoindan on the DNA damage induced by a peroxynitrite-donor, SIN-1

	Tail length (μm)	
Control	2.22 ± 5.54	
Treated with SIN-1 alone	6.61 ± 9.95	
Pretreatment with		
(−)Desmethylselegiline		(−)Aminoindan
1 μM	1.70 ± 8.77*	10.35 ± 11.4
100 nM	0.32 ± 2.26*	8.57 ± 8.57
10 nM	2.18 ± 6.83*	6.79 ± 8.39

Each value represents the mean ± SD of three independent experiments with 200 cells. The cells were incubated with (−)desmethylselegiline or (−)aminoindan 20 min before the addition of 10 μM SIN-1, and the DNA damage was quantified by the comet assay. * Statistically different from the cells treated with SIN-1 alone, by Sheffe's F test ($p < 0.05$)

(R)enantiomer, but they still prevented apoptotic DNA damage, as summarized in Table 1. (R)(−)-Desmethylselegiline was effective as rasagiline, but aminoindan did not reduce the DNA damage as summarized in Table 2. After pre-treatment with 1 μM and 100 nM desmethylselegiline, the number of DNA-damaged cells was reduced to 4–8% of the total from that in cells treated SIN-1 alone, 35%, or those pre-treated with aminoindan, 31–38%.

The effects of clorgyline, a selective inhibitor of MAO-A, and pargyline, a non-selective inhibitor of MAO-A and -B, on the DNA damage by SIN-1 were examined. DNA damage was quantitatively assayed by the comet assay, and the damage was expressed as the tail length. As shown in Fig. 3, clorgyline did not protect the cells from apoptotic DNA damage, and pargyline with a similar chemical structure to deprenyl suppressed the damage, only at 1 μM. In addition, clorgyline and pargyline at the concentration higher than 10 μM did not protect the cells, but even increased DNA damage.

Mechanism of protection by rasagiline

To determine the effect of incubation timing, cells were treated with 1 μM rasagiline, before, simultaneously or after the addition of 10 μM SIN-1. As summarized in Table 3, rasagiline protected the cells, only when it was added before the treatment with SIN-1. The time course study revealed that protection of rasagiline required at least 20 min pretreatment before incubation with SIN-1.

The effect of SIN-1 on mitochondrial membrane potential, $\Delta\psi m$, was studied by use of a fluorescent probe, JC-1. JC-1 as monomer shows green fluorescence and insides the intact mitochondrial membrane it aggregates to be detected as red fluorescence. Figure 4 shows the fluorescent photos representing $\Delta\psi m$ in the cells. In cells treated with 100 μM SIN-1, the red fluorescence of J-aggregates declined in a time-dependent way, whereas in control cells it did not change for 60 min, as shown in Fig. 4. After 20 min

Table 3. Effects of timing of rasagiline treatment on the DNA damage induced by a peroxynitrite-donor, SIN-1

	Comet length (μm)	Tail length (μm)
Control	17.8 ± 2.9	1.75 ± 6.5
Treated with SIN-1	22.6 ± 7.3	6.32 ± 7.01
Treatment with 1 μM rasagiline		
Before SIN-1 addition	18.3 ± 3.3*	1.05 ± 3.26*
Simultaneously	22.8 ± 6.0	4.53 ± 7.09
After SIN-1 addition	21.3 ± 7.0	5.27 ± 7.21

Each value represents the mean ± SD of three independent experiments with 200 cells. The cells were incubated with rasagiline 20 min before, simultaneously or 20 min after the addition of 10 μM SIN-1. After 3 hr treatment, DNA damage was quantified by the comet assay. *Statistically different from the cells treated with SIN-1 in the absence of rasagiline, by Sheffe's F test ($p < 0.05$)

pre-incubation with 1 μM rasagiline, the cells were treated with SIN-1, and rasagiline completely suppressed the loss of $\Delta\psi m$, and the fluorescence intensity was almost the same as before the SIN-1 treatment or in control cells (Fig. 4). The quantitative analyses of the fluorescence intensity confirmed the stabilization of $\Delta\psi m$ against the loss by SIN-1, as summarized in Table 4.

To examine whether rasagiline could scavenge peroxynitrite directly, generation of peroxynitrite was quantitatively analyzed in the presence of rasagiline by use of a fluorescent indicator, H_2DCFDA. As summarized in

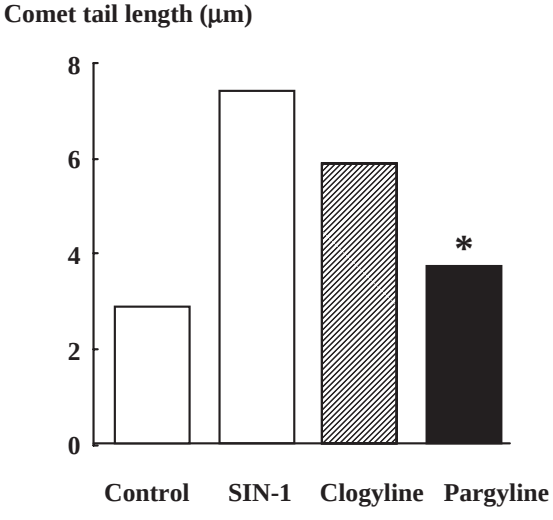

Fig. 3. The effect of clorgyline and pargyline on DNA damage induced by SIN-1. SH-SY5Y cells were incubated for 20 min with 1 μM of clorgyline or pargyline, and then treated with 10 μM SIN-1 for 3 hr. DNA damage was quantified by measurement of the comet tail. Each column represents the mean of 200 cells. In the cells pre-incubated with pargyline, the comet length was reduced and the difference from the cells treated with SIN-1 alone was statistically significant, $p < 0.01$

Table 4. The effect of rasagiline pretreatment on the loss of $\Delta\psi m$ induced by SIN-1

SH-SY5Y cells	Fluorescence intensity of J-aggregates (Relative intensity/cell)
Control cells before incubation	246.6 ± 15.7 (100%)
Control cells after 60 min incubation	286.3 ± 50.4 (116.1%)
Cells treated with SIN-1	67.7 ± 2.8* (27.5%)
Cells pre-treated with rasagiline, then SIN-1	225.9 ± 21.9 (91.6%)

The cells were incubated with or without 10 μM SIN-1 for 60 min, with or without pre-incubation with 10 μM rasagiline. $\Delta\psi m$ was measured by staining with JC-1, and the fluorescence intensity (arbitrary unit/cell) of J-agglutinates was quantified by use of NIH image software. The cell numer was counted by phase-contrast microscopy. The number represents the mean ± SD of measurements of 4 fields with 200 cells. *Statistically different from other samples, $p < 0.001$

Table 5, rasagiline did not scavenge peroxynitrite generated from SIN-1, even at the concentration of 100 μM.

Discussion

This paper presents the data that the anti-parkinson drug, rasagiline and its (S)-enantiomer are some of the most potent neuroprotective reagents so far studied using SH-SY5Y cells. The structure-activity relationship suggests that the presence of a propargylamine group is essential for the protection, as shown by the lack in the neuroprotective activity of aminoindan, the metabolite of rasagiline and TVP-1022. Furthermore MAO inhibition is not an essential factor, since TVP-1022, S-isomer of rasagiline is a poor MAO inhibitor (Finberg et al., 1999; Youdim et al., 2001b, 1999). Pargyline with one carbon chain length less than deprenyl in the alkyl group showed the neuroprotective properties only at 1 μM, but cytotoxic at 10 μM. This result suggests that the adequate distance between a propargylamine and a hydrophobic group is

Table 5. Effect of rasagiline on the generation of peroxynitrite from SIN-1

	2′,7′-Dichlorofluorescein produced (pmol/min)
SIN-1 (100 μM)	9.09 ± 0.713
SIN-1 + Rasagiline	
100 μM	8.75 ± 0.55
10 μM	9.86 ± 0.56
1 μM	10.03 ± 0.44
0.1 μM	11.02 ± 0.44

SIN-1 (100 μM in the final concentration) was added to 50 μM H_2DCFDA solution in the absence and presence of rasagiline (100 − 0.1 μM), and the production of 2′,7′-DCF by peroxynitrite was quantitatively assayed fluorometrically. There was no statistical difference in peroxynitrite generation between SIN-1 alone and the presence of rasagiline

also required. The absence of protection by clorgyline indicates that the structure of the hydrophobic group is also important for the neuroprotection. Recently, we found that N-(2-heptyl)-N-methylpropargylamine protected SH-SY5Y cells from apoptosis induced by a dopaminergic neurotoxin, N-methyl(R)salsolinol (Maruyama et al., 2001). These results indicate that three groups of propargylamines have similar neuroprotective function; rasagiline (a secondary cyclic benzylamine) (Youdim et al., 2001a,b), selegiline (a tertiary alkylamine) and N-(2-heptyl)-N-methylpropargylamine (a tertiary branched alkylamine). All these propargylamines are inhibitors of MAO with specific affinity to type B (Yu et al., 1992), suggesting that the propargylamine residue may bind to a selective site of or similar to the catalyzing site in MAO-B. However, SH-SY5Y cells contain only type A MAO (Maruyama et al., 1997b), indicating that the binding protein is not MAO-B and inhibition of MAO-B activity is not involved in the neuroprotection. This is further supported by the observation that not only rasagiline but also its (S)-enantiomer (TVP-1022) showed neuroprotection, since the (S)-enantiomer(TVP-1022) has little MAO-B inhibitory activity (Youdim et al., 2001b). The MAO B or MAO A inhibitory action of TVP-1022 is between 2–4 fold (100–10,000 times) less than rasagiline. At present, we have not determined the target protein of rasagiline and TVP-1022 but one candidate may be glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or mitochondrial transition permeability pore which constitutes the voltage dependent ion channel. GAPDH was proposed to be a possible binding site of CGP 3466, a non MAO inhibitory propargyl compound and selegiline (Kragten et al., 1998), and be involved in the apoptotic cascade as suggested by Ishitani and Chuang (1996). However, Youdim et al. (2001b, 1999) have reported that the antiapoptotic actions of both rasagiline and TVP-1022 is dependent on new protein synthesis because the transcriptional inhibitor (actinomysin) and translational inhibitor (cylcoheximide) blocked the antiapoptotic action of both drugs in PC12 cells. The nature of protein(s) synthesis is not known, but Western blot measurements have shown that both drugs prevent the decrease seen in BCL-2 and Cu-Zn superoxide dismutase (SOD1) during serum- and NGF-withdrawal in partially neuronally differentiated PC12 cells (Youdim et al., 1999).

The protection by rasagiline requires the incubation in prior to the treatment with peroxynitrite, suggesting that intracellular process to suppress the apoptotic death process or activate the anti-apoptotic process in the cells should be initiated by rasagiline. Recently, reduction of $\Delta\psi m$ is proposed to play a major role to initiate apoptosis (Kroemer et al., 1998). Using a fluorescence probe, JC-1, $\Delta\psi m$ was found to decline by incubation with SIN-1. Pretreatment with rasagiline prevented the collapse of $\Delta\psi m$, which is consistent with previous observation by use of selegiline, rasagiline and TVP-1022 (Wadia et al., 1998). Another proposed mechanism is the induction of new synthesis of neuroprotective proteins or expression of immediate early genes involved in the anti-apoptotic process. Tatton et al. (1994) reported that selegiline reduced apoptosis in PC12 cells induced by withdrawal of serum and growth factor, and transcription induction was required for its anti-apoptotic effect (Tatton and Chalmers-Redman, 1996). Other reported mechanisms

were enhancement of the synthesis of neurotrophic substances, such as nerve growth factor (Tatton et al., 1994), and anti-oxidant enzymes, such as SOD and catalase (Tatton et al., 1994). Indeed chronic rasagiline treatment significantly increases the activities of SOD and catalase in the brain (striatum, hippocampus and cortex), heart and liver (Carrillo et al., 2000). Selegiline was reported to scavenge reactive oxygen species, such as hydroxyl radical (Cohen and Spina, 1989; Riederer and Youdim, 1986). However, rasagiline did not scavenge peroxynitrite directly as reported here, and the time course studies proved that rasagiline required only 20 min pre-incubation before peroxynitrite treatment. Our results suggest that the mechanism underlying the anti-apoptotic activity by rasagiline may be different from that by selegiline, and that it may not be related to the new synthesis of proteins. The protection of $\Delta\psi m$ indicates that rasagiline may attenuate the initiating point in the apoptotic cascade, since it inhibits the activation of caspase 3 initiated by SIN-1 and 6-hydroxydopamine (Maruyama et al., 2000a,b).

A series of N-propargylamine derivatives were reported not only to protect dopaminergic neurons, but also noradrenergic (Zhang et al., 1995), and cholinergic neurons (Bronzetti et al., 1992) against cell deaths induced by various stimuli. Recently, rasagiline and TVP-1022 were reported to protect rat brain against ischemic damage (Speiser et al., 1998) and mouse brain against closed head injury (Huang et al., 1999). These results suggest that rasagiline may be neuroprotective for several types of neurons against apoptosis elicited during the process of neurodegenerative diseases and possibly in aging.

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