Table 1
Anti-apoptotic function of *R*-(−)-BPAP analogues on apoptosis induced by *N*-methyl(*R*)salsolinol

| | Number of apoptotic cells ([1]; % of the total, [2];% of apoptotic cells in *N*M(*R*)Sal-treated cells) | | | | | |
|---|---|---|---|---|---|---|
| | Control[1] | *N*M(*R*)Sal[1] | 1 μM[2] | 100 nM[2] | 10 nM[2] | 1 nM[2] |
| *R*-(−)FPFS-1169 | 3.2 | 36.8 | 61.7 ± 11.6* | 65.4 ± 7.5* | 52.7 ± 14.5* | 108.7 ± 1.5 |
| *S*-(+)-FPFS-1170 | 5.1 | 30.9 | 67.8 ± 9.5* | 63.2 ± 1.9* | 73.7 ± 4.4* | 79.6 ± 11.9 |
| *R*-(+)-FPFS-1179 | 3.0 | 28.9 | 128.8 ± 16.0 | 125.2 ± 14.2 | 120.5 ± 16.7 | 144.5 ± 30.1 |
| *S*-(−)-FPFS-1181 | 5.6 | 32.0 | 91.7 ± 6.3 | 89.1 ± 11.5 | 112.9 ± 14.2 | 101.4 ± 13.5 |
| *R*-(+)-FPFS-1180 | 4.5 | 29.6 | 77.9 ± 6.9* | 81.1 ± 7.5* | 75.8 ± 7.3* | 62.6 ± 10.8* |
| *S*-(−)-FPFS-1182 | 3.41 | 18.7 | 92.5 ± 2.3 | 94.3 ± 6.2 | 117.9 ± 0.1 | 97.3 ± 14.5 |
| *R*-(−)-FPFS-1183 | 4.7 | 29.1 | 79.7 ± 14.2 | 66.8 ± 8.0* | 76.9 ± 18.0 | 97.3 ± 16.8 |
| *S*-(+)-FPFS-1184 | 3.45 | 34.2 | 71.3 ± 8.3* | 128.8 ± 20.9 | 114.7 ± 9.1 | 99.6 ± 15.3 |

*Statistically different from *N*M(*R*)Sal-treated cells, $p < 0.05$.

at 4 °C with anti-human Bcl-2 (100) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-β-actin antibody as control (Sigma, St. Louis, MO, USA). The membranes were incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA) at room

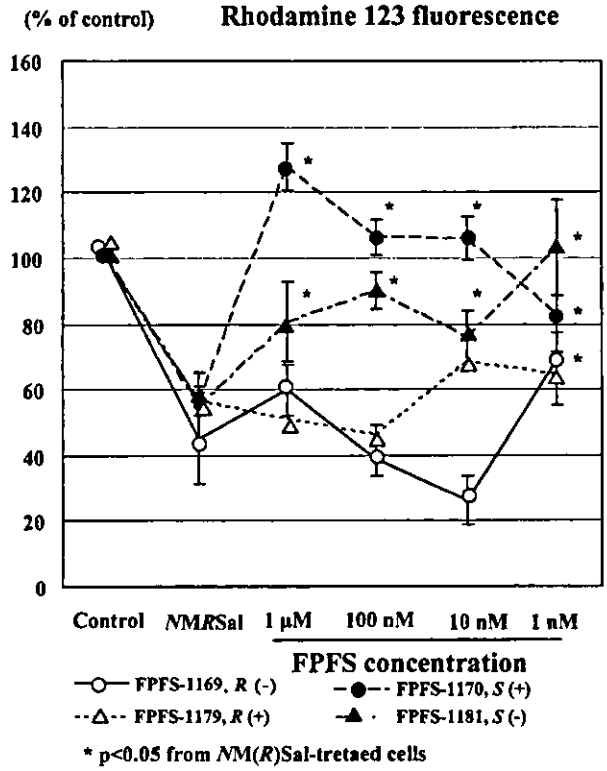


Fig. 2. The effects of BPAP derivatives on ΔΨm decline induced by *N*M(*R*)Sal in SH-SY5Y cells. As described in Materials and methods, the cells were treated with FPFS-1169, FPFS-1170, FPFS-1179 and FPFS-1181, then with *N*M(*R*)Sal, and the fluorescence intensity of Rhodamine 123 was expressed as percentage of control. Each point and bar represent the mean and SD of three experiments. *; Difference from the cells treated with *N*M(*R*)Sal alone was statistically significant, $p < 0.05$.

temperature. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA), and quantified by computer-assisted image analysis with the NIH imaging software.

*Statistics*

Experiments were repeated 4 to 8 times, 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

*Anti-apoptotic function of BPAP derivatives*

Fig. 1 shows the chemical structure of (−)-BPAP analogues used in these experiments. Apoptosis was induced in 19–37% of SH-SY5Y cells after treatment with 250 μM *N*M(*R*)Sal, whereas necrotic

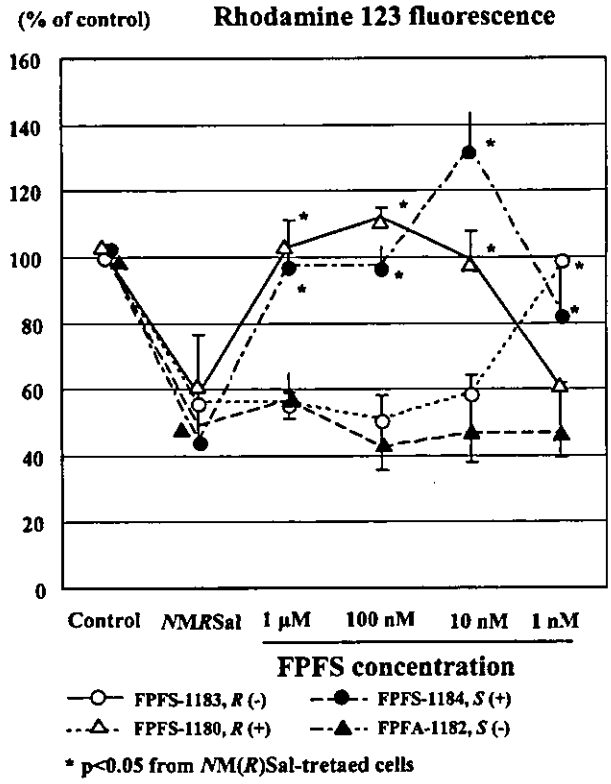


Fig. 3. The effects of BPAP derivatives on ΔΨm decline induced by *N*M(*R*)Sal in SH-SY5Y cells. The cells were treated with FPFS-1180, FPFS-1182, FPFS-1183 and FPFS-1184, as described in legend for Fig. 2.

cells were virtually negligible. As summarized in Table 1, *R*-(−)- and *S*-(+)-BPAP (the development number; FPFS-1169 and FPFS-1170) and FPFS-1180 reduced the number of apoptotic cells to 50–60% of that of *N*M(*R*)Sal-treated cells. *R*-(−)1-(Benzofuran-2-yl)-2-aminopentane (FPFS-1183) and the *S*-(+)-enantiomer (FPFS-1184) protected the cells from apoptosis only at 100 nM and 1 μM, respectively.

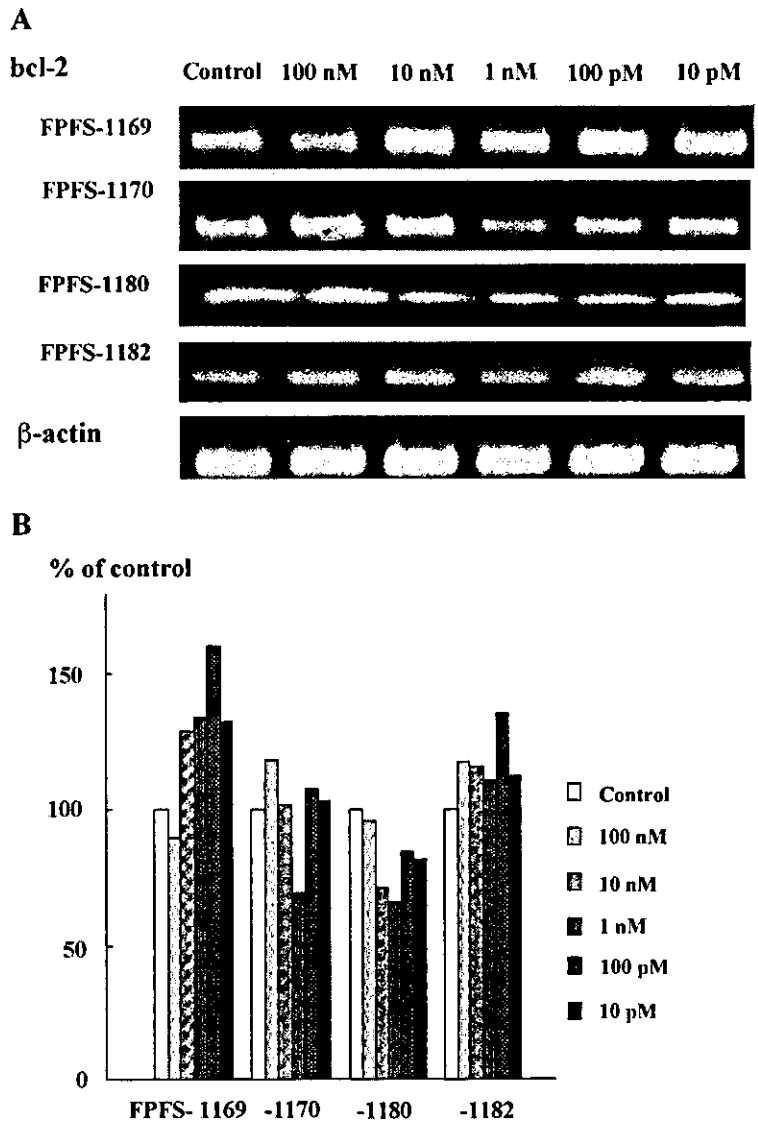


Fig. 4. The effect of BPAP derivatives on mRNA levels of bcl-2 in SH-SY5Y cells. The cells were cultured in the presence of 100 nM–10 pM BPAP derivatives for 24 h, and mRNA levels were measured, as written in Materials and methods. β-Actin mRNA was used as control. (A) Gel electrophoresis of bcl-2 and β-actin mRNA. (B) One of the quantitative data of mRNA amounts.

*R*-(+)-FPFS-1179 and *S*-(−)-FPFS-1181 containing both a propargyl and a propyl group at the 1-amino group did not protect the cells from apoptosis.

*The stabilization of $\Delta\Psi m$*

*N*M(*R*)Sal induced PT in SH-SY5Y cells, and the fluorescence of pre-loaded Rhodamine 123 reduced. The effects of BPAP derivatives on the stabilization of $\Delta\Psi$m were studied, as shown in Figs. 2 and 3. After 2 h treatment with 250 μM *N*M(*R*)Sal, the fluorescence intensity reduced to about 50% of

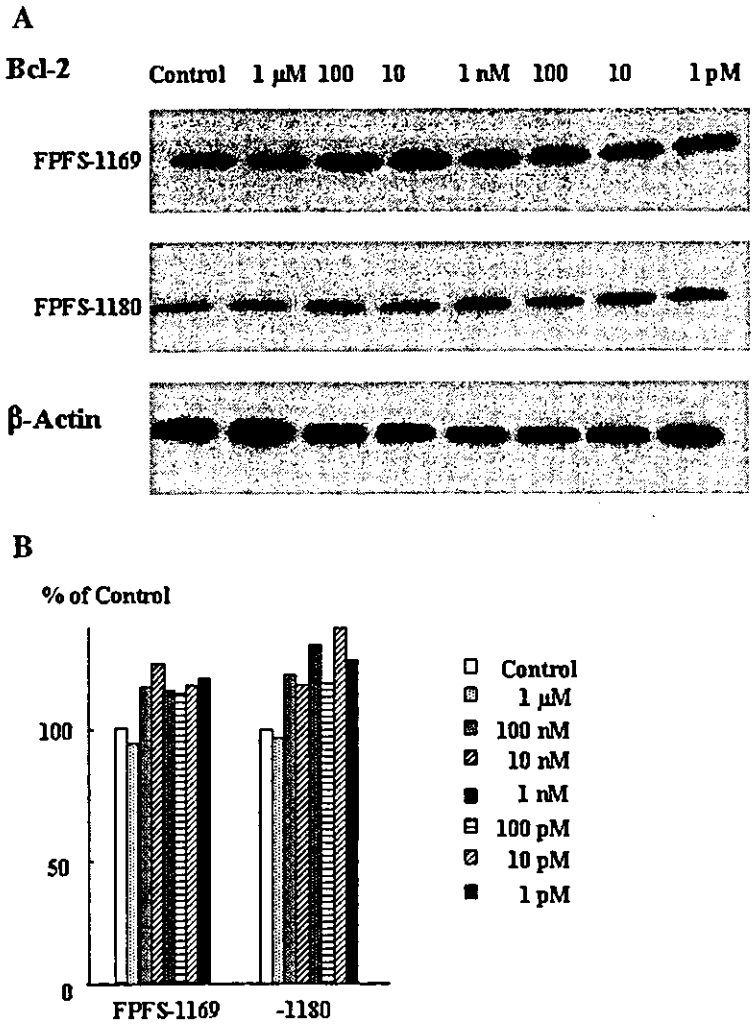


Fig. 5. The effects of BPAP derivatives on the protein level of Bcl-2. SH-SY5Y cells were treated with 1 μM–1 pM BPAP derivatives, and the Bcl-2 protein levels were determined by Western blot analysis. β-Actin was used as an internal control. (A) Western blots of Bcl-2 and β-Actin. (B) One of the quantitative data of Bcl-2 protein amounts.

control, and *S*-(+)-FPFS-1170, *R*-(+)-FPFS-1180 and *S*-(+)-FPFS-1184, the analogues with dextrorotation, prevented the reduction at 1 μM – 10 nM, whereas the corresponding enantiomers with levorotation, *R*-FPFS-1169, *S*-FPFS-1182, *R*-FPFS-1183, did not. Neither *R*-(+)-FPFS-1179 nor *S*-(−)-FPFS-1181 prevented the ΔΨm reduction.

*Induction of anti-apoptotic bcl-2 mRNA and protein*

The effects of BPAP derivatives on the mRNA level of apoptosis-preventing and survival-promoting bcl-2 were examined by RT-PCR method. As shown in Fig. 4, *R*-(−)-FPFS-1169 and *R*-(+)-FPFS-1180 increased mRNA level of bcl-2 at 10 nM – 10 pM, whereas *S*-(+)-FPFS-1170 and *S*-(−)-FPFS-1182 did not. Fig. 4B shows one of the quantitative data of blc-2 mRNA in the cells treated with (−)-BPAP analogues.

Bcl-2 protein levels in SH-SY5Y cells treated with BPAP derivatives were examined by Western blot analysis. As shown in Fig. 5, *R*-(−)-FPFS-1169 and *R*-(+)-FPFS-1180 increased the Bcl-2 levels at 100 nM–1 pM.

## Discussion

This paper presents that a series of benzofuran derivatives prevented apoptosis induced by an endogenous neurotoxin, *N*M(*R*)Sal, through the stabilization of PT pore closed state and the induction of bcl-2. As reported previously with another neuroprotective agent, rasagiline, prevention of apoptosis by these compounds was confirmed from reduction of apoptotic cells staining with Hoechst 33342 and from prevention of nucleosomal DNA fragmentation.

The studies on the structure-activity relationship show that benzofuran derivatives with dextrorotation, *S*-FPFS-1170, *R*-FPFS-1180 and *S*-FPFS-1184, prevented the ΔΨm decline, whereas the corresponding derivatives with levorotation, *R*-FPFS-1169, *S*-FPFS-1182 and *R*-FPFS-1183, did not affect the ΔΨm. However, *R*-(−)-FPFS-1169, *S*-(+)-FPFS-1170, and *R*-(+)-FPFS-1180 could protect the cells from apoptosis. Their neuroprotective potency may be due to the induction of anti-apoptotic Bcl-2, as shown here with *R*-(−)-FPFS-1169 and *R*-(+)-FPFS-1180. Our previous results using rasagiline, and isolated mitochondria and SH-SY5Y cells (Maruyama et al., 2001a, 2001b; Akao et al., 2002a) indicate the presence of a binding site enantio-specific for (*R*)-enantiomers in the outer membrane of mitochondria. The stabilization of PT pore-closed state by benzofuran derivatives depended on the absolute stereo-chemical structure, suggesting the presence of a binding site of BPAP derivatives with dextrorotation. The presence of both a propargyl and a propyl group at the 1-amino group in *R*-(+)-FPFS-1179 and *S*-(−)-FPFS-1181 abolished the potency to suppress ΔΨm decline and apoptosis, suggesting that the presence of these two residues inhibited the binding to the target protein in mitochondria. The induction of bcl-2 mRNA and BCL-2 protein was increased at the lower concentrations (Figs. 4B and 5B), which may be comparable with our previous results with rasagiline. The effects of rasagiline concentration on the potency to induce GDNF showed an inverted U-shape dose-response curve (Maruyama et al., 2004). The mechanism of these results remains to be clarified, but the binding site(s) may have such an affinity to these neuroprotective drugs.

Rasagline (Akao et al., 2002b) and (−)deprenyl (Tatton et al., 2000) induced Bcl-2 and GDNF (Maruyama et al., 2004), a dopamine neuron-specific neurotrophic factor, in cultured cells, and increased

the enzymatic activities of superoxide dismutase and catalase in dopamine neurons of rat brains (Carrillo et al., 2000). Neuroprotective agents were found to induce anti-apoptotic and pro-survival genes though the activation of transcription factors. Rasagiline activates a nuclear transcription factor, NF-κB, which mediates the induction of pro-survival genes encoding Bcl-2, GDNF and anti-oxidant enzymes in general. At present, it remains to be clarified whether BPAP derivatives activate transcription factors, such as NF-κB, to induce anti-apoptotic genes. In addition, the relation of the gene induction to catecholaminergic-serotonergic enhancing effects should be further examined to elucidate the mechanism behind neuroprotection by a series of BPAP derivatives.

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# Neuromelanin inhibits enzymatic activity of 26S proteasome in human dopaminergic SH-SY5Y cells

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**Summary.** Recently, impairment of the ubiquitin-proteasome system is suggested to be responsible for the neuronal death in ageing and Parkinson's disease. The specific degeneration of dopamine neurons containing neuromelanin (NM) suggests that NM itself may be involved in the cellular dysfunction and death, even though the direct link has never been reported. We examined the effects of NM isolated from the human substantia nigra on the proteasome activity in human dopaminergic SH-SY5Y cells. NM reduced the activities of 26S proteasome, as shown *in situ* using a green fluorescent protein homologue targeted to 26S proteasome and also *in vitro* using ubiquitinated lysozyme as a substrate. However, NM did not affect 20S proteasome activity *in vitro*. NM reduced the amount of PA700 regulatory subunit of 26S proteasome, but did not affect that of α- and β-subunits of 20S proteasome. These results suggest that NM may inhibit the ubiquitin-26S proteasome system, and determine the selective vulnerability of dopamine neurons in ageing and related disorders.

**Keywords:** Dopamine neuron, neuromelanin, 26/20S proteasome, ubiquitin, ubiquitin-proteasome system, proteasome sensor protein.

## Abbreviations

*AMC* 7-Aminomethylcoumarin, *DMSO* dimethyl sulfoxide, *DTT* dithiothreitol, *ECF* enhanced chemofluorescence, *GFP* green fluorescent protein, *Lyso* lysozyme, *MCA* 4-methyl-coumaryl-7-amide, *MEM* minimum essential medium: $MPP^+$ 1-methl-4-phenylpyrimidium ion, *NM* neuromelanin, *PBS* phosphate-buffered saline, *PD* Parkinson's disease, *PSI* carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [Z-Ile-Glu(Obu$^t$)-Ala-Leu-H (aldehyde)], *SDS* sodium dodecyl sulfate, *SN* substantia nigra, *UCH-L1* ubiquitin C-terminal hydrolase L1, *UP* ubiquitin-proteasome, *ZsGFP* a green fluorescence protein homologue from *Zoanthus sp.*, *Z-LLE-MCA* carboxy-L-leucyl-L-leucyl-L-glutamyl-MCA, *Z-LLVY-MCA* carbobenzoxy-L-leucyl-L-leucyl-L-valyl-L-tyrosine-MCA.

Neuromelanin (NM) is present in the neurons of the brainstem, especially the substantia nigra (SN) and the locus coeruleus, which are preferentially affected in ageing and Parkinson's disease (PD) (Emborg et al., 1998; Mann and Yates, 1974), suggesting that NM may be involved in the vulnerability of these neurons (Hirsch et al., 1988; Kastner et al., 1992). However, the direct link between NM and degeneration of dopamine neurons has never been proved. The role of NM was discussed either in a preventing or promoting way to the degeneration of nigro-striatal dopamine neurons. NM was reported to increase the vulnerability of SN neurons (Youdim et al., 1994; Offen et al., 1997), and iron(III)-melanin complex was cytotoxic to dopaminergic neurons *in vitro* (Jellinger et al., 1992; Mochizuki et al., 1993; Double et al., 2002). Increased production of reactive oxygen species was proposed to account for the cytotoxicity of NM-metal conjugates (Ben-Shachar et al., 1991; Zareba et al., 1995). More recently NM was reported to increase the secretion of cytokines and nitric oxide from microglia, which might be related to degeneration of dopamine neurons in PD (Wilms et al., 2003). On the other hand, NM has been proposed to be neuroprotective, by scavenging redox active metals (Fe, Cu and Mn), toxic metals (Cd, Hg and Pb) (Youdim et al., 1994; Zecca et al., 1994), pesticides (Lindquist et al., 1987), beta-carbolines (Oestergren et al., 2004), and other toxins, such as 1-methyl-4-phenylpyridinium ion ($MPP^+$) (D'Amato et al., 1986) and 1,2(*N*)-dimethyl-6,7-dihydroxyisoquinolinium ion, an oxidation product of an endogenous dopaminergic neurotoxin, *N*-methyl-(*R*)salsolinol [1,2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] (Naoi et al., 1994). In addition, NM synthesis reduces accumulation of cytotoxic quinone produced by dopamine oxidation (Sulzer et al., 2000).

The neuronal death in PD was considered to be due to increased oxidative stress, mitochondrial dysfunction, and accumulation of oxidatively modified proteins in the cells. Recently in the familial parkinsonism, mutations in α-synuclein (Polymeropoulus et al., 1997), parkin, an E3 ubiquitin-protein ligase (Kitada et al., 1998; Shimura et al., 2000), and ubiquitin C-terminal hydrolase L1 (UCH-L1) (Leroy et al., 1998) were reported. These results suggest the contribution of the ubiquitin-proteasome (UP) system to neurodegeneration also in sporadic form of PD. Increased number of evidences suggest that impaired

UP system leads to accumulation of oxidatively-modified and/or ubiquitinated protein, and might account for the formation of Lewy body, the pathological hallmark of PD (McNaught et al., 2002a). Actually, proteasome subunits were found to co-localize in Lewy body (Ii et al., 1997), in addition to proteins associated with the UP system, α-synuclein, UCH-L1, and parkin (Gai et al., 2000; Shimura et al., 2001). Recently, aggresome formed in response to the accumulation of abnormal proteins in the cells is suggested to be a precursor of Lewy body in PD (McNaught et al., 2002b). These results suggest again that reduced activity of proteasome system may play a key role in induction of cell death through accumulation of abnormal proteins.

In the UP system, 20S proteasome is composed of 4 rings to make a cylindrical structure made of seven either α or β subunits of 20S. Binding of a regulatory complex called 19S (ATPase, PA700) to both the end of the 20S cylinder produces 26S proteasome. 26S proteasome degrades protein substrates, such as polyubiquitinated proteins and ornithine decarboxylase, in an ATP-dependent process, whereas 20S proteasome degrades oxidized proteins in ubiquitination- and ATP-independent manner.

In this paper, the effects of NM on proteasome were studied in SH-SY5Y cells by use of NM purified from the SN of control human brains, as suggested by one of us (P. R.). To measure the activities of 26S and 20S proteasome, respectively, we used *in vitro* assays for 26S proteasome using ubiquitinated lysozyme (Lyso) and synthesized fluorescent peptides, and *in situ* assays by use of SH-SY5Y cells transfected with a green fluorescence protein homologue from reef coral, *Zoanthus sp.* (ZsGFP) fused to the degradation domain for 26S proteasome with ornithine decarboxylase. The results are discussed in relation to the possible involvement of NM in dysfunction and death of dopamine neurons in ageing and PD through inhibiting the UP system.

## Materials and methods

### *Materials*

7-Aminomethylcoumarin (AMC) and substrates for proteasome, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methyl-coumaryl-7-amide (MCA) (Suc-LLVY-MCA, a substrate for chymotrypsin-like activity), benzyloxycarbonyl-L-leucyl-L-leucyl-L-glutamic acid-MCA (Z-LLE-MCA, for peptidyl-glutamyl peptide hydrolase-like activity), and an inhibitor of proteasome, benzyloxycarbonyl-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinal [PSI, Z-Ile-Glu(Obu$^t$)-Ala-Leu-H (aldehyde)] were purchased from Peptide Institute (Osaka, Japan). L-Cysteine and Lyso were purchased from Sigma (St. Louis, MO, USA). Anti-Lyso polyclonal antibody was obtained from Chemicon International (Temecula, CA, USA), anti-polyubiquitin monoclonal antibody from NBT (Tokyo, Japan). Antibodies against α- and β-subunit of 20S proteasome, and the proteasome activator, PA700 complex, were purchased from Calbiochem (San Diego, CA, USA). A proteasome sensor vector, pZsProSensor-1, was purchased from BD Biosciences (Palo Alto, CA, USA), and minimum essential medium (MEM) and other reagents were from Nacalai Tesque (Kyoto, Japan).

### *Preparation of NM*

NM was isolated from the SN, dissected from brains of neurologically normal adult individuals within 40 h *post mortem* at −15°C, as described previously (Dzierzega-Lecznar et al., 2004; Gerlach et al., 1995; Wakamatsu et al., 2003). Proteinase K-treated NM is essentially free of

adhering proteins (Dzierzega-Lecznar et al., 2004), to remove iron NM was further incubated in EDTA (Double et al., 2003). The identity and purity of this NM sample has been assessed by elemental analysis, amino acid analysis and electron paramagnetic resonance spectroscopy as previously reported (Zecca et al., 1996, 2000). NM was dissolved in distilled water containing 15 mM L-cysteine and 10% dimethyl sulfoxide (DMSO) (L-Cyst-DMSO solution) to be 0.5 mg/ml in the final concentration. NM suspension was sonicated for 20 min and then shaken gently for 4 days for rehydration at room temperature under protection from light.

### *Establishment of SH-SY5Y cells expressing a proteasome sensor vector*

Transfectant with a proteasome sensor vector was established using a pZsProSensor-1 eukaryotic expression vector, designed to express ZsGFP fused to the degradation domain of mouse ornithine decarboxylase, a specific substrate for 26S proteasome. ZsGreen fluorescence increases in the cells expressing the proteasome sensor vector, depending on its intracellular accumulation due to reduction of 26S proteasome activity. SH-SY5Y cells were transfected with pZsProSensor-1 by the lipofection technique according to the manufacturer's protocol (Gibco, Life Technologies, Invitrogen, Calsbad, CA, USA). Selection was started 3 days after the transfection using the Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan), supplemented with 5% newborn calf serum containing 100 μg/ml of G418 (Gibco, Life Technologies) in 5% $CO_2$ −95% atmosphere at 37°C. G418-resistant clones were isolated by limiting dilution. To confirm the expression of the proteasome sensor vector, the cells were cultured in the presence or the absence of 100 μM PSI for 24 h. The living cells were harvested, gathered by centrifugation, washed with phosphate-buffered saline (PBS), and then, the cells were suspended with PBS to be $5\times10^5$ cell/ml. The intensity of the fluorescence of ZsGreen was measured by a spectrofluorophotometer, RF-5300 (Shimadzu, Kyoto, Japan) with excitation at 493 nm and emission at 505 nm.

### *Measurement of in situ 26S proteasome activity using ZsGFP-expressed cells*

The *in situ* activity of 26S proteasome was estimated in SH-SY5Y cells transfected with the proteasome sensor vector (PSV-SH-SY5Y cells). PSV-SH-SY5Y cells were cultured in 6-well poly-L-lysine coated flasks as described above. NM suspension was mixed with the culture medium to be 0.05–1 μg/ml and incubated at 37°C for 2 h. The culture medium was changed with the medium containing L-Cyst-DMSO solution without (control) or with NM and the cells were cultured for 1 to 3 days. Accumulation of NM and ZsGFP in PSV-SH-SY5Y cells after treatment with NM was observed morphologically using a fluorescence microscope (Olympus BX60; Olympus, Tokyo, Japan) as reported previously (Maruyama et al., 2001). The fluorescence of ZsGFP in the living cells was measured as described above. The protein amount was measured according to Bradford (1976) and the fluorescence intensity of the cells was expressed as arbitrary fluorescence unit/mg protein. The cytotoxicity of NM was examined by morphological observation of the cells.

### *Analysis of in vitro 26S proteasome activity using polyubiquitinated Lyso*

To estimate the direct effects of NM on *in vitro* activity of 26S proteasome, a substrate selective for 26S proteasome, ubiquitin-conjugated Lyso, was prepared according to Shringarpure et al. (2003). Authentic Lyso dissolved in distilled water (1 mg/ml) was heated at 100°C for 5 min and then, cooled immediately. Heat-denatured Lyso was conjugated with ubiquitin using Ubiquitin protein conjugating kit (Calbiochem, San Diego, CA, USA) according to the manufacture's instruction. In brief, Lyso was incubated with the reaction mixture containing full compliment of purified conjugation enzymes (E1, E2 and E3s), 150 μM of ubiquitin solution for 4 h. The sample was stored at −20°C until analysis.

Enzyme samples were prepared from SH-SY5Y cells as reported previously (Shamoto-Nagai et al., 2003). Cells were washed twice and suspended in 50 mM Tris-HCl buffer, pH 8.0, homogenized and centrifuged at 14,000 g for 60 min. Glycerol was added to the supernatant to be the

final concentration of 20%. The sample was concentrated by approximate 3 folds using a 300 kDa cut off membrane filter (Pall, Ann Arbor, Michigan, USA) (Rodgers and Dean, 2003). All the procedure was carried out at 4°C.

The *in vitro* activity of ubiquitin-26S proteasome system was determined by measuring degradation of ubiquitin-conjugated Lyso. The enzyme sample (100 μg) was incubated with NM in L-Cyst-DMSO solution or L-Cyst-DMSO solution as control in at 37°C for 30 min, then with mixture of ubiquitinated Lyso at 37°C for 4 h. The reaction was terminated by adding the 1/4 volume of Laemmli's sample buffer (100 mM Tris-HCl containing 4% SDS, 12% β-mercaptoethanol and 20% glycerol). The protein was subjected to SDS-PAGE using 12.5% polyacrylamide gel (WAKO, Tokyo, Japan) and immunoblotting was performed as reported previously (Shamoto-Nagai et al., 2003) using anti-Lyso or anti-polyubiquitin antibodies and enhanced chemofluorescence (ECF) Western blotting kit (Amersham Biosciences, Piscataway, NJ, USA). The fluorescence intensity at 540 nm was quantified with excitation at 488 nm using a Fluoroimager 595 (Amersham Biosciences, Piscataway, NJ, USA). The amounts of ubiquitinated Lyso and free Lyso were quantified using antibody against polyubiquitin and Lyso, respectively, and NIH Image software.

### *Proteasome activity in NM-treated cells*

SH-SY5Y cells were cultured in 6-well poly-L-lysine coated flasks after addition of the L-Cyst-DMSO solution with or without NM (0.05–1 μg/ml in the final concentration). After 1 or 3 days, the cells were mechanically harvested and the enzyme sample was prepared, as described above. The activities of proteasome were measured using synthetic substrates (Shamoto-Nagai et al., 2003). The enzyme sample was incubated with 50 μM of Z-LLVY-MCA or Z-LLE-MCA for 30 min at 37°C, and the fluorescence intensity of AMC cleaved from the substrate was measured in a RF-5300 spectrofluorophotometer with excitation at 380 nm and emission at 440 nm.

### *Assay for the in vitro activity of 20S proteasome*

The activity of 20S proteasome was measured *in vitro* as reported previously (Okada et al., 1999; Shamoto-Nagai et al., 2003). Enzyme preparation (50 μg protein) was treated with L-Cyst-DMSO in the presence or absence of NM (0.75–7.5 μg/ml) in the reaction mixture [1 mM dithiothreitol (DTT), 0.5 mM EDTA 2Na, 2 mM ATP in 50 mM Tris-HCl buffer, pH 8.0] at 37°C for 30 min, and then the substrate, Z-LLVY-MCA or Z-LLE-MCA, was added to be 50 μM in the final concentration. After further incubation for 30 min at 37°C, the reaction was terminated by adding the same volume of 100 mM Tris-HCl buffer, pH 8.0, containing 1% SDS. AMC fluorescence was measured at 440 nm with excitation at 380 nm, as described above. The activity of the proteasome was expressed as pmoles of AMC produced per min per mg protein.

### *Western blot analyses of proteasome subunits in the cells incubated with NM*

After treatment with NM for 1 or 3 days, the cells were gathered, washed with PBS, and lyzed in the RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Fifty μg of protein was subjected to SDS-polyacrylamide electrophoresis using 12.5% polyacrylamide gel (Bio Craft, Tokyo, Japan), and blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Proteasome subunits were visualized using polyclonal antibody against α and β subunits of 20S proteasome and PA700 complex as described previously (Shamoto-Nagai et al., 2003).

### *Statistics*

Experiments were repeated at least 3 times. The data was expressed as mean ± SD and the difference was evaluated by analysis of variance (ANOVA) followed by Scheffe's F-test. A p value less than 0.05 was estimated to be statistically significant.

## Results

### *NM reduced in situ 26S proteasome activity in SH-SY5Y cells*

After treatment with NM, the accumulation of ZsGFP was observed in cytoplasm by fluorescence microscopy (Fig. 1A). The increased ZsGFP fluorescence in the cells was quantified in a RF-5300 spectrophotofluorometer. As shown in Fig. 1B, the fluorescence intensity increased in a dose- and time-dependent way. The ZsGFP fluorescence increased in the cells treated with 0.05 and 0.1 μg/ml of NM and further increased after 3 days' incubation in the presence of 0.1 μg/ml NM. NM at these concentrations neither affected cell proliferation, nor induced morphological changes in SH-SY5Y cells.

### *NM directly inhibited in vitro ubiquitin-26S proteasome activity*

The direct effect of NM on ubiquitin-26S proteasome activity was examined using the enzyme samples prepared from SH-SY5Y cells, and ubiquitinated

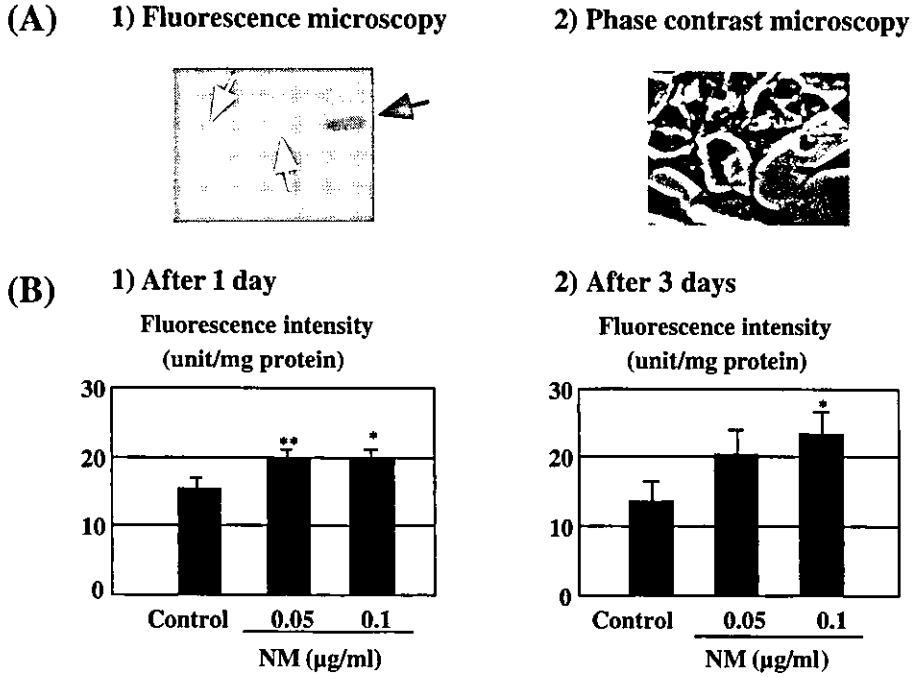


**Fig. 1.** Effects of neuromelanin (NM) on the *in situ* activity of 26S proteasome in SH-SY5Y cells transfected with the proteasome sensor vector (PSV-SH-SY5Y cells). A Morphological observation of PSV-SH-SY5Y cells treated with NM. The cells were cultured in the presence of 0.1 μg/ml of NM for 3 days and accumulation of ZsGFP (white arrows) and NM (black arrow) was observed by fluorescence microscopy (1) and phase contrast microscopy (2). B Quantitation of ZsGFP fluorescence in PSV-SH-SY5Y cells treated with 0.05 and 0.1 μg/ml of NM for 1 to 3 days. The fluorescence intensity was quantified using a fluorospectrophotometer with excitation at 493 nm and emission at 505 nm, and expressed as arbitrary fluorescence unit/mg protein. The column and bar represent mean and SD of 3 experiments. After the treatment with NM, the fluorescence intensity increased in a dose- and time-dependent manner compared to Control treated with L-Cyst-DMSO solution. $^{*}p<0.05$ and $^{**}p<0.01$ by ANOVA compared to Control

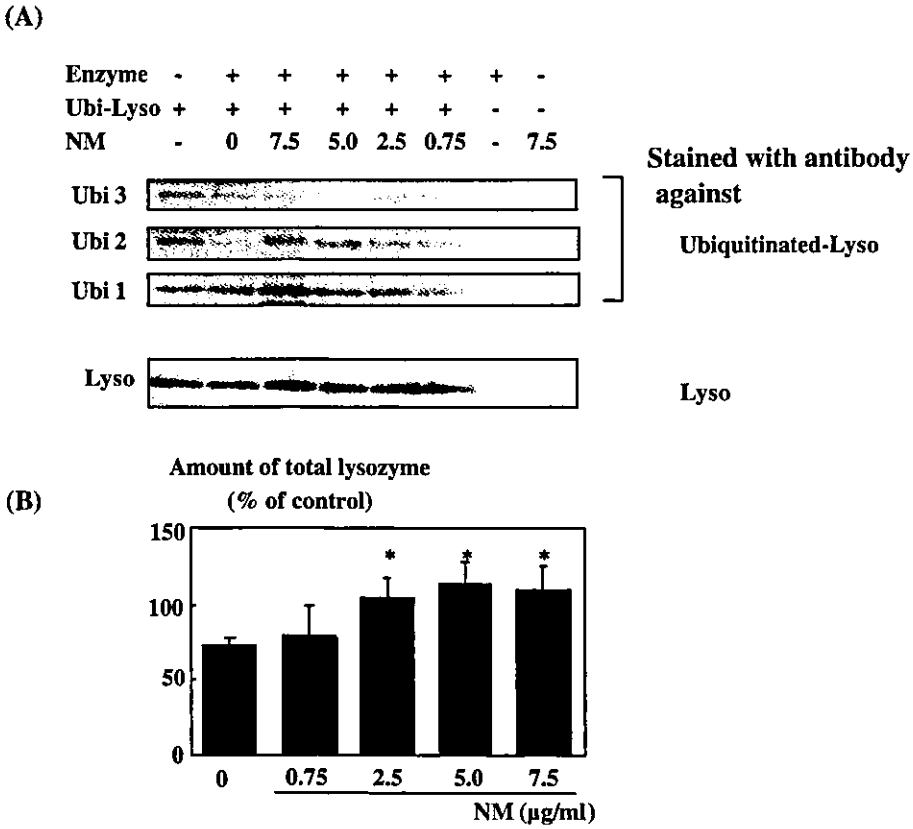


**Fig. 2.** Effects of neuromelanin (NM) on the *in vitro* activity of 26S proteasome. **A** The effects of NM on the activity measured with polyubiquitinated lysozyme (Lyso), a specific substrate for 26S proteasome. Mono-, di- and tri-ubiquitinated Lyso (Ubi 1, Ubi 2, Ubi 3), was prepared as described in the Materials and methods. Western blotting shows the degradation of ubiquitinated (Ubi) and non-ubiquitinated free Lyso after incubation with the enzyme preparation pre-treated with L-Cyst-DMSO solution in the absence or presence of NM. Ubiquitinated Lyso (Ubi 1, Ubi 2 and Ubi 3) was visualized with anti-polyubiquitinated antibody, and Lyso with anti-Lyso antibody. NM treatment reduced the degradation of ubiquitinated Lyso, especially Ubi 2 and Ubi 3, significantly. **B** The effect of NM on the total amount of Lyso (ubiquitinated and free). The amounts were quantified by measuring proteins stained with anti-Lyso antibody and using NIH imaging as described in the Materials and methods. The column and bar represent mean and SD of 3 independent experiments. NM significantly inhibited the ubiquitin-26S proteasome dependent degradation of Lyso significantly. $^{*}p<0.05$ by ANOVA compared to control treated by L-Cyst-DMSO solution without NM (0)

Lyso as a substrate specific for 26S proteasome. Figure 2A shows the immunoblotting of mono-, di- and tri-ubiquitinated Lyso and non-ubiquitinated Lyso. In the enzyme preparation and the NM suspension, ubiquitinated proteins were not detected. After 4 h incubation with the enzyme preparation, the amounts of ubiquitinated Lyso, especially di- and tri-ubiquitinated Lyso, were decreased significantly. In this system the ubiquitin ligase activity was not inhibited, and the amount of Lyso itself decreased. The total amounts of Lyso (free and ubiquitinated) were increased with NM, indicating that NM inhibited the Lyso

degradation by ubiquitin-26S proteasome system (Fig. 2B). The inhibition was increased by NM from 0.75 to 2.5 μg/ml, and reached to a plateau, indicating the soluble NM might be saturated.

*Effects of NM on proteasome activities in cells*

In the cells treated with NM for 1–3 days, the proteasome activities were measured using synthetic substrates, LLVY-MCA for chymotrypsin-like activity or LLE-MCA for peptidyl-glutamyl peptide hydrolase-like activity without addition of ATP. The activity represented mainly that of 20S proteasome, but the contribution of 26S could not be completely excluded. As shown in Fig. 3, the catalytic activity towards LLVY-MCA and LLE-MCA reduced after the incubation with NM, but the inhibition was not so marked. Reduction of the activity was more prominent in the cells treated with NM for 1 day, and after 3 days the reduced activity tended to be restored.

*NM did not inhibit in vitro 20S proteasome activity directly*

The proteasome activity in the enzyme preparation from control SH-SY5Y cells was measured with the synthetic substrates without addition of ATP. NM at the concentrations up to 7.5 μg/ml in the reaction mixture did not inhibit the enzyme activity *in vitro*, as shown in Fig. 4.

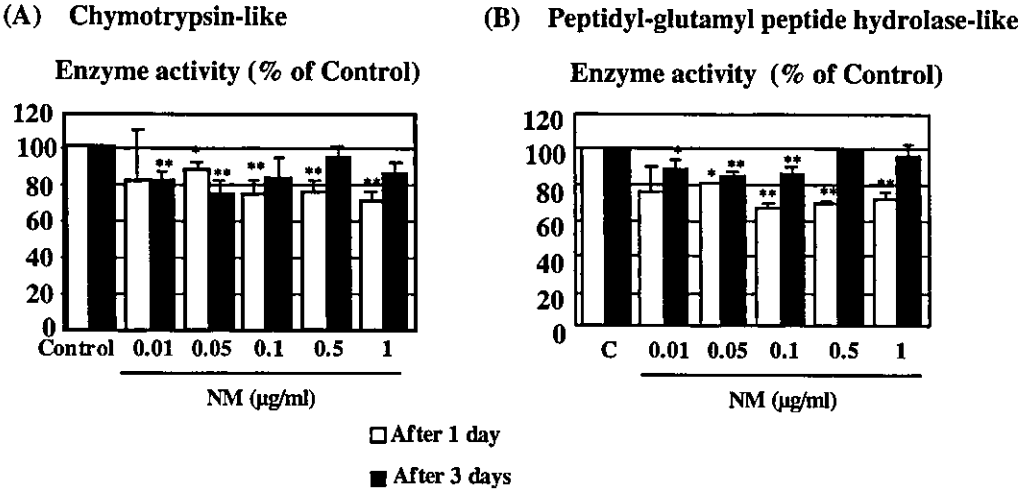


**Fig. 3.** Effects of neuromelanin (NM) on the proteasome activities in the cells treated with NM. The cells were cultured with L-Cyst-DMSO solution in the absence (Control) or presence of 0.01–1 μg/ml of NM for 1 and 3 days, then the enzyme sample was prepared. The chymotrypsin-like (**A**) and peptidyl-glutamyl peptide hydrolase-like (**B**) proteasome activities were measured with synthetic substrates. The column and bar represent mean and SD of 3 independent experiments. The activity was expressed as percent of Control. Open column and filled column; the proteasome activity in the cells after 1 day and 3 days treatment with NM, respectively. $^{*}p<0.05$ and $^{**}p<0.01$ by ANOVA compared to control without NM treatment (**C**)

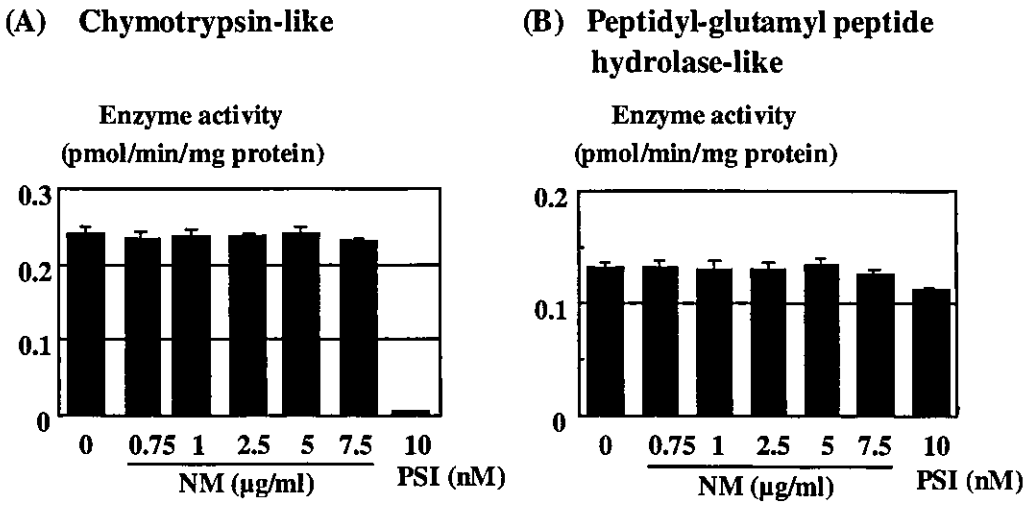


**Fig. 4.** Effects of neuromelanin (NM) on *in vitro* activities of chymotrypsin-like or peptidyl-glutamyl peptide hydrolase-like proteasome. The activities in the enzyme preparation were measured in the absence and presence of NM (0.75–7.5 μg/ml at the final concentrations), using artificial substrates, as described in Materials and Methods. The enzyme activity was expressed as pmole AMC produced/min/mg protein. The column and bar represent mean and SD of 4 independent experiments. NM did not reduce the chymotrypsin-like (**A**) or peptidyl-glutamyl peptide hydrolase-like (**B**) activity, but a proteasome inhibitor, PSI (10 nM) inhibited chymotrypsin-like proteasome activity completely

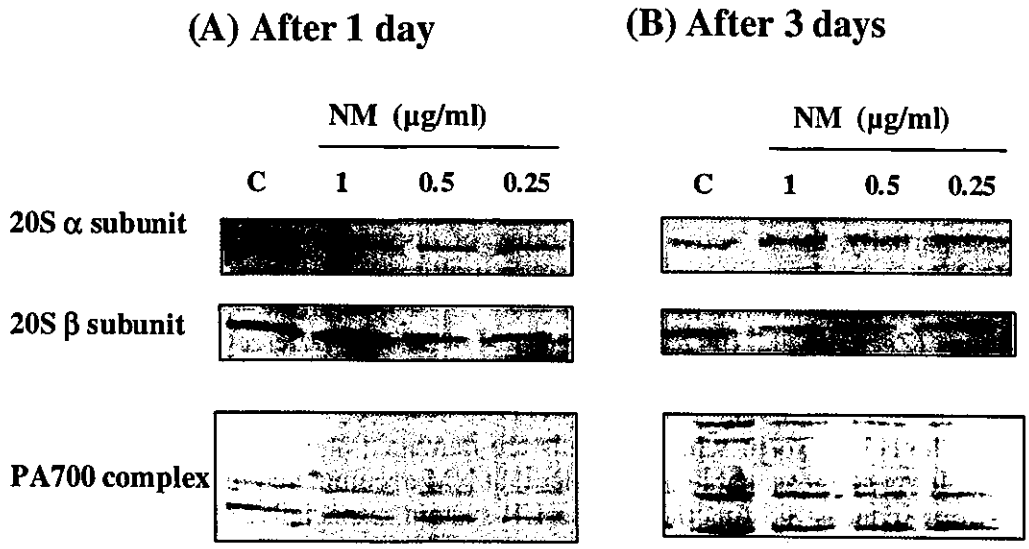


**Fig. 5.** Effect of neuromelanin (NM) on the protein levels of α- and β-subunits of 20S proteasome and PA700 complex of 26S proteasome. The cells were incubated with L-Cyst-DMSO solution in the absence or presence of NM (0.25–1 μg/ml) for 1 and 3 days and the cell lysate was subjected to Western blot analysis, using antibody against 20S α- and β-subunits and PA700 complex, respectively. NM reduced the level of PA700 complex, but did not affect the level of 20S α- and β-subunits in SH-SY5Y cells after 3 days' culture

### *NM reduced the protein level of PA700 complex, but not 20S α- and β-subunit*

To examine the effect of NM on the protein levels of proteasome subunits, proteins in the cells treated with NM at 0.25–1 μg/ml for 1–3 days were analyzed using polyclonal antibodies against α- and β-subunits of 20S proteasome, and a proteasome activator PA700 complex. As shown in Fig. 5, the protein level of PA700 complex was reduced significantly after 3 days of treatment, whereas NM did not affect the protein levels of α and β subunits of 20S under the conditions examined.

## Discussion

The results presented here clearly demonstrate that NM inhibits 26S proteasome activity *in situ* and *in vitro* whereas it did not inhibit the *in vitro* activity of 20S proteasome, nor reduced the protein levels of 20S α- and β-subunit in SH-SY5Y cells. NM treatment reduced the *in vitro* activity of 26S proteasome measured by use of ubiquitinated Lyso as a substrate, which was confirmed further by the increased fluorescence of the proteasome sensor protein, ZsGFP. In addition, NM reduced ubiquitination of free Lyso by E1, E2 and E3 ligase *in vitro* as shown by increased level of free Lyso in the reaction mixture. The inhibition of ubiquitin-26S proteasome system by NM may be due to the direct inhibition of the enzyme activities, by binding of soluble NM components to the active sites. In addition, NM reduced the protein level of a proteasome activator, PA700 complex, which forms active 26S proteasome by binding to the 20S proteasome and makes it possible to degrade polyubiquitinated proteins. The result presented here may be relevant to the recent report that the levels of the PA700 subunit and α-subunit of 20S proteasome were significantly reduced in the SN of PD brains (McNaught et al., 2003). However, the detailed molecular mechanism underlying the reduction of 26S proteasome activity by NM remains to be clarified.

The involvement of NM in impaired function of UP system may explain, at least partially, the selective vulnerability of dopamine neurons in ageing and PD. NM is a polymeric molecule having multilayer structure with planar overlapped sheets consisting of dihydroxyindole, benzothiazine rings and aliphatic groups (Zecca et al., 1992; Wakamatsu et al., 2003; Dzierzega-Lecznar et al., 2004). NM accumulates continuously in dopamine neurons of SN during aging reaching very high values in elderly (Zecca et al., 2002), and it is not secreted from the cells, except when the cells are damaged and phagocytosed by glial cells. It may be reasonable to consider that NM accumulated in nigral dopamine neurons reduces the function of the UP system, especially 26S and increases accumulation of ubiquitinated and denatured proteins. Indeed, Lewy bodies were found in the SN in subjects who had no Parkinsonian symptoms or signs during life and were suggested that this "Incidental Lewy body" represents early, subclinical PD (Forno and Langston, 1993). In normal ageing the accumulation of NM does not cause neuronal degeneration probably because some agents block the interaction between NM and proteasome however in PD it is possible that these blocking molecules are removed or not expressed, therefore

NM could inhibit proteasome and trigger the cascade ending into neuronal death.

Under the conditions used in this study, NM treatment did not induce the cell death in SH-SY5Y cells, indicating that other factors may be involved in the final cell death. The mitochondrial dysfunction, and reduction of ATP synthesis may further enhance the inhibition of the UP activity in dopamine neurons, since the activity of 26S, but not 20S, depends on ATP level. Recently we found that mitochondrial dysfunction caused by rotenone, a complex I inhibitor, increased modification of the 20S proteasome subunit with acrolein, and reduced the activity of proteasome, through binding of aggregated oxidized protein to the catalytic site (Shamoto-Nagai et al., 2003). These results suggest that the mitochondrial dysfunction, increased oxidative stress and impairment of the UP system may interact with each other, resulting in the cell death of dopamine neurons in ageing and PD.

The induction of cell death by accumulated modified protein may be due to occupation of limited space in cells, change in the signal transduction, or by inhibition of transcription of important genes, but it requires further studies for full elucidation. In addition, it is not clear whether NM itself or NM components, including quinones, products of dopamine, oxidized products of lipid and protein, or trace metals was truly cytotoxic and inhibited proteasome activity. Further molecular studies will elucidate full mechanistic features of the involvement of NM in degeneration of dopamine neurons in the brain.

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