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A novel thiol antioxidant that crosses the blood brain barrier protects dopaminergic neurons in experimental models of Parkinson's disease

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Abstract

It is believed that oxidative stress (OS) plays an important role in the loss of dopaminergic nigrostriatal neurons in Parkinson's disease (PD) and that treatment with antioxidants might be neuroprotective. However, most currently available antioxidants cannot readily penetrate the blood brain barrier after systemic administration. We now report that AD4, the novel low molecular weight thiol antioxidant and the *N*-acytel cysteine (NAC) related compound, is capable of penetrating the brain and protects neurons in general and especially dopaminergic cells against various OS-generating neurotoxins in tissue cultures. Moreover, we found that treatment with AD4 markedly decreased the damage of dopaminergic neurons in three experimental models of PD. AD4 suppressed amphetamine-induced rotational behaviour in rats with unilateral 6-OHDA-induced nigral lesion. It attenuated the reduction in striatal dopamine levels in mice treated with 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP). It also reduced the dopaminergic neuronal loss following chronic intrajugular administration of rotenone in rats. Our findings suggest that AD4 is a novel potential new neuroprotective drug that might be effective at slowing down nigral neuronal degeneration and illness progression in patients with PD.

Introduction

The motor manifestations of Parkinson's disease (PD) are due mainly to the progressive loss of dopaminergic neurons in the substantia nigra (SN). It is believed that oxidative stress (OS) plays a crucial role in the pathogenesis of many neurodegenerative diseases, especially PD (Jenner & Olnaw, 1996; Simonian & Coyle, 1996; Gilgun-Sherki *et al*., 2001; Rao & Balachandran, 2002). Indeed, many studies showed that overproduction of reactive oxygen species (ROS) leads to OS enhancement resulting in functional alterations in proteins (Floor & Wetzel, 1998), lipids (Dexter *et al*., 1989) and DNA (Zhang *et al*., 1999). Lipid damage, in turn, leads to loss of membrane integrity and increased permeability to ions such as calcium, which can promote excitotoxicity (Beal, 1998).

Further evidence that OS participates in the loss of nigral neurons in PD comes from studies using the toxins 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) and rotenone that generate ROS *in vitro* and *in vivo* (Sriram *et al*., 1997; Dajas-Bailador *et al*., 1998; Gassen *et al*., 1998; Wong *et al*., 1999; Betarbet *et al*., 2000; Gao *et al*., 2002). 6-OHDA is a neurotoxin that selectively destroys the dopaminergic projections to the striatum originating from the SN (Hudson *et al*., 1993), and was shown to produce OS *in vitro* (e.g. Gassen *et al*., 1998) and in rats *in vivo* (e.g. Dajas-Bailador *et al*., 1998). MPTP is a dopaminergic toxin that causes nigral cell loss and clinical symptoms similar to those of sporadic PD. Its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP+), is taken up into dopaminergic terminals by the plasma-membrane dopamine transporter (Akaneya *et al*., 1995; Przedborski *et al*., 2000). Both MPTP and MPP+ induced toxicity is linked, in part, to OS (Sriram *et al*., 1997; Wong *et al*., 1999). Chronic treatment with rotenone, an inhibitor of mitochondrial complex I activity, was demonstrated to cause selective loss of dopaminergic neurons, associated with cytoplasmic inclusions containing ubiquitin and α-synuclein, and also ATP depletion, oxidative damage, and death (Betarbet *et al*., 2000; Sherer *et al*., 2003a,b).

Based on the above, it would be reasonable to propose that exogenous antioxidants may be effective in diminishing the cumulative damaging effects of OS in PD and thus slow down nigral neuronal degeneration and disease progression. Consequently, various antioxidants have been investigated as a potential treatment for PD, but the therapeutic use of most of these compounds is *a priori* restricted as they do not cross the blood brain barrier (BBB) (reviewed in Gilgun-Sherki *et al*., 2001). We therefore developed a novel low molecular weight thiol compound (AD4) that is able to penetrate the brain following systemic administration and is also partially metabolized to the antioxidant *N*-acetyl cysteine (Atlas *et al*., 1999). AD4 was shown to be a potent free radical scavenger that protects cells against various pro-oxidants and neurotoxins (Offen *et al*., 2004). Furthermore, using AD4 in an animal model for multiple sclerosis, i.e.

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experimental autoimmune encephalomyelitis (EAE), we found that it reduces axonal damage, inflammation and demyelination in the central nervous system (CNS) (Offen *et al.*, 2004). Here, we report that AD4 prevents degeneration of neurons in culture and in several experimental models of PD.

Materials and methods

Drugs

AD4 was synthesized as described by Atlas *et al.* (1999). Glutathione reductase (GRX), GSSG, 5,5-dithiobis 2-nitrobenzoic acid (DTNB), nicotinamide adenine dinucleotide phosphate (NADPH), 2-vinylpyridine (2-VP), triethanolamine (TEA), buthionine sulfoximine (BSO), dopamine, levodopa, 6-hydroxydopamine (6-OHDA) hydrobromide, 1-methyl-4-phenylpyridinium ion (MPP+), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and amphetamine were obtained from Sigma, Israel, and dissolved in phosphate buffered saline (PBS).

The composition of HEPES buffer was 120 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 5 mM $NaHCO_3$, 6 mM glucose, 1 mM $CaCl_2$, and 10 mM HEPES.

Cell cultures

Human neuroblastoma SHSY5Y (NB) cells and rat pheochromocytoma (PC12) cells (ATCC) were maintained in Dulbeco's Modified Eagle's Medium (DMEM), supplemented with 8% fetal calf serum (FCS) and 8% horse serum, penicillin (25 μg/mL), streptomycin (25 μg/mL), and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For protection studies, experimental cells (100 μL of 3×10^5 cells/mL) were subcultured (in 2% FCS serum) to poly L-lysine-coated 96-well microtiter plates (Corning).

Mesencephalic neuron-enriched cultures

Primary rat ventral mesencephalic neuronal cultures were prepared as follows. Ventral mesencephalic tissues were dissected from 13/14-day-old Sprague–Dawley rats after decapitation, and were added to 100% Hanks BSS solution containing 6 g/L of D-glucose. Tissues were then added to 1 mL of Punk's A solution and DNAse 1 : 100, respectively, and dissociated with a mild mechanical trituration. The supernatant was removed after centrifugation at 1000 r.p.m. for 10 min at 4 °C. The pellet was resuspended with a treatment medium containing 25% Hank's BSS, 25% MEM, 5% Nu-serum and 6 g/L glucose. Cells were seeded to 24-well (5×105 per well) culture plates precoated with poly D-lysine (10 μg/mL) in a humidified atmosphere of 5% CO_2 and 95% air.

Cell viability

Cell survival was evaluated by the neutral red assay, a selective staining for lysosomes in living cells (Borefreund & Purner, 1984; Zhang *et al.*, 1990; Ishiyama *et al.*, 1996; Offen *et al.*, 2000). For staining, the medium was washed out and 100 μL of 0.34% neutral red (dissolved 1 : 100 in DCCM-1 medium) were added to wells and incubated for 2 h at 37 °C. The wells were then washed with PBS containing 1 mM $MgCl_2$ and 200 μL Sorenson buffer (0.1 M disodium citrate with 0.1 M HCl in a proportion of 1.6 : 1, respectively, dissolved 1 : 1 in ethanol) were added. Absorption intensity was measured by an ELISA reader (wave length 590 nm). (Borefreund *et al.*, 1984; Zhang *et al.*, 1990; Ishiyama *et al.*, 1996; Offen *et al.*, 2000).

Evaluation of intracellular glutathione levels

Determination of reduced glutathione (GSH) and glutathione disulphide (GSSG) was performed according to Griffith (1980). Briefly, after preparation of the samples from cultured cells, supernatant solutions were taken and equally halved. 2-VP was applied to one half in order to determine GSSG (30 min, 25 °C) and the other half was used to measure total glutathione (30 min, 0 °C). The reaction was terminated by applying a mixture of TEA : H_2O (1 : 1). Samples were then mixed with a working solution, [comprising stock buffer 125 mM Na-phosphate, 6.3 mM Na-EDTA, adjusted to pH 7.5) as follows: (i) 1 mM DTNB; (ii) 1 mM NADPH, and (iii) approximately 1 unit of glutathione reductase per 10 μL], placed on an ELISA reader (wave length 405 nm) for 20 min with a sampling interval of 2 min. A standard curve was generated under conditions similar to those described by Tietze (1969).

Preparation of synaptosomal fractions

Preparation of synaptosomal fractions was performed according to LeBel & Bondy (1990). Briefly, mice were killed by decapitation, the brains excised rapidly on ice, and the cerebellum, cortex, and brainstem dissected out. Each region was put into a microcentrifuge tube, placed at −20 °C for 24 h, and stored at −70 °C until crude synaptosomal preparation. Each region was weighed and homogenized in ten volumes of 0.32 M sucrose. The crude nuclear fraction was removed by centrifugation at $1800 \times \boldsymbol{g}$ for 10 min at 2 °C. The resulting supernatant fraction was centrifuged at 31 500 ***g*** for 10 min to yield the synaptosomal pellet. The pellet was then suspended in HEPES buffer at a concentration of 0.0185 g/mL (Lebel *et al.*, 1990).

Assay for reactive oxygen species

Quantification of the formation rate of ROS was determined as previously described (Lebel *et al.*, 1990). Briefly, synaptosomal fractions were diluted 1 : 10 with 40 mM Tris (pH 7.4) and loaded with 5 μM 2,7-dichlorofluorescein (DCF, in methanol) for 15 min at 37 °C. Following loading, the fluorescence was recorded prior to (initial) and after an additional 1-h (final) incubation. The formation of the fluorescent-oxidized derivatives of DCF was monitored in a cuvette holder (thermostatically maintained at 37 °C) as follows: DCF, excitation wavelength, 488 nm (bandpass 5 nm), and emission wavelength, 525 nm (bandpass 20 nm). DCF formation was quantified from separate standard curves in methanol (0.05–1 μM) (Lebel *et al.*, 1990).

Animals

Male Sprague–Dawley rats (Harlan, Israel) weighing 250–300 g were used for the 6-OHDA-lesion experiment. Two-month-old male Lewis rats (Harlan, Israel), weighing 300–350 g, were used for rotenone study. Eight-week-old 20–25 g female C57/bL mice (Harlan, Israel) were used for the MPTP experiment. All animals were housed in standard conditions; constant temperature (22 ± 1 °C), humidity (relative, 30%), 12-h light : 12-h dark cycle, free access to food and water. Surgical procedures were performed under the supervision of the Animal Care Committee at the Rabin Medical Center and at Tel Aviv University, Tel Aviv, Israel.

Minipump implantation

Pump preparation and implantation was performed as described previously (Betarbet *et al*., 2000) with modifications. Briefly, Alzet osmotic minipumps (2 ML4; Durect, USA) were filled with rotenone to obtain a final delivery of 5 mg/kg/day (calculated according to average body weight), dissolved in equal volumes of DMSO and polyethyleneglycol (PEG). Pumps were attached to a cannula (pe60, Durect, USA) and incubated in sterile 0.9% (w/v) saline at 37 °C for at least 4 h prior to implantation. Rats were anaesthetized by chloral hydrate, 350 mg/kg intraperitoneally (i.p.). Pumps were implanted under the skin on the back of the rats and inserted into the right jugular vein. At the end of operation, rats were allowed to recover in isolated cages and were later returned to their cages. Rats were monitored for behavioural changes, weight loss and overall health.

6-OHDA lesions

Rats were anaesthetized with chloral hydrate, 350 mg/kg i.p., and secured in a stereotaxic frame (Stoelting, USA). Two groups, of nine animals each, were unilaterally injected with 6-OHDA hydrobromide (12 μg in 6 μL saline with 0.01% ascorbate) that was prepared fresh and kept on ice until injection to prevent auto-oxidation. A special drill was used to place a single burr hole at the appropriate site with the following coordinates: posterior 4.8 mm, lateral 1.8 mm, dorsoventral 8.1 mm, with respect to bregma and dura, based on the stereotaxis atlas (Paxinos & Watson, 1986). 6-OHDA (6 μL, 1 μL/min) was injected using a Hamilton 10 μL syringe with a 26-gauge needle. At the completion of the injection, the needle was left in place for another 3-min period and then withdrawn at 1 mm/min in order to prevent a vacuum. The burr hole was cleaned and the skin was closed (Perese *et al*., 1989). One group received AD4 (120 mg/kg, i.p.) 15 min prior to the 6-OHDA injection, while the other group was treated with saline using an identical protocol.

Measurement of rotational behaviour

Lesioned rats were tested for rotational behaviour induced by an intraperitoneal (i.p.) injection of amphetamine (5 mg/kg) 14 days after the 6-OHDA lesion**.** This test is widely used as a reliable index of dopamine depletion in the striatum (Hefti *et al*., 1982; Carman *et al*., 1991; Hudson *et al*., 1993; Thomas *et al*., 1994; Pavon *et al*., 1998). The clockwise turnings of each animal were measured visually, in turn, in a round tool (40-cm diameter) for 2 min every 10-min cycle, for a total duration of 2 h.

MPTP injections

MPTP hydrochloride (25 mg/kg, i.p.) was administered in 0.1 mL of saline once a day for 5 days. Four animals in each group received MPTP or MPTP with 1 g/kg/day of AD4, given via drinking water for 21 days, starting at day one of the MPTP injections. Control mice received saline throughout the experimental period (Dehmer *et al*., 2000).

Measurement of striatal dopamine content

High-performance liquid chromatography (HPLC) with electrochemical detection was used to measure dopamine. Briefly, three weeks after the last injection of MPTP, mice were killed by decapitation. Brains were rapidly removed and the left and right striata were dissected out freehand on an ice-cold plate, immediately frozen on dry ice, and stored at −80 °C until analysis. On the day of the assay, each sample was weighed, and then homogenized on ice in 50 volumes of 0.2 M perchloric acid. After centrifugation (10 000 × ***g*** for 15 min at 4 °C), the supernatants were filtered through a nylon syringe filter (Gelman ACRO LC3A, 0.45 μm). An aliquot of filtrate was injected into the HPLC system (Waters, Milford, MA, USA) equipped with a C18 reverse phase, 3-μ LUNA column (100 mm × 2 mm, Phenomenex, Torrance, CA, USA). The sample was eluted by a mobile phase made of 25 mM NaH_2PO_4, 50 mM Na-citrate, 0.03 mM EDTA, 10 mM diethylamine HCl and 2.2 mM sodium octyl sulphate (pH 3.2), 30 mL/L methanol and 22 mL/L dimethylacetamide at a flow rate of 0.4 mL/min. DA peak was determined by electrochemical detection at a potential of 0.6 V. The DA content in the sample was calculated by extrapolating the peak area from a standard curve (range 1–200 pg of DA) constructed under the same conditions during each run by the Maxima Workstation (Waters) (Petroske *et al*., 2001).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Jackson-Lewis & Liberatore, 2000) with modification. Briefly, at the end of treatment, rats (four per group) were anaesthetized with chloral hydrate (350 mg/kg), then perfused transcardially with 50 mL of normal saline followed by 150 mL of 4% paraformaldehyde in 0.1 M sodium-phosphate buffer (pH 7.1). Brains were quickly removed, fixed for 72 h at 4 °C and cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 3 days at 4 °C. The brains were frozen by immersion in dry ice-cooled 2-methylbutane and stored at −70 °C until sectioned. For each rat, cryostat-cut sections (25 μm) throughout the entire ventral midbrain were collected free floating and adjacent sections were stained for tyrosine hydroxylase (TH). Briefly, sections, fixed with 100% methanol then 100% acetone for 2 min, were first rinsed (3 × 5 min) with 0.1 M PBS (pH 7.4), then immersed in a solution of 3% H_2O_2−10% methanol for 5 min, followed by incubation with 5% normal goat serum (NGS) for 60 min. Sections were then incubated on a shaker with the primary antibody, rat anti-TH (1 : 1000, v/v; Calbiochem) in 0.1 M PBS, pH 7.4, containing 2% NGS and 0.3% Triton X-100, for 48 h at 4 °C. After rinsing in PBS, biotinylated secondary goat anti-rabbit IgG (1 : 200, v/v; Vector, Burlington, CA, USA) in 0.1 M PBS, pH 7.4, containing 2% NGS was added and the sections were incubated for 60 min at room temperature followed by incubation in avidin–biotin peroxidase complex (Vector) for 60 min. Visualization was performed by incubation in 3,3−diaminobenzidine–glucose–glucose oxidase for 10 min. All sections were then washed for 3 × 5 min in PBS, mounted on 0.1% gelatin-coated slides, dried, dehydrated in graded ethanol, cleared in xylene, and coverslipped.

Microscopes

Cells cultures were analysed using an Olympus IX70-S8F2 microscope (Olympus, Tokyo, Japan). An Olympus BX52TF microscope was used to analyse slides for histopathology. ViewfinderLite™ software, with a DP50 microscope digital camera system attached to the microscopes, was used to acquire images and the StudioLite™ software was used to edit and analyse images recorded (Olympus, Tokyo, Japan).

Image analysis technique

Image analysis was performed on four representative areas of each well using the Image Pro-Plus software (Media Cybernetics, USA).

The analysis of brain slices was used to quantify the entire area of the SNpc, was photographed in a series of nine frames (a total of 45 pictures for each brain). They were assessed by two examiners, double blind.

Isolation and enzymatic activity assays of brain mitochondria

At the end of experiment, rats were killed in CO_2. Brains were rapidly dissected out and plunged in ice-cold buffer containing 120 mM HEPES, 250 mM sucrose, 1 mM EDTA, 10 mM KCL. The rinsed brain samples were homogenized into the same buffer for 1 min. The homogenates were then centrifuged at $1000 \times \boldsymbol{g}$ for 10 min at 4 °C and samples were taken for protein determination. The subsequent supernatant was centrifuged at $14\,000 \times \boldsymbol{g}$ for 10 min followed by two washings of the mitochondrial pellet in a large volume of the above-mentioned buffer (JA-20 motor and J2-HC centrifuge, Beckman). The final mitochondria pellet was then suspended in the medium and used to determine enzymatic activity. Mitochondrial protein concentrations were estimated according to the BCA protein assay kit (Pierce, Rockford, IL, USA).

The activities of complex I (NADH ubiquinone oxidoreductase) and complex IV (cytochrome *c* oxidase) were measured according to Birch-Machin & Turnbull (2001). Citrate synthase activity was measured according to Shepherd & Garland (1969). All enzyme activities were expressed as nanomoles per minute per milligram of protein, except for cytochrome *c* oxidase, which was expressed as the first-order rate constant (k/min/mg of protein).

Statistical analysis

All data presented as means ± standard error of the mean (SEM). Significance of the differences between the rotational behavioural data following amphetamine administration was analysed by the two-tail Student's *t*-test (Fig. 6). The one-way ANOVA (SPSS, version 11.5) was used in order to analyse the data presented in Figs 1–5 and 7–9, with Scheffe *posthoc* tests for multiple comparisons in each case. All the *in vitro* experiments were performed at least twice, in triplicate, and a representative figure is shown. For the *in vivo* experiments at least two experiments were performed with four animals in each group as a minimum. In all tests, significance was assigned when $P < 0.05$.

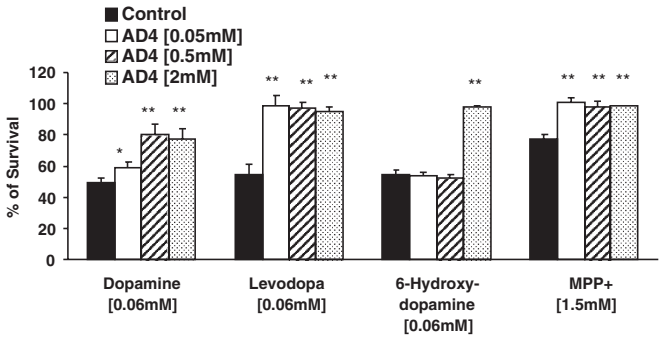

FIG. 1. AD4 (0.05–2 mM) protects neuroblastoma cells against toxicity of levodopa, dopamine, 6-OHDA (0.06 mM) and MPTP (1.5 mM). Cell survival was estimated by neutral red assay. Results are expressed as percentage of the control (vehicle) cells, mean ± SEM of triplicate wells. *$P < 0.05$, compared to neurotoxin treatment; **$P < 0.01$, compared to control.

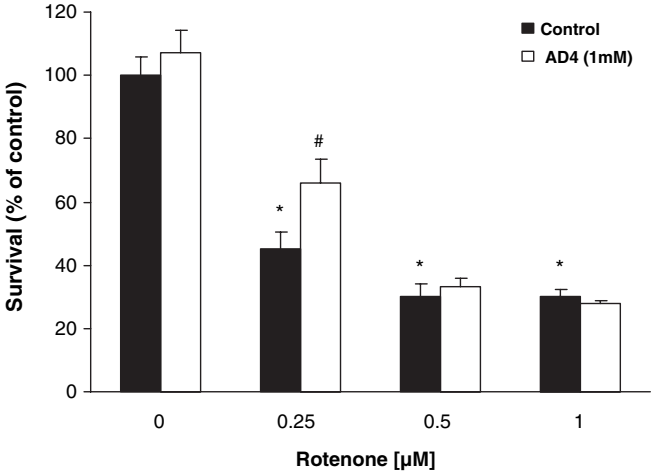

FIG. 2. AD4 protects PC12 cells from rotenone toxicity. PC12 cells were treated for 24 h with vehicle or rotenone (0.25–1 μM). AD4 (1 mM) was added to cells 5 h before rotenone. Cell survival was estimated by neutral red assay. Results are expressed as percentage of the control (vehicle) cells, mean ± SEM of triplicate wells. *$P < 0.05$, compared to control; #$P < 0.05$ compared to untreated (no rotenone).

AD4 treatments

In the 6-OHDA model, AD4, dissolved in saline, was injected i.p. (120 mg/kg), 15 min before 6-OHDA injection. In the MPTP model, AD4 was given via the drinking water (1 g/kg/day) for the whole period (21 days). In the rotenone model, AD4 was also administered in the drinking water (100 mg/kg/day) for the duration of the experiment (28 days) water intake of each rat was approximately (50 mL/day).

Results

Protection against oxidation by AD4 in vitro

In order to examine the ability of AD4 to neutralize free radicals, we performed a simple experiment that measured the prevention of dopamine oxidation *in vitro*. When suspended in a cell-free DMEM medium (without serum) dopamine (100 mM) is oxidized and polymerized in several hours to form the black pigment of oxidized dopamine (neuromelanin). However, in the presence of AD4 (2 mM), oxidation was delayed and the level of polymerization, as indicated by absorption at 960 nm, was reduced (data not shown).

We further investigated AD4 efficacy to reduce the neurotoxic effects of ROS-producing agents such as levodopa, dopamine, 6-OHDA and MPP+, known to be toxic to dopaminergic neurons in cell culture. As shown in Fig. 1, survival of neuroblastoma cells exposed to levodopa (0.06 mM), dopamine (0.06 mM), 6-OHDA (0.06 mM) or MPP+ (1.5 mM) was reduced by 50% ($P < 0.01$). However, cotreatment with AD4 (0.05–2 mM) raised the survival rates by 20–50% ($P < 0.05$) in a dose-dependent manner, as determined by the neutral red method (Fig. 1).

AD4 (1 mM) was added to PC12 cells (2×10^4 per well) 5 h prior to 24 h exposure to rotenone, a complex I inhibitor that is selectively toxic to dopaminergic cells. As shown in Fig. 2, rotenone (0.25–1 μM) reduced cell survival, as indicated by neutral red, to 45 ± 5% and 30 ± 2% ($P < 0.05$) compared to cells treated with vehicle (DMSO) alone. AD4 treatment significantly increased survival to 66 ± 7% in cells treated with 0.25 μM rotenone ($P < 0.05$, compared to rotenone treatment). Similar results were obtained with higher concentrations of

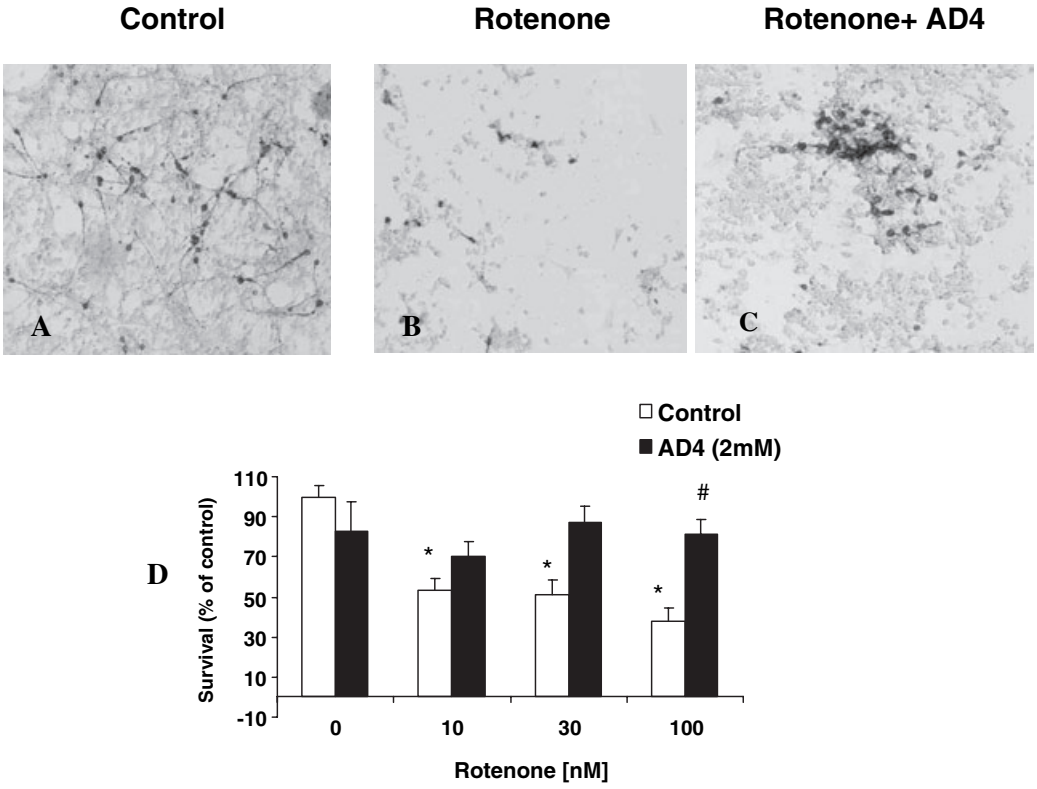

FIG. 3. AD4 pretreatment protects primary dopaminergic cultures against rotenone toxicity. Primary dopaminergic cultures were prepared from rat embryos (day 13/14). (A) Control cultures were immunoassayed with anti-TH. (B) Cultures treated with rotenone (100 nM) for 2 days. (C) Cultures pretreated with AD4 (2 mM) one hour before rotenone administration. (D) Percentage of the TH-positive stained cells compared to the control cultures. The results are presented as mean ± SEM of triplicate wells. *$P < 0.001$, compared to the control; #$P < 0.006$ compared to rotenone treatment.

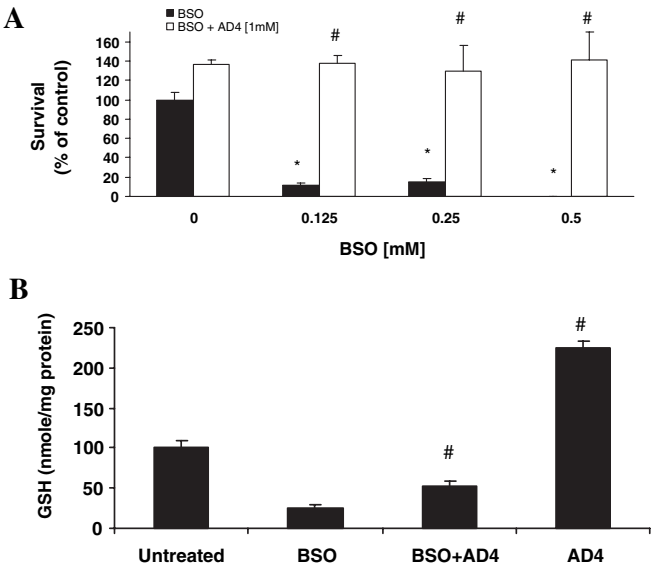

FIG. 4. AD4 (1 mM) raises the survival and GSH levels in control and BSO-treated (0.125–0.5 mM) neuroblastoma cells. The survival of AD4- and BSO-treated cells was measured by the neutral red method (A). GSH was measured according to Tietze (1969) (see Materials and methods) (B). Results are expressed as percentage of the control cells, mean ± SEM of triplicate wells. *$P < 0.01$, compared to control; #$P < 0.01$ compared to BSO treatment.

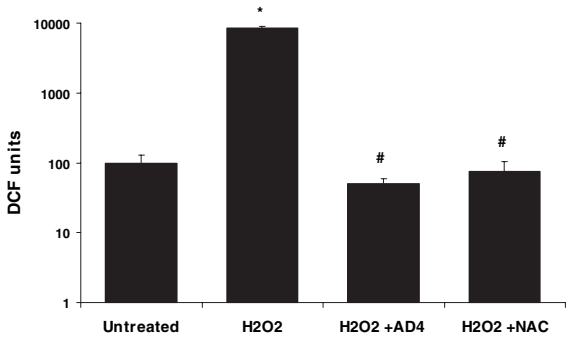

FIG. 5. *N*-acetyl cysteine (NAC) and AD4 2 mM, inhibits the hydrogen peroxide (10 mM)-induced ROS generation in mouse brain synaptosomes. ROS generation was measured by DCF accumulation (see Materials and methods). Results are expressed as arbitrary units, mean ± SEM of triplicate wells. *$P < 0.01$, compared to control; #$P < 0.01$ compared to BSO treatment.

AD4. However, concentrations higher than 10 mM had toxic effects (data not shown), similar to other thiol-containing substances (Offen *et al*., 1996). In addition, higher concentrations of rotenone (0.5 μM and 1 μM), were highly toxic and therefore AD4 was not effective in improving cell survival (data not shown).

The protection of AD4 against rotenone was also examined in dopaminergic cultures. Cultures were treated with AD4 (2 mM) 1 h

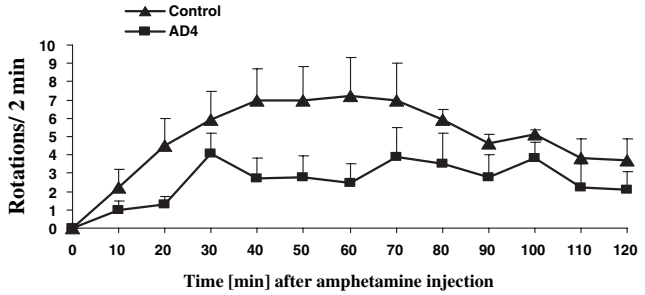

FIG. 6. AD4 (120 mg/kg, i.p.) administration to rats, 15 min before the unilateral injection of 6-OHDA, markedly reduced amphetamine-induced rotations. The rotations were measured for 2 h in a round tool (Control, $n = 12$; AD4, $n = 9$; $P < 0.001$).

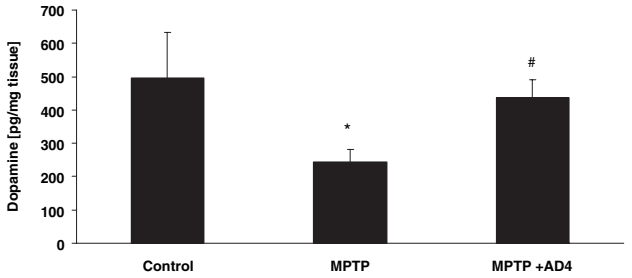

FIG. 7. AD4 protects against MPTP–induced toxicity in C57/bL mice ($n = 10$ in each group). Dopamine content in the striatum of MPTP (25 mg/kg/day, i.p. once a day daily for 5 days) treated with AD4 (via drinking water, 1 g/kg/day for all study period), was measured by HPLC. *$P < 0.05$, compared to control; #$P < 0.05$ compared to BSO treatment.

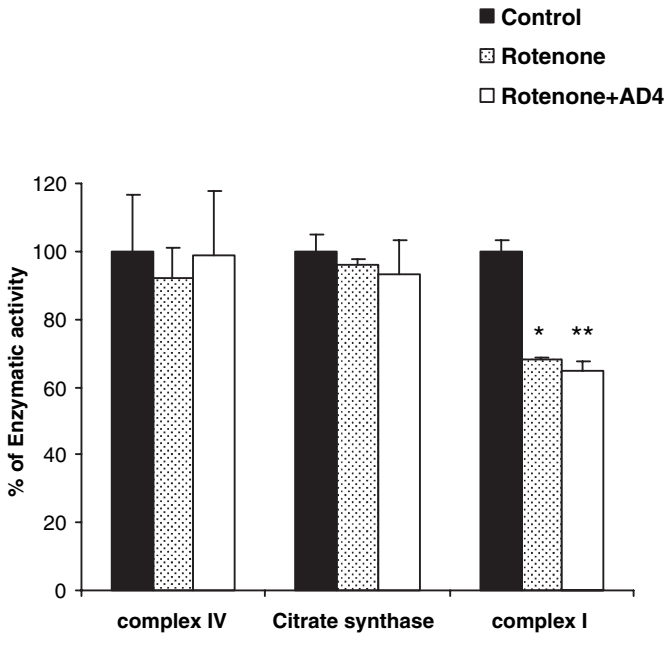

FIG. 8. Chronic rotenone treatment selectively reduces complex I (NADH-ubiquinone oxidoreductase) activity in rat brains. Rats were treated with rotenone (5 mg/kg/day) for 28 days or vehicle (PEG: DMSO). AD4 (100 mg/kg/day) was added in drinking water for the duration of the study period. Brain mitochondria were isolated and the activity of complex I (NADH-ubiquinone oxidoreductase), complex IV (cytochrome *c* oxidoreductase) and citrate synthase was assayed (see Materials and methods). Results are presented as mean ± SEM ($n = 4$, in each group). *$P < 0.008$, compared to control and **$P < 0.005$ compared to rotenone treatment.

before rotenone treatment (10–100 nM) for two days. At the end of experiment, cultures were immunostained with anti-TH. As shown in Fig. 3B, the number of TH immunoreactive (IR) neurons was reduced by rotenone (100 nM) treatment as compared to controls (Fig. 3A). However, the number of TH-IR neurons was preserved in cultures treated with AD4 (2 mM) (Fig. 3C). Quantification of the stained neurons revealed that rotenone reduced the number of TH-IR neurons in a dose-dependent manner (Fig. 3D). Treatment with rotenone (10 nM) reduced the number of TH-IR neurons to 53 ± 6% ($P < 0.001$, compared to control). Although AD4 pretreatment increased cell survival to 70 ± 7% it was not statistically significant ($P = 0.1$). The number of TH-IR neurons treated with rotenone (30–100 nM) was further reduced to 51 ± 7 and 38 ± 6, respectively ($P < 0.001$, compared to control). Nevertheless, AD4 pretreatment significantly improved the survival of TH-IR cells to 87 ± 7% and 81 ± 7%, respectively ($P < 0.006$, compared to rotenone alone).

NAC is known to promote the synthesis of reduced glutathione (GSH), a key factor in the cellular redox balance. To study whether AD4 might enhance cellular GSH levels, we treated neuroblastoma cells for 48 h with BSO, a selective inhibitor of glutathione synthesis. As shown in Fig. 4A, BSO (0.125–0.5 mM) reduced cell survival rates by 80–100% ($P < 0.01$). In addition, BSO treatment (0.25 mM) reduced GSH levels by 75% ($P < 0.01$, Fig. 4B). Co-treatment with AD4 elevated the survival rate of BSO-treated cells by more than 100% (Fig. 4A) and raised their GSH levels by 25% ($P < 0.01$, Fig. 4B). Interestingly, AD4 markedly increased GSH levels by 2.5-fold, in naive nontreated cells (Fig. 4B) suggesting that it could be a precursor of GSH.

Addition of AD4 was also effective against general pro-oxidants such as hydrogen peroxide. Exposure of mouse brain synaptosomes to hydrogen peroxide (10 mM) resulted in elevations of ROS generation, as measured by DCF accumulation. Pretreatment with the antioxidants NAC (2 mM) or AD4 (2 mM), reduced the hydrogen peroxide-induced ROS generation down to control levels ($P < 0.01$, Fig. 5).

Protective effects of AD4 in vivo

We explored the protective effects of AD4 *in vivo*, looking for neuroprotection in three animal models of PD. In the first model, we used 6-OHDA-lesioned rats manifesting amphetamine-induced rotational behaviour. Rotation intensity is directly proportional to the extent of the unilateral 6-OHDA nigral lesion (Hudson *et al*., 1993). Administration of amphetamine (5 mg/kg, i.p.) to 6-OHDA-lesioned rats ($n = 12$), elevated the rotation rate towards the lesion site (320 ± 30 for 2 h). In contrast, AD4 injection (120 mg/kg, i.p.) 15 min before the 6-OHDA lesion ($n = 9$), markedly reduced the amphetamine-induced rotations for 2 h by 49%; $P < 0.001$, Fig. 6).

In a second animal model for PD, MPTP (25 mg/kg/day, i.p., once daily for 5 days) was administered to C57/bL mice and the striatal dopamine levels were measured using HPLC-EC. We found that MPTP treatment reduced dopamine content in the striatum by 51% ($P < 0.05$), while in animals cotreated with AD4 via drinking water (1 g/kg/day), the striatal dopamine levels remained similar to control (Fig. 7).

In a third PD model, rats were treated chronically with rotenone (5 mg/kg/day for 28 days) administered through the jugular vein by a minipump (see Materials and methods). AD4 was given via drinking water (100 mg/kg/day) for the duration of the experiment. At the end of treatment, striatal mitochondrial enzymes were determined and nigral dopaminergic neurons were immunostained and counted. As shown in Fig. 8, complex I activity in brain extracts, was reduced by 30% ± 1% in rats chronically treated with rotenone as compared to

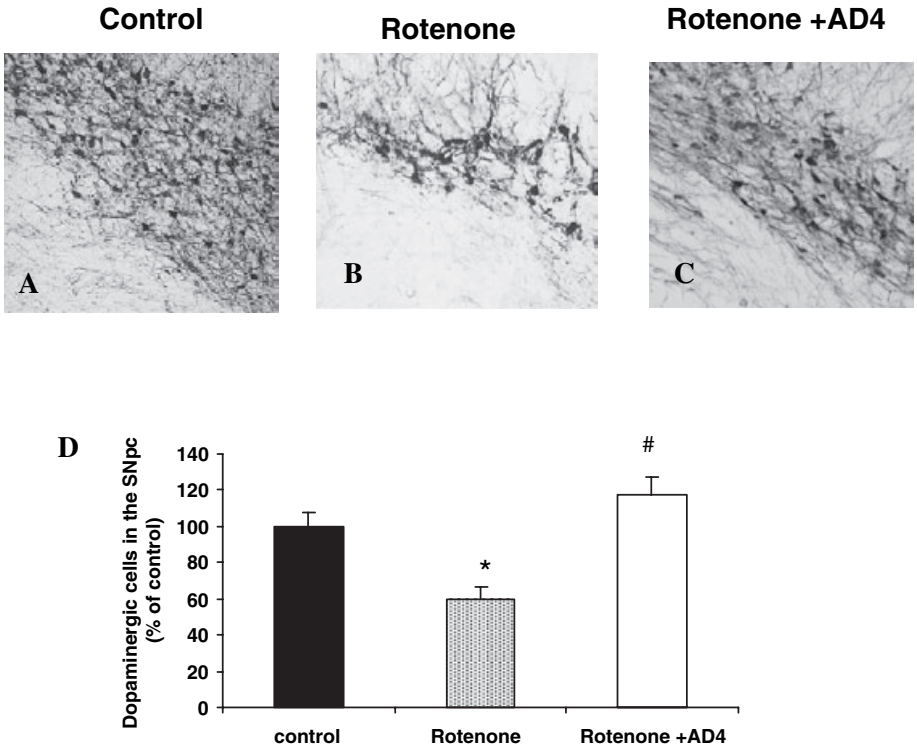

FIG. 9. AD4 increases the survival of dopaminergic neurons in the SN of rats chronically treated with rotenone. Rats ($n = 4$, in each group) were treated for 28 days with rotenone (5 mg/kg/day) alone or with AD4 (100 mg/kg/day) in drinking water for all study period. Dopaminergic cells in the SN were immunostained with anti-TH in the control rats (A), in rats treated with rotenone alone (B) or in rats treated with rotenone and AD4 (C). TH-positive neurons results are expressed as percentage of the control, mean ± SEM (D). *$P < 0.02$, compared to control, #$P < 0.003$ compared to rotenone treatment. The sections were processed in the same experiment, in which the enzymatic activity was evaluated, and normalized to untreated rats.

controls (vehicle PEG/DMSO) ($P < 0.008$). Rats that were chronically treated with rotenone and AD4 (100 mg/kg/day), showed a reduction in complex I activity (35 ± 3%, $P < 0.005$) without any reduction in the activities of citrate synthase or complex IV (cytochrome *c* oxidoreductase) after rotenone treatment, alone or with AD4. In addition, AD4 did not alter rat survival or weight loss following rotenone exposure (data not shown).

TH immunostaining of dopaminergic neurons in the SN of rotenone-treated rats demonstrated a marked reduction compared to control rats (Fig. 9A and B). However, in rats orally treated with AD4 (100 mg/kg/day), the number of TH-IR neurons remained unchanged (Fig. 9C). Quantification of the TH-IR neurons in the SN revealed that rotenone reduced the number of TH-IR neurons by 40 ± 8% ($P < 0.02$, compared to control) while co-administration with AD4, completely abolished the neuronal loss induced by rotenone (117 ± 10%, $P < 0.003$ compared to rotenone) similar to control ($P < 0.3$, Fig. 9D).

Discussion

Our study shows that AD4, our novel BBB-crossing antioxidant, protected against the toxicity of ROS-producing agents such as 6-OHDA, dopamine, levodopa, MPP+ and rotenone in neuronal and mescenphalic dopaminergic cell cultures. AD4 raised both the survival and GSH levels in BSO-treated neuroblastoma cells, indicating that it can also act as a precursor of the natural intracellular antioxidant, GSH. It neutralized the auto-oxidation of dopamine to dopamine-melanin and ROS production in mouse brain synaptosomes exposed to H_2O_2, a general pro-oxidant. Our *in vivo* studies showed that pretreatment with AD4 reduced damage to dopaminergic neurons in three different animal models of PD. 6-OHDA, injected unilaterally into the SN, enters the cell via the dopamine transporter and produces free radicals such as nitric oxide that impair mitochondrial respiration (Tolwani *et al*., 1999). The induced oxidative stress eventually leads to dopaminergic cell damage in the nigrostriatal pathway, resulting in the amphetamine-induced rotational behaviour, measured post lesion. Although this model does not mimic the slow and chronic progression of Parkinson disease, it is a useful *in vivo* method to analyse the survival of the dopaminergic cells and terminals. Many studies have previously shown that behaviour analysis correlates with the number of TH-immunoreactive cells remaining in the SN. Our findings indicate that AD4 can rescue the dopaminergic cells and their terminals. MPTP in humans causes a severe, irreversible syndrome that very closely resembles idiopathic PD. The mouse MPTP model is also highly relevant as it replicates some of the clinical features as well as some of the biochemical hallmarks of the disease. The MPTP toxic metabolite MPP+ also enters the dopaminergic nerve terminals via the dopamine transporter and induces an acute dopaminergic cell loss, probably at least in part, by the generation of oxidative stress (Schober, 2004). As reported, damage is reflected in reduction of striatal dopamine levels caused by injury and axonopathy of the dopaminergic terminals in the MPTP model. Furthermore, the loss of dopamine content in the terminal is not necessarily associated with a reduction in the number of dopaminergic neurons in the SN and thus their measurement provides an evaluation of only the striatal dopamine content performed by HPLC analysis. The fact that AD4 prevented the reduction in the striatal dopamine content is indicative of its neuroprotective property. Our efforts were invested mainly in the rotenone model of PD for the

following reasons. Firstly, it was shown that chronic, systemic exposure to the pesticide through jugular vein cannulation in rats reproduced many features of PD (Betarbet *et al*., 2000). Secondly, chronic treatment and slow disease progression mimics the natural history of the disease in humans and might be more relevant to PD pathophysiology. Thirdly, in contrast to MPTP and 6-OHDA, which are artificial substances absent from the body and the environment, rotenone is a common environmental toxin. This fact suggests the relevance of this model to the natural pathogenesis of PD in humans. The clear-cut results obtained in our *in vitro* and *in vivo* studies including the examination of over 100 slides (per group), demonstrated that AD4 prevented the chronic rotenone-induced neurodegeneration.

Nigral cells are particularly exposed to OS due to dopamine metabolism, which gives rise to the accumulation of reactive oxygen species (ROS) and production of various oxidation products that may polymerize to form quinones and neuromelanin, which may also be toxic (Jenner, 1998; Offen *et al*., 1999). We have shown that dopamine (Ziv *et al*., 1994; Offen *et al*., 1995) and also its precursor levodopa, the most common therapy for PD (Ziv *et al*., 1997; Mytilineou *et al*., 2003), are toxic to various cell cultures, causing OS-induced apoptosis, although they are nontoxic *in vivo* (Melamed *et al*., 2000; Mytilineou *et al*., 2003). The concept that OS is pathogenetically involved is supported by postmortem studies showing that in SN of PD patients, there are increased levels of lipid peroxidation products (Dexter *et al*., 1989), transition metal iron (Sofic *et al*., 1991), oxidized glutathione (GSSG) and decreases in reduced glutathione (GSH) (Damier *et al*., 1993; Sian *et al*., 1994; Jenner & Olanow, 1998; Fitzmaurice *et al*., 2003).

As OS has been implicated in the pathogenesis of neurodegenerative diseases and nigral degeneration in PD (Fahn & Cohen, 1992; Jenner, 1998), it seems plausible to suggest that treatment with exogenous antioxidants might neutralize and diminish its deleterious effects. Indeed, several antioxidants e.g. vitamins E and C, melatonin and alpha-lipoic acid, have already been used in the 6-OHDA and MPTP experimental models of PD and also in PD patients. However, their therapeutic efficacy was minimal, probably limited by their capacity to cross the BBB (reviewed in Gilgun-Sherki *et al*., 2001). To improve penetration into the BBB, we synthesized a series of low molecular weight BBB-penetrating thiol antioxidants (Atlas *et al*., 1999). AD4, as the lead molecule, was recently shown to chelate Cu^{2+} and scavenge ROS in red blood cells. In addition, it significantly inhibited JNK and p38 mitogen-activated protein kinase phosphorylation concurrently with the reduction in ROS levels (Offen *et al*., 2004). Furthermore, it suppressed EAE, the animal model of multiple sclerosis (Offen *et al*., 2004).

Thiol antioxidants such as GSH are scavengers of many ROS species (Aruoma *et al*., 1988) and may interact directly with cysteine residues in proteins to ensure their maintenance in the reduced state that is required for preservation of protein structure and activity. Indeed, we have shown that thiol-containing compounds such as NAC and GSH inhibited apoptosis of PC12 cells caused by various neurotoxins (Offen *et al*., 1996). GSH is the most abundant intracellular nonprotein thiol compound in mammalian cells (Sies, 1999), and it plays a crucial role as the scavenger of toxic free radicals and detoxification of xenobiotics. Other functions of GSH include maintenance of thiol redox potential in cells by reducing the thiol groups of proteins, transport and storage of cysteine and also by acting as a cofactor in certain isomerization reactions (reviewed in Meister & Anderson, 1983; Dringen *et al*., 2000; Schultz *et al*., 2000). Indeed, GSH depletion by BSO, an inhibitor of gamma-glutamyl-cysteine synthase, the rate limiting enzyme in GSH synthesis, can cause cell damage and enhances dopamine toxicity (Offen *et al*., 1996). In addition, alternations in glutathione metabolism is involved in a variety of disease states such as protein energy malnutrition (PEM), seizures, haemoglobinopathies (reviewed in Reid & Jahoor, 2001), and also in neurodegenerative disorders such as PD, Alzheimer's disease and amyotrophic lateral sclerosis (reviewed in Bains & Shaw, 1997). Additionally, postmortem studies observed significant (> 60%) reductions in GSH levels in the SNpc of PD patients stressing the importance of glutathione for dopaminergic cell survival. Therefore, administration of glutathione directly or indirectly, e.g. via a precursor of GSH, such as NAC (Martinez *et al*., 1999; Banaclocha, 2001), might be effective in diminishing the cumulative destructive effect of OS. Indeed, in a small, open-labelled clinical study, GSH was shown to reduce patient's disability with early, untreated PD (Sechi *et al*., 1996). Although these results show the efficacy of GSH, the study sample was small and uncontrolled, and the penetration of GSH into the brain was not monitored.

As AD4 is converted to GSH, after it penetrates the brain it might counteract cellular degeneration through several mechanisms. Firstly, it can act by raising the intracellular concentration of cysteine, an antioxidant and a precursor for GSH biosynthesis (Aruoma *et al*., 1989; Ruffmann & Wendel, 1991; Offen *et al*., 1996; Deneke, 2000). Secondly, it stimulates cytosolic enzyme activities involved in the glutathione cycle, such as glutathione reductase, which enhances the rate of glutathione regeneration, and replenishes other soluble and protein thiol-groups (Aruoma *et al*., 1988). In addition, AD4 protects, similar to NAC, by inhibition of ROS-induced apoptotic cell death (Sandstrom *et al*., 1994). Other mechanisms of action might include protection of the mitochondrial respiratory chain proteins from oxidative damage and preservation of mitochondrial bioenergetic capacity. Our data indicated that increase of GSH levels by only 25% caused dramatic effect in cell survival (see Fig. 4). This nondirect correlation might be explained by the powerful protective effect of GSH against ROS-induced apoptosis and its protection against OS-induced mitochondrial damage (Offen *et al*., 1996; Offen *et al*., 2004).

In our *in vitro* experiments, low doses of AD4 appeared to be less protective against 6-OHDA, a neurotoxin that selectively destroys the dopaminergic projections to the striatum (Hudson *et al*., 1993; see Fig. 1). This can be explained by the high toxicity of 6-OHDA compared to other neurotoxins such as dopamine and levodopa (Offen *et al*., 2000; Kim *et al*., 2001). In our *in vivo* experiment we examined whether AD4 had a neuroprotective effect in the 6-OHDA lesion rat model of PD. We found that AD4 markedly reduced the amphetamine-induced rotations when it was given before the 6-OHDA injection (see Fig. 6). As it was shown in many studies, there is high correlation between the intensity of the rotations and the number of dopaminergic cells in the SN and the dopamine levels in the striatum (Hefti *et al*., 1982; Carman *et al*., 1991; Hudson *et al*., 1993; Thomas *et al*., 1994; Pavon *et al*., 1998). Thus, the fact that AD4 inhibited the rotation behaviour probably correlates with dopamine levels and dopaminergic cell survival.

We found that AD4 (1 mM) was highly protective against the toxicity of rotenone at 0.25 μM, but not at higher doses (0.5 μM and 1 μM). Thus, we can speculate that AD4 protects the cells against free radical production induced by mild complex I inhibition, caused by low doses of rotenone, but not against high doses that cause a complete complex I inhibition and bioenergetic defect (Sherer *et al*., 2003a,b). The higher toxicity of rotenone in primary dopaminergic cultures as compared to PC12 cells, shown in our study, may be explained by the presence of microglia in the culture known to markedly increase the susceptibility to rotenone (Gao *et al*., 2002). *In vivo*, AD4's protective effect was examined in a

model of PD developed by Betarbet *et al*. (2000) where rotenone was administered chronically to rats via the jugular vein. Using this model, we found that rotenone (5 mg/kg) selectively reduced brain complex I (NADH ubiquinone oxidoreductase) activity but did not induce any change in complex IV (cytochrome *c* oxidoreductase) and citrate synthase activities, while lower doses of rotenone (< 5 mg/kg), as reported by other studies (Betarbet *et al*., 2000; Sherer *et al*., 2003a,b), were not effective in reducing brain complex I activity.

According to Greenamyre *et al*., (2001), modest complex I impairment (25–50% decrease in activity) may not have any direct impact on mitochondrial ATP levels or $\Delta\Psi M$. However, partial inhibition of complex I activity as a result of interference with electron transfer, markedly increases the production of H_2O_2. Our measurements of the mitochondrial oxygen consumption of brain extracts in rotenone-treated rats revealed that, given in doses that caused complex I reduction, rotenone did not inhibit respiration and probably did not impair ATP levels (data not shown). Thus, rotenone toxicity, seen in the dopaminergic neurons, is probably due to the generated free radicals as a result of complex I inhibition. Indeed, data presented in Fig. 8 indicate that AD4 does not neutralize the rotenone-induced reduction of complex I activity. However, AD4 treatment did reduce rotenone's toxic effect, as shown in Fig. 9D. The fact that AD4 treatment did not change complex I inhibition caused by rotenone, but still protected the cells against the free radicals production induced by this inhibition, emphasizes the potential of AD4 as a neuroprotective antioxidant.

In addition to a loss of dopaminergic neurons caused by rotenone, Betarbet *et al*. (2000) reported on cytoplasmic inclusions that contain ubiquitin and α-synuclein, hypokinetic and unsteady movements. In our study, we also evaluated motor activity in rats treated chronically with rotenone. However, behavioural tests such as open-field and rotor-rod did not show any significant differences between the rotenone treated and control groups (data not shown).

In conclusion, we have demonstrated that AD4, a novel thiol and BBB-penetrating antioxidant, prevented dopaminergic damage caused *in vitro* and *in vivo* by 6-OHDA, MPTP and the complex I inhibitor, rotenone. The advantage of AD4 is its ability to penetrate the brain and to function within the CNS. We believe AD4 may be evaluated as a novel neuroprotective agent for preventing PD progression.

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Abbreviations

BBB, blood brain barrier; BSO, buthionine sulfoximine; DCF, 2,7-dichlorofluorescein; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine; MPP+, 1-methyl-4-phenylpyridinium ion; NAC, *N*-acytel cysteine; OS, oxidative stress; PD, Parkinson's disease; PBS, phosphate buffered saline; ROS, reactive oxygen species; SN, substantia nigra.

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