

MPP⁺-induced cytotoxicity in neuroblastoma cells: Antagonism and reversal by guanosine

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Abstract Guanosine exerts neuroprotective effects in the central nervous system. Apoptosis, a morphological form of programmed cell death, is implicated in the pathophysiology of Parkinson's disease (PD). MPP⁺, a dopaminergic neurotoxin, produces in vivo and in vitro cellular changes characteristic of PD, such as cytotoxicity, resulting in apoptosis. Undifferentiated human SH-SY5Y neuroblastoma cells had been used as an in vitro model of Parkinson's disease. We investigated if extracellular guanosine affected MPP⁺-induced cytotoxicity and examined the molecular mechanisms mediating its effects. Exposure of neuroblastoma cells to MPP⁺ (10 μM–5 mM for 24–72 h) induced DNA fragmentation in a time-dependent manner ($p < 0.05$). Administration of guanosine (100 μM) *before, concomitantly with* or, importantly, *after* the addition of MPP⁺ abolished MPP⁺-induced DNA fragmentation. Addition of MPP⁺ (500 μM) to cells increased caspase-3 activity over 72 h ($p < 0.05$), and this was abolished by pre- or co-treatment

with guanosine. Exposure of cells to pertussis toxin prior to MPP⁺ eliminated the anti-apoptotic effect of guanosine, indicating that this effect is dependent on a Gi protein-coupled receptor, most likely the putative guanosine receptor. The protection by guanosine was also abolished by the selective inhibitor of the enzyme PI-3-K/Akt/PKB (LY294002), confirming that this pathway plays a decisive role in this effect of guanosine. Neither MPP⁺ nor guanosine had any significant effect on α-synuclein expression. Thus, guanosine antagonizes and reverses MPP⁺-induced cytotoxicity of neuroblastoma cells via activation of the cell survival pathway, PI-3-K/Akt/PKB. Our results suggest that guanosine may be an effective pharmacological intervention in PD.

Keywords Apoptosis · Caspase-3 · Cell survival · Cytotoxicity · DNA fragmentation · Guanosine · 1-methyl-4-phenyl-pyridinium (MPP⁺) · Parkinson's disease · SH-SY5Y human neuroblastoma cells

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Abbreviations

ABTS	2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]
AIF	apoptosis-inducing factor
AO-EB	acridine orange-ethidium bromide
BCA	bicinchoninic acid
Caspases	cysteine-dependent aspartyl proteases
CHAPS	3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid
CREB	cAMP response element binding protein
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum

FOXO	Forkhead box transcription factor, class O
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LY294002	[2-(4-morpholinyl)-8-phenyl-1(4 <i>H</i>)-benzopyran-4-1-hydrochloride]
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase
MPP ⁺	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF- κ B	nuclear factor - κ B
PD	Parkinson's disease
PD98059	[2-(2-amino-3-methoxyphenyl)-4 <i>H</i> -1-benzopyran-4-1]
PI-3-K	phosphatidylinositol-3-OH kinase
PKB	protein kinase B
PTX	pertussis toxin
ROS	reactive oxygen species

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused primarily by selective degeneration of dopaminergic neurons in the substantia nigra pars compacta [1, 2]. Apoptosis, a morphological form of programmed cell death [3], has been implicated in the pathophysiology of PD [4–6]. The biochemical pathways that mediate apoptosis may be initiated by extrinsic (e.g. activation of the death receptors) or intrinsic factors (e.g. mitochondrial insult) [7] and result in the activation of a set of cysteine-dependent aspartyl proteases (caspases) [8]. Both apoptotic pathways stimulate the effector enzyme, caspase-3 [7, 9, 10], which in turn activates a specific nuclear DNase that degrades DNA into 200 base pair oligonucleosomal fragments, a hallmark of apoptosis [11, 12]. Apoptosis-related alterations have been reported in the dopaminergic brain regions in post-mortem tissues of PD patients, including increased caspase-3 activity in the substantia nigra [13, 14], and elevated immunoreactivity of the pro-apoptotic protein, Bax [15], indicating that apoptosis may play a role in the pathophysiology of PD.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been used extensively to study PD [16, 17]. MPTP selectively targets and damages the dopaminergic neurons causing parkinsonism in humans and other primates [18, 19]. MPTP readily crosses the blood-brain barrier and is converted into the active metabolite, 1-methyl-4-phenylpyridinium (MPP⁺) [17]. MPP⁺ is taken up by the dopaminergic neurons and accumulates in the mitochondria, where it inhibits complex I of the electron transport chain and ultimately causes neuronal cell death [5]. The mechanisms of MPP⁺-induced cytotoxicity most likely involve oxidative stress [20]. Several groups reported that treatment of mice with MPTP induces a Parkinson-like syndrome [16],

accompanied by apoptosis of the dopaminergic neurons in the substantia nigra [21] and up-regulation of α -synuclein, a pre-synaptic protein, which plays an important role in PD pathogenesis [22]. In vitro studies using human SH-SY5Y neuroblastoma cells have produced comparable results [23, 24]. Exposure of these cells to MPP⁺ led to fragmentation of nuclear DNA [25], activation of caspase-3 [23, 26] and to up-regulation of α -synuclein [27–29]. In cultured SH-SY5Y cells dopamine-dependent cytotoxicity was enhanced by the accumulation of α -synuclein [30]. Therefore, targeting the molecular pathways activated during apoptosis may lead to novel treatment strategies for PD [5, 31].

Guanosine and other non-adenine-based purines exert neuroprotective effects in the central nervous system [32]. In vitro guanosine also protects rat astrocytes from staurosporine-induced apoptosis [33] and SH-SY5Y cells from β -amyloid-induced apoptosis [34]. In both cases, the anti-apoptotic effect of guanosine was mediated by stimulation of the phosphatidylinositol-3-kinase (PI-3-K)/Akt/protein kinase B (PKB) and mitogen-activated protein kinase (MAPK) cell survival pathways [33, 34]. These pathways were activated via the pertussis toxin-sensitive Gi protein-coupled putative guanosine receptor that we have recently characterized pharmacologically [35–37].

In PD, symptoms appear only after the majority of the dopaminergic neurons in the substantia nigra pars compacta have degenerated, so putative neuroprotective drugs for PD should be tested in models in which the neurodegenerative process is already underway [38]. Therefore, we examined the effects of guanosine on MPP⁺-induced caspase-3 activation and DNA fragmentation in SH-SY5Y human neuroblastoma cells not only when it was added prior to, or at the same time as MPP⁺, but also when it was added to the cultures up to 48 h *after* MPP⁺ treatment, when the pro-apoptotic pathways were already activated. We also studied the molecular mechanisms that mediate the neuroprotective effects of guanosine.

Materials and methods

Tissue culture and treatment of human SH-SY5Y neuroblastoma cells

Human SH-SY5Y neuroblastoma cells (ATCC, Manassas, VA, USA) were cultured using 45% minimum essential medium, 45% F12 Hamilton's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 U/ml of streptomycin at a pH of 7.4 (all from Invitrogen, Burlington, ON, Canada). Culture medium was changed every 3–4 days, and cells were maintained in a humidified 5% CO₂ atmosphere at 37°C and subcultured at a ratio of 1:20 every 7–10 days. Culture

medium was changed to 1% FBS for 24 h before the start of each experiments. All experiments were performed using 70–80% confluent cultures.

Apoptosis was induced by adding MPP^+ (Sigma-Aldrich) to SH-SY5Y neuroblastoma cells, using a fresh stock solution of MPP^+ (10 mM in reverse osmosis purified H_2O). In all experiments, guanosine (Sigma-Aldrich) was dissolved in 0.1 M NaOH and added to the culture medium at a final concentration of 0.001 M NaOH. Some cells (control) were exposed to 0.001 M NaOH. In experiments in which the protective effect of guanosine was tested, guanosine (100 μ M) was added to SH-SY5Y neuroblastoma cells either: 1 h prior to the administration of MPP^+ (*pre-treatment*), or at the same time as MPP^+ (*co-treatment*) or 24 or 48 h after the addition of MPP^+ (*post-treatment* at 24 h, or post-treatment at 48 h, respectively; Fig. 1). Guanosine and MPP^+ remained in the culture medium for the duration of the experiment (24, 48 or 72 h). In the first *post-treatment* experimental group at 24 h, cells were exposed to MPP^+ for 24 h, followed by MPP^+ plus guanosine for an additional 24 h (Fig. 1). In the second *post-treatment* experimental group at 48 h, cells were treated with MPP^+ for 48 h, followed by MPP^+ plus guanosine for an additional 24 h (Fig. 1).

In experiments in which enzyme inhibitors were tested, SH-SY5Y cells were pre-treated with various agents for 20 min prior to the addition of guanosine. These treatments included: the potent and selective inhibitor of PI-3-K, [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-1-hydrochloride] (LY294002; IC_{50} =1.4 μ M; Sigma), or the selective

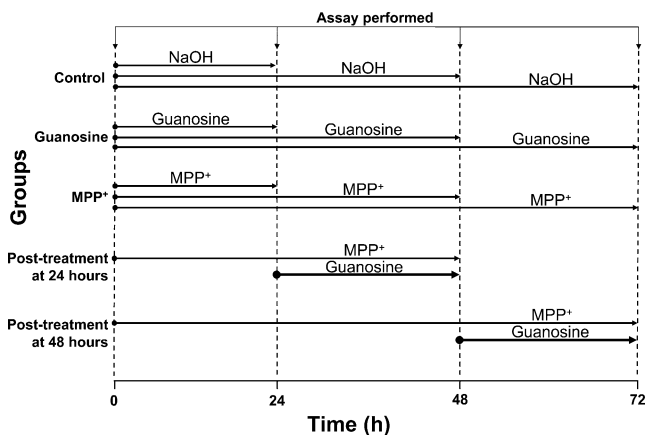


Fig. 1 Experimental design for guanosine post-treatment of SH-SY5Y neuroblastoma cells added at 24 or 48 h after MPP^+ . SH-SY5Y neuroblastoma cells were exposed to either vehicle, or guanosine (100 μ M) or MPP^+ (500 μ M) for various times. For *post-treatment* experiments with guanosine at 24 h, cells were exposed to MPP^+ for 24 h, followed by MPP^+ plus guanosine for an additional 24 h (total MPP^+ exposure = 48 h). For *post-treatment* experiments with guanosine at 48 h, cells were exposed to MPP^+ for 48 h followed by MPP^+ plus guanosine for a further 24 h (total MPP^+ exposure = 72 h). Once added, guanosine and MPP^+ remained in the culture medium for the duration of the experiment. DNA fragmentation, or caspase-3 activity, was determined at 24, 48 or 72 h as described in the “Materials and methods” section

inhibitor of MAP kinase kinase (MEK), [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-1] (PD98059; IC_{50} =2 μ M; Sigma). In experiments using the ADP-ribosylating factor of the inhibitory guanosine nucleotide binding protein (Gi), pertussis toxin [PTX, 200 ng/ml; in 50% glycerol, 0.5 M NaCl, and 0.05 M Tris-glycine (pH 7.5); Sigma] was added overnight (16 h). LY294002 and PD98059 were dissolved in and added to the culture medium at a final concentration of 0.01% dimethyl sulfoxide (DMSO).

Evaluation of cell death and DNA fragmentation

In initial experiments, cell death was determined using acridine orange-ethidium bromide (AO-EB, Sigma-Aldrich) staining. Cells were seeded onto 6-well plates (diameter 10 mm; 2.5×10^4 cells/well) and exposed to MPP^+ . After 24, 48 or 72 h, cells were washed three times with phosphate-buffered saline (PBS) and AO-EB solution (6 μ M/ml) was added to the wells. Cells were examined using a fluorescence microscope (Nikon, Tokyo, Japan). The number of fragmented nuclei and/or condensed chromatin was determined by counting >200 cells. The percentage of apoptotic cells is defined as: (total number of cells with apoptotic nuclei/total number of cells) \times 100.

In subsequent experiments, DNA fragmentation was evaluated by the oligonucleosomal ELISA (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics, Laval, QC, Canada) and carried out according to the manufacturer’s instructions. This assay measures the amount of oligonucleosomal fragments and is a marker of apoptosis [39]. Cells were exposed to MPP^+ alone, or in combination with guanosine or pre-treated with the various inhibitors as described above. Following various treatments, cells were isolated for analysis of DNA fragmentation as described by Pettifer et al. [34]. For the oligonucleosomal ELISA, 10,000 viable cells/treatment were lysed and centrifuged (200 g for 10 min) to isolate fragmented oligonucleosomal DNA. The cytosolic fractions of cell lysates were transferred into streptavidin-coated microplate wells, and a mixture of biotin-linked anti-histone antibody and peroxidase-linked anti-DNA antibody was added and incubated for 2 h at room temperature. Plates were washed with incubation buffer to remove the unfixed anti-DNA antibody and the peroxidase activity was determined spectrophotometrically with 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) as the substrate (absorbance of 405 nm). The amount of DNA fragmentation is expressed as a percentage of the positive control provided with the kit.

Determination of caspase-3 activity

Caspase-3 activity was determined using the caspase-3 colourimetric assay (Sigma-Aldrich). Cells were exposed to

different treatments as described above; then cells were isolated, re-suspended in 100 $\mu\text{l}/10^7$ cells of lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 5 mM 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS), 5 mM dithiothreitol (DTT)) and incubated on ice for 20 min. Cell lysates were centrifuged (18,000 g for 10 min at 4°C) and the supernatants (5 $\mu\text{l}/\text{well}$) were transferred to 96-well plates. A selective caspase-3 inhibitor (*N*-acetyl-Asp-Glu-Val-Asp-CHO; 10 $\mu\text{l}/\text{well}$ at 200 μM) was added to supernatants to control for the nonspecific hydrolysis of the substrate. Purified caspase-3 human recombinant protein, provided with the assay, was used (5 $\mu\text{l}/\text{well}$ at 5 $\mu\text{g}/\text{ml}$) as a positive control for caspase-3 activity. Cell lysates were incubated in an assay buffer [40 mM HEPES, pH 7.4, 1% CHAPS, 10 mM DTT, 4 mM ethylenediaminetetraacetic acid (EDTA)], containing *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroaniline (10 $\mu\text{l}/\text{well}$ at 2 mM) overnight at 37°C and the formation of *p*-nitroaniline was measured at 405 nm, using a microtiter plate reader. The activity of caspase-3 was calculated from the absorbance values using a calibration curve, and it is expressed as μg of substrate cleaved/min per ml. As the number of cells in 1 ml of cell lysate is adjusted to 1×10^8 cells, this caspase-3 activity is generated by 1×10^8 cells.

Measurement of α -synuclein protein concentration by ELISA

The concentration of α -synuclein protein in SH-SY5Y neuroblastoma cells was determined by an ELISA (BioSource, San Diego, CA, USA). Cells were exposed to various treatments as described above and subsequently lysed using a cell extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate; BioSource, San Diego, CA, USA), containing protease inhibitors [1 mM phenylmethanesulfonyl fluoride, 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin; Sigma]. Cells were incubated on ice in cell extraction buffer for 30 min and centrifuged (14,000 g for 10 min at 4°C). Aliquots of supernatants (25 μl) were removed for the determination of the total protein concentration using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). We measured α -synuclein protein concentration using an ELISA kit according to the manufacturer's instructions. Briefly, cell lysates (diluted 1:10) were added to α -synuclein antibody-coated wells in duplicate, allowed to bind and then were treated with the anti- α -synuclein antibody. This was followed by the addition of an anti-rabbit IgG-horse radish peroxidase-linked antibody, and by the addition of a stabilized chromogen

(tetramethylbenzidine). The reaction was terminated by adding 1 M HCl (stop solution) provided with the kit, and the absorbance of each well was read at 450 nm. Standard curves were prepared using purified recombinant α -synuclein protein (100 ng/ml) provided with the kit. The concentration of the α -synuclein protein in each sample was determined from a nonlinear regression, and it is expressed as a ratio of α -synuclein protein (in ng/ml) to total protein concentration (in ng/ml).

Statistical analysis

Data obtained in experiments testing the concentration dependence of the protective effect of guanosine on MPP⁺-induced DNA fragmentation (Fig. 3) and the effect of PTX, and inhibitors of the PI-3-K, and the MAPK pathways on DNA fragmentation (Fig. 6 and Table 1) were analyzed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keul post-hoc test. Two-way ANOVA, followed by a Bonferroni post-hoc test, was used to analyze the data obtained from experiments evaluating the effects of guanosine, administered at different time points on MPP⁺-induced DNA fragmentation (Fig. 4) and on MPP⁺-induced caspase-3 activation (Fig. 5). A *p* value of 0.05 or less was considered statistically significant. Results represent the mean \pm SEM of a minimum of three independent experiments.

Results

MPP⁺ induces DNA fragmentation in SH-SY5Y neuroblastoma cells

SH-SY5Y neuroblastoma cells were exposed to different concentrations of MPP⁺ (10–500 μM) for various times (0–72 h) to optimize the experimental conditions and to confirm that MPP⁺-induced DNA fragmentation was concentration and time dependent (Fig. 2). DNA fragmentation in untreated control cells was around 10% and remained unchanged for up to 72 h (Fig. 2). Treatment of cells with 500 μM MPP⁺ increased DNA fragmentation significantly after 48 h ($p < 0.01$) and 72 h ($p < 0.001$; Fig. 2) compared to untreated controls. Since exposure of cells to 500 μM MPP⁺ for 48 h induced robust DNA fragmentation, these conditions were used in the following studies.

Pre-treatment of SH-SY5Y neuroblastoma cells with guanosine attenuates MPP⁺-induced DNA fragmentation

SH-SY5Y cells were exposed to various concentrations of guanosine (1–300 μM) for 1 h, followed by the addition of 500 μM MPP⁺ for a further 48 h, and DNA fragmentation

was determined at 49 h by oligonucleosomal ELISA. As we observed in the previous experiment the addition of MPP⁺ (500 μ M) led to significantly increased DNA fragmentation compared to controls ($p < 0.001$; Fig. 3). Pre-treatment of cells for 1 h with various concentrations of guanosine (10–300 μ M) significantly reduced MPP⁺-induced DNA fragmentation ($p < 0.05$) for all four concentrations of guanosine compared to cells treated with MPP⁺ alone (Fig. 3). Based on these results, we used 100 μ M guanosine in all subsequent experiments.

Post-treatment with guanosine protects SH-SY5Y neuroblastoma cells against MPP⁺-induced DNA fragmentation

We next examined whether guanosine exerted any effect on MPP⁺-induced DNA fragmentation when it was added to neuroblastoma cells after apoptosis was already in progress. Cells were pre-treated with 100 μ M guanosine prior to MPP⁺ or co-treated with guanosine plus MPP⁺ or, as described in Fig. 1, guanosine was added as *post-treatment*. DNA fragmentation was determined in cells exposed to various treatments after 24, 48 and 72 h of MPP⁺ administration (Fig. 4). The addition of MPP⁺ led to a significant increase in DNA fragmentation compared to controls at 48 h ($p < 0.01$) and 72 h ($p < 0.001$) (Fig. 4). Guanosine alone had no significant effect on DNA fragmentation (Fig. 4). *Pre-treatment* of cells with guanosine significantly reduced DNA fragmentation after 48 and 72 h compared to MPP⁺ treatment alone, at the respective time points ($p < 0.05$, at both time points; Fig. 4). Similarly, *co-treatment* of cells with guanosine plus MPP⁺ significantly reduced DNA fragmentation after 48 and 72 h compared to cells treated with MPP⁺ alone at these times

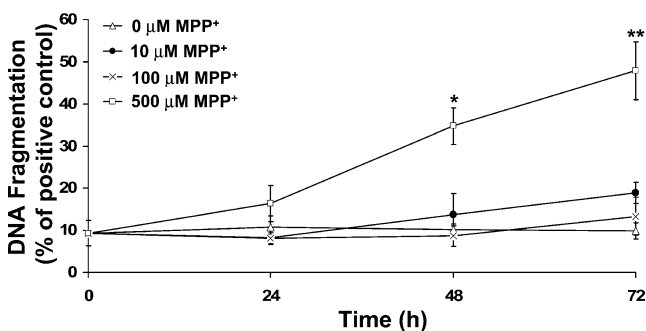


Fig. 2 MPP⁺ induces DNA fragmentation in SH-SY5Y cells. MPP⁺ (10, 100 or 500 μ M) was added to SH-SY5Y cells and DNA fragmentation determined after 24, 48 or 72 h by oligonucleosomal ELISA as described in the “Materials and methods” section. Data are presented as the percentage of DNA fragmentation relative to a positive control ($n > 3$). MPP⁺ (500 μ M) increased DNA fragmentation significantly at 48 h ($*p < 0.01$) and at 72 h ($**p < 0.001$) compared to that of untreated cells at zero time

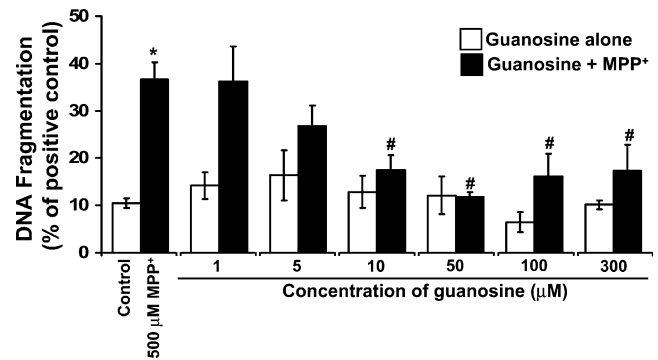


Fig. 3 Pre-treatment of SH-SY5Y cells with guanosine decreases MPP⁺-induced DNA fragmentation. SH-SY5Y cells were exposed to MPP⁺ (500 μ M) for 48 h to induce DNA fragmentation. Some cells were pre-treated with guanosine (1–300 μ M) for 1 h prior to MPP⁺. Guanosine and MPP⁺ remained in the cultures for the duration of the experiment. DNA fragmentation was determined after 48 h by oligonucleosomal ELISA as described in the “Materials and methods” section and is expressed as the percentage of the positive control ($n > 3$). MPP⁺ increased DNA fragmentation significantly at 48 h compared to that of untreated controls ($*p < 0.001$). Pre-treatment with guanosine (10–300 μ M) reduced significantly the MPP⁺-induced DNA fragmentation compared to that in cells treated with MPP⁺ for the same time ($#p < 0.05$, at all four concentrations)

($p < 0.05$, at both time points; Fig. 4). Addition of guanosine 24 h after MPP⁺ (*post-treatment at 24 h*) or 48 h after MPP⁺ (*post-treatment at 48 h*) reduced DNA fragmentation significantly compared to cells treated with MPP⁺ alone for 48 or 72 h, respectively ($p < 0.05$, for both treatments; Fig. 4). These results demonstrate that *pre-treatment* or *co-treatment* of cells with guanosine and MPP⁺ attenuates MPP⁺-induced DNA fragmentation, and guanosine added 24 or 48 h after the start of the DNA fragmentation (*post-treatment*) can actually reverse this process.

Guanosine inhibits MPP⁺-induced caspase-3 activity

Exposure of SH-SY5Y cells to MPP⁺ leads to apoptosis via caspase-3 activation [23, 26]. We therefore examined if under our experimental conditions treatment of neuroblastoma cells with MPP⁺ activated caspase-3 and whether the addition of guanosine to MPP⁺-treated cells at various times (*pre-treatment*, *co-treatment* or *post-treatment*) had any effect on the activity of this enzyme. In untreated control cells caspase-3 activity was low and remained unchanged for up to 72 h (Fig. 5). Addition of guanosine to cells had no significant effect on caspase-3 activity (Fig. 5). In MPP⁺-treated cells caspase-3 activity increased significantly from 24 to 72 h ($p < 0.001$, at all time points; Fig. 5). *Pre-treatment* of cells with guanosine prior to MPP⁺ addition significantly reduced caspase-3 activity after 48 or 72 h compared to that in cells treated with MPP⁺ alone for the same time ($p < 0.01$, at both time points; Fig. 5). Similar results were also obtained when cells were *co-treated* with

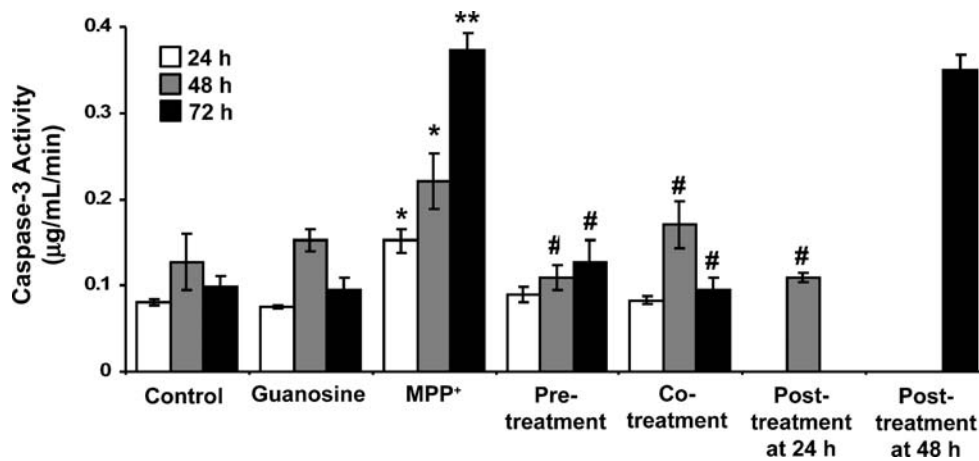


Fig. 4 Guanosine antagonizes and reverses MPP⁺-induced DNA fragmentation in SH-SY5Y cells. SH-SY5Y cells were exposed to MPP⁺ (500 µM) for 48 h to induce DNA fragmentation. Some cells were treated with guanosine (100 µM) either for 1 h prior to MPP⁺ (*pre-treatment*), or at the same time as MPP⁺ (*co-treatment*) or 24 or 48 h after MPP⁺ (*post-treatment*, see Fig. 1). DNA fragmentation was determined after 24, 48 or 72 h of MPP⁺ addition by oligonucleosomal ELISA as described in the “Materials and methods” section and it is expressed as a percentage of the positive control ($n > 3$). MPP⁺ induced a significant increase in DNA fragmentation at 48 h ($*p < 0.01$) and at 72 h ($**p < 0.001$) compared to that of untreated control cells at the same time. *Pre-treatment* or *co-treatment* of cells with guanosine significantly reduced MPP⁺-induced DNA fragmentation at 48 and 72 h compared to that in cells treated with MPP⁺ alone at the same time ($\#p < 0.05$ at both time points, for both treatments). *Post-treatment* with guanosine at 24 or 48 h also reduced MPP⁺-induced DNA fragmentation at 48 and 72 h compared to that in cells treated with MPP⁺ alone at the same time ($\#p < 0.05$, at both time points)

guanosine and MPP⁺ for 48 and 72 h ($p < 0.01$, at both time points; Fig. 5). *Post-treatment with guanosine at 24 h* (see Fig. 1) also reduced caspase-3 activity significantly at 48 h compared to that in cells treated with MPP⁺ alone at this time point ($p < 0.01$; Fig. 5). When guanosine was added to

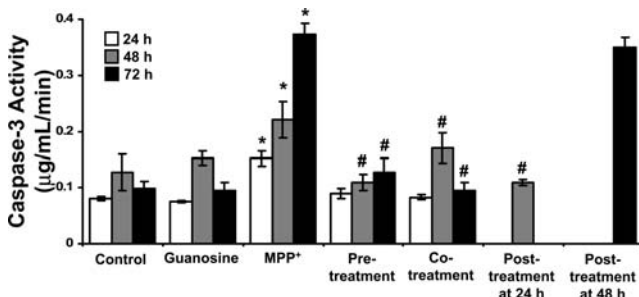


Fig. 5 Guanosine inhibits MPP⁺-induced caspase-3 activation in SH-SY5Y cells. SH-SY5Y cells were exposed to MPP⁺ (500 µM) for 48 h to induce to induce caspase-3 activation. Some cells were treated with guanosine (100 µM) either for 1 h prior to MPP⁺ (*pre-treatment*), or at the same time as MPP⁺ (*co-treatment*) or 24 or 48 h after MPP⁺ (*post-treatment*, see Fig. 1). Caspase-3 activity was determined as described in the “Materials and methods” section and is expressed as the amount of *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroaniline cleaved by 1×10^8 cells, in µg/min per ml ($n > 3$). MPP⁺ significantly increased caspase-3 activity from 24 to 72 h ($*p < 0.001$, at all three time points) compared to that of control cells at the same time. *Pre-treatment* or *co-treatment* of cells with guanosine significantly reduced MPP⁺-induced caspase-3 activity, at 48 and 72 h, compared to that in cells treated with MPP⁺ alone at the same time ($\#p < 0.01$, at both time points, for both treatments). *Post-treatment* with guanosine at 24 h reduced MPP⁺-induced caspase-3 activity at 48 h compared to that in cells treated with MPP⁺ alone at the same time ($\#p < 0.01$). Guanosine added to cultures 48 h after MPP⁺ had no effect on caspase-3 activity

cells 48 h after the addition of MPP⁺ (*post-treatment at 48 h*), caspase-3 activity determined after 72 h was unaffected compared to MPP⁺ treatment alone at this time point (Fig. 5). These results thus demonstrate that *pre-treatment*, *co-treatment* or *post-treatment at 24 h* of neuroblastoma cells with guanosine prevents MPP⁺-induced activation of caspase-3. Administration of guanosine to cells 48 h after MPP⁺ however has no effect on caspase-3 activity.

Guanosine has no effect on α -synuclein protein expression in MPP⁺-treated neuroblastoma cells

Several authors reported that addition of MPP⁺ to SH-SY5Y cells increased α -synuclein protein expression [27, 29]. We therefore determined α -synuclein protein concentration in neuroblastoma cells under our experimental conditions (500 µM MPP⁺ for 48 h) and evaluated the effects of guanosine on the expression of this protein in MPP⁺-treated cells. The ratio of α -synuclein protein/total protein in untreated control cells after 48 h was very low and exposure of neuroblastoma cells to MPP⁺ for 48 h had no significant effect on the expression of this protein (data not shown). Treatment of cells with guanosine prior to the addition of MPP⁺, or at the same time as MPP⁺ or 24 h after MPP⁺ had no significant effect on α -synuclein protein expression (data not shown). These results indicate that under the present experimental conditions neither MPP⁺ nor a combination of guanosine plus MPP⁺ has any effect on α -synuclein protein expression.

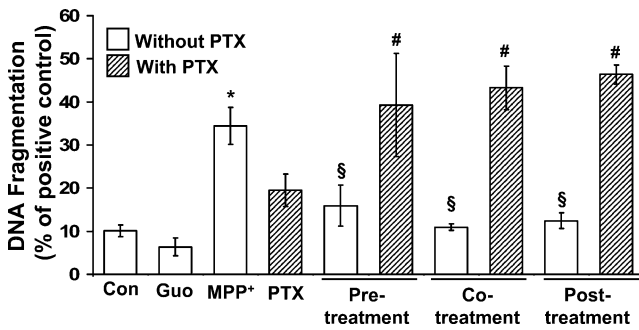


Fig. 6 Pertussis toxin abolishes the anti-apoptotic effect of guanosine in SH-SY5Y cells. SH-SY5Y cells were exposed to MPP⁺ (500 μ M) for 48 h to induce DNA fragmentation. Some cells were treated with guanosine (100 μ M) either for 1 h prior to MPP⁺ (*pre-treatment*), or at the same time as MPP⁺ (*co-treatment*) or 24 h after MPP⁺ (*post-treatment*, see Fig. 1). Pertussis toxin (PTX, 200 ng/ml for 16 h) was added to some cultures prior to *pre-treatment* or *co-treatment* or *post-treatment* with guanosine at 24 h. DNA fragmentation was determined at 48 h and is expressed as a percentage of the positive control ($n > 3$). MPP⁺ increased DNA fragmentation significantly at 48 h ($*p < 0.001$) compared to that of untreated control cells at the same time. *Pre-treatment*, or *co-treatment* or *post-treatment* of cells with guanosine at 24 h reduced significantly MPP⁺-induced DNA fragmentation compared to that in cells treated with MPP⁺ alone at the same time ($§p < 0.01$). PTX reversed the protective effect of guanosine. DNA fragmentation in cells exposed to PTX plus *pre-treatment*, or *co-treatment* or *post-treatment* with guanosine was significantly higher compared to that of cells without PTX ($#p < 0.05$, for all three treatments). These values were comparable to that obtained in cells treated with MPP⁺ alone

Pertussis toxin abolishes the anti-apoptotic effect of guanosine

We reported recently that specific binding sites exist for guanosine in rat brain membranes [35], in cultured rat astrocytes [36] and in SH-SY5Y neuroblastoma cells (Traversa and Caciagli, 2005, personal communications). We have further shown that guanosine binding in these cells is sensitive to treatment with PTX [37, 40], indicating that the putative guanosine receptor is coupled to Gi protein. We therefore tested whether the anti-apoptotic effect of guanosine in the MPP⁺-treated cells is mediated via this putative guanosine receptor. Cells were exposed to PTX (200 ng/ml for 16 h) prior to the various treatments with guanosine and MPP⁺ (*pre-treatment*, *co-treatment* or *post-treatment* at 24 h) and DNA fragmentation was determined at 48 h. As in previous experiments, MPP⁺ induced a significant increase in DNA fragmentation and the addition of guanosine alone had no significant effect on this process (Fig. 6). Exposure of neuroblastoma cells to PTX alone increased DNA fragmentation slightly under basal conditions, but this was not statistically significant. *Pre-treatment*, *co-treatment* or *post-treatment* at 24 h of cells with guanosine and MPP⁺ significantly reduced DNA fragmentation compared to cells treated with MPP⁺ only ($p < 0.01$, for all three treatments; Fig. 6). Addition of PTX

to neuroblastoma cells prior to any of the three guanosine plus MPP⁺ treatments reversed the anti-apoptotic effect of guanosine (Fig. 6). These results demonstrate that regardless of the time of its addition to MPP⁺-treated cells, the protective effect of guanosine is abolished by PTX. These findings are consistent with the interpretation that these effects are mediated by a Gi protein-coupled receptor, most likely the putative guanosine receptor.

Guanosine protects against MPP⁺-induced apoptosis by activating the cell survival pathways

We have shown earlier that guanosine activates the PI-3-K/Akt/PKB and MAPK pathways in neuroblastoma cells [34]. We therefore evaluated if the protective effects of guanosine against MPP⁺-induced DNA fragmentation were also mediated via these signaling pathways. Cells were exposed to either LY294002, or to PD98059, selective inhibitors of PI-3-K or MEK, respectively, for 20 min prior to various treatments with guanosine and MPP⁺ (*pre-treatment*, *co-treatment* or *post-treatment* at 24 h). Exposure of neuroblastoma cells to LY294002 alone had no significant effect on DNA fragmentation under basal conditions. Although in cells exposed to PD98059 alone DNA fragmentation increased slightly, this was not statistically significant (Table 1). As in previous experiments, the addition of MPP⁺ induced significant DNA fragmentation and treat-

Table 1 Effect of selected inhibitors of cell survival pathways on the anti-apoptotic effect of guanosine in SH-SY5Y cells

Treatment	DNA fragmentation (% of positive control)		
	No inhibitor	LY294002 10 μ M	PD98059 10 μ M
Control	10.1 \pm 1.3	16.3 \pm 3.1	24.0 \pm 4.7
MPP ⁺ (500 μ M)	34.5 \pm 4.3 ^a	–	–
Guanosine pre-treatment	15.9 \pm 4.8	38.6 \pm 10.3 ^b	38.3 \pm 9.1 ^b
Guanosine co-treatment	11.0 \pm 0.7	32.9 \pm 5.7 ^b	28.7 \pm 10.4 ^{n.s.}
Guanosine post-treatment	12.4 \pm 1.8	48.2 \pm 4.3 ^c	28.4 \pm 4.8 ^{n.s.}

SH-SY5Y cells were exposed to MPP⁺ (500 μ M) for 48 h to induce DNA fragmentation. Some cells were treated with guanosine (100 μ M) either for 1 h prior to MPP⁺ (*pre-treatment*), or at the same time as MPP⁺ (*co-treatment*) or 24 h after MPP⁺ (*post-treatment*, see Fig. 1). Some cultures were exposed to LY294002 (inhibitor of PI-3-K) or to PD98059 (inhibitor of MAP kinase kinase, MEK) for 20 min prior to the addition of guanosine. DNA fragmentation was determined at 48 h ($n = 3$) and is expressed as a percentage of the positive control. LY294002 abolished the protective effect of guanosine in cells *pre-treated*, *co-treated* or *post-treated* with this nucleoside. PD98059 also abolished the protective effect of guanosine in cells *pre-treated* with this nucleoside, but had no significant effect in cells *co-treated* or *post-treated* with guanosine

^a $p < 0.001$ compared to control

^b $p < 0.01$ compared to same treatment without inhibitor

^c $p < 0.05$ compared to same treatment without inhibitor

n.s. not significant

ment of cells with guanosine under various conditions significantly reduced DNA fragmentation compared to cells treated with guanosine (Table 1).

Exposure of cells to LY294002 prior to *pre-treatment*, or *co-treatment* or *post-treatment* at 24 h with guanosine and MPP⁺ reversed the protective effect of guanosine. DNA fragmentation in cells exposed to LY294002 plus guanosine plus MPP⁺ was significantly reduced compared to cells treated with guanosine plus MPP⁺ without the inhibitor ($p < 0.05$, for *pre-treatment* and *co-treatment*; $p < 0.01$, for *post-treatment* at 24 h; Table 1). Addition of PD98059 to cells before *pre-treatment* with guanosine and MPP⁺ also abolished the protective effect of guanosine ($p < 0.05$; Table 1). But this inhibitor had no significant effect on MPP⁺-induced DNA fragmentation in cells *co-treated* or *post-treated* at 24 h with guanosine and MPP⁺ (Table 1). These results demonstrate that the protective effects of guanosine are abolished by LY294002 in MPP⁺-treated cells regardless of the time of its administration and are consistent with a role of the PI-3-K/Akt/PKB cell survival pathway in guanosine-mediated reduction of DNA fragmentation. In contrast, the effect of PD98059 is limited to pre-treatment of cells with guanosine prior to MPP⁺, thus indicating that the MAPK pathway contributes to the protective effects of guanosine only when it is added before MPP⁺.

Discussion

We have shown earlier that pre-treatment with guanosine protects different cell types against apoptosis [33, 34]. Here, we report that guanosine not only protects human SH-SY5Y neuroblastoma cells against MPP⁺-induced apoptosis, but more importantly, it can reverse MPP⁺-induced cytotoxicity after the pro-apoptotic pathways have already been activated. Thus, these findings are novel and demonstrate for the first time that guanosine can in fact ‘rescue’ neuroblastoma cells from apoptosis when it is added to cells under experimental conditions that are ‘clinically relevant’ to the treatment of PD.

Undifferentiated dopaminergic human SH-SY5Y neuroblastoma cells have been used widely to study the molecular pathways that mediate MPP⁺-induced apoptosis [23–29]. MPTP, after conversion to MPP⁺, activates the same intracellular pathways *in vivo* and *in vitro* as in idiopathic PD [2]. Thus, MPP⁺ is taken up by dopaminergic neurons, where it inhibits complex I of the mitochondrial electron transport chain. This leads to enhanced production of reactive oxygen species (ROS) and decreased synthesis of ATP [2]. Prolonged exposure to MPP⁺ results in the up-regulation of the pro-apoptotic protein Bax, and following its translocation to the mitochondria it promotes the release of mitochondrial cytochrome *c*. This may be initiated by the

DNA damage caused by MPP⁺. Cytochrome *c*, together with pro-caspase-9, is incorporated into the complex, the apoptosome, which activates caspase-9, and this enzyme in turn activates caspase-3 [2, 41]. At the same time, the anti-apoptotic protein, Bcl-2, is down-regulated [42]. Taken together these data demonstrate that exposure of dopaminergic cells to MPP⁺ leads to cytotoxicity and cell death by apoptosis [2, 17]. Our findings are consistent with previous reports that MPP⁺ induces DNA fragmentation and activation of caspase-3 in SH-SY5Y cells [23, 25]. Interestingly, we detected no significant change in α -synuclein protein expression in MPP⁺-treated cells (data not shown). This may be the result of the relatively low MPP⁺ concentration (500 μ M) and short duration of our experiments (48 h). Others reported that high concentrations of MPP⁺ (5 mM for 12 h [29]) or prolonged exposure of neuroblastoma cells to lower concentrations of MPP⁺ (5 μ M for 4 days [28] or 1 mM for 3 days [27]) actually increased α -synuclein protein concentration. Mutations of this protein have been associated with a familial form of PD [43] and the wild-type protein is a major constituent of Lewy bodies [44]. In MPTP-treated mice α -synuclein expression is up-regulated [45], and in SH-SY5Y cells accumulation of this protein contributes to dopamine-dependent apoptosis [30]. Under our experimental conditions that were optimized to detect DNA fragmentation, we observed no change in α -synuclein protein expression.

The molecular mechanisms by which guanosine exerts its protective effects are complex. Results from the present study show that the effects of both *pre-treatment* and *co-treatment* with guanosine are pertussis toxin sensitive, and these may depend on the putative plasma membrane localized guanosine receptor [35–37] that is coupled to Gi proteins. There may be other possible explanations, which account for the effects of pertussis toxin on MPP⁺-induced DNA fragmentation. Since pertussis toxin enters cells and ADP-ribosylates the α -subunit of Gi proteins [46, 47], it may also attenuate signaling by other Gi protein-coupled receptors, resulting in the elevation of intracellular cAMP [48, 49] and this in turn may activate cell survival pathways, such as the MAPK [56]. Guanosine may also enter neuroblastoma cells via the equilibrative or the concentrative nucleoside transporters [50], cause an alteration in intracellular concentrations of guanine nucleotides [40] and this in turn may affect G protein activity.

However, as we have shown before, guanosine binding to its cognate receptor leads to rapid activation of the cell survival pathways, PI-3-K/Akt/PKB and MAPK [33, 34], and these play a critical role in protecting neurons against cell death [51–56]. Although these signaling pathways are most often triggered by growth factor binding to their cognate receptors, G protein-coupled receptors have also been shown to activate them [57–60]. Thus, binding of

guanosine to its Gi protein-coupled putative receptor may activate the class IB PI-3-K (PI-3-K γ), via the dissociated β,γ -subunits of Gi proteins, which promote the synthesis of phosphatidylinositol (3,4,5) trisphosphate at the plasma membrane and recruit the protein kinase, Akt/PKB [60, 61]. Activation of Akt/PKB by phosphorylation at Thr 308 and Ser 473 regulates cell survival and apoptosis by both transcription-dependent and transcription-independent pathways [7, 54, 61]. Thus, Akt/PKB may suppress apoptosis by phosphorylating and inactivating the pro-apoptotic protein Bad [62], inhibiting the release of mitochondrial cytochrome *c* [63] and inhibiting the activation of caspase-9 and caspase-3 [64]. Expression of cell survival genes may also be triggered by the PI-3-K/Akt/PKB pathway, via activation of the transcription factors CREB and NF- κ B, and inhibition of Forkhead transcription factors of the FOXO family [54].

Similarly, activation of the MAPK pathway, specifically the extracellular signal-regulated kinases 1 and 2 (ERK1/2), also prevent apoptosis and promote cell survival by both transcription-dependent and transcription-independent pathways [55, 56, 65, 66]. The specific downstream targets of the ERK1/2 pathway include inhibition of caspase-9 by phosphorylation [67], suppression of the pro-apoptotic protein Bad by phosphorylation and activation of the transcription factor CREB [55]. Guanosine binding to its putative receptor may also stimulate this pathway via the dissociated β,γ -subunits of Gi proteins [59]. Reports suggest that the PI-3-K/Akt/PKB and MAPK pathways do not function independently, but there is significant cross-talk between them [57, 68]. It has been suggested that whereas the PI-3-K/Akt/PKB pathway is the main signaling pathway for maintaining trophic support by pro-survival factors, ERK1/2 mediates protection against damage-induced cell death [56]. Our results are thus consistent with the interpretation that protection of cells from MPP⁺-induced DNA fragmentation by guanosine *pre-treatment* is dependent on the activation of both these pathways. Following *co-treatment* with guanosine and MPP⁺, however, it is the PI-3-K/Akt/PKB pathway, which plays the essential role.

In the present study, we also examined the effect of guanosine on MPP⁺-induced DNA fragmentation by exposing cells to guanosine *after* caspase-3 activity was elevated and DNA fragmentation was already underway (*post-treatment*). This strategy is novel and is based on a recent proposal by Meissner et al. [38], who advocate that potential neuroprotective agents for the treatment of PD should be tested under conditions that are relevant to the clinical situation. Because clinical symptoms of PD are apparent only after the majority of nigrostriatal neurons have died, patients do not present for treatment until after the process of cell death is well established [38]. Most *in vitro* studies have been conducted by adding potential

neuroprotective agents to cells prior to MPP⁺-triggered apoptosis [69–71] or at the same time as MPP⁺ [27, 72, 73], making these results irrelevant to the treatment of PD, where the process of neurodegeneration has already begun.

Addition of guanosine to cells *after* MPP⁺-induced DNA fragmentation is already in progress may involve additional protective mechanisms. As DNA fragmentation is a late event in apoptosis [11, 12], some cells with damaged DNA may actually undergo DNA repair and survive [74, 75]. The preferred survival strategy of a cell is to attempt to restore any DNA damage by using a complex set of DNA repair pathways [74]. Only when the damage to the DNA becomes excessive and the repair capacity of the cell is overwhelmed will it undergo apoptosis. Therefore, adding guanosine 24 or 48 h after DNA fragmentation has been initiated may activate some of the DNA repair mechanisms, antagonizing and reversing the MPP⁺-induced DNA fragmentation. The protective effects of guanosine in these cells thus may include activation of both cell survival and DNA repair pathways.

In contrast, *post-treatment* with guanosine had no significant effect on MPP⁺-induced activation of caspase-3. As activation of this enzyme requires a cleavage of the interdomain region of the inactive dimer of caspase-3, once this process is initiated it cannot be reversed [8–10]. So addition of guanosine to cells after proteolytic cleavage of this enzyme will not alter caspase-3 activity.

Recently administration of MPTP to mice has been reported to promote caspase-independent cell death of dopaminergic neurons [76]. This “apoptosis-like” programmed cell death [77] is mediated by the release of the apoptosis-inducing factor (AIF) from the mitochondria and its subsequent translocation to the nucleus, where it induces DNA fragmentation [78–80]. If this process plays a significant role in MPP⁺-induced cell death of neuroblastoma cells, DNA fragmentation will still be reversed by the protective action of guanosine, but caspase-3 activation will not be affected.

Several authors suggested recently that targeting programmed cell death in neurodegenerative diseases may lead to successful therapy [5, 31]. Drugs that will be effective in PD should protect the dopaminergic cells from apoptotic insult by either interrupting the signaling pathways that mediate cell death or activating the cell survival pathways. Our present results show that the non-adenine-based purine nucleoside guanosine, when added to MPP⁺-treated neuroblastoma cells, promotes their survival by inhibiting caspase-3 activation and DNA fragmentation via activation of the PI-3-K/Akt/PKB pathway. Furthermore, guanosine exerts these effects not only when it is pre- or co-administered with MPP⁺, but even when it is added to cells *after* caspase-3 is activated and DNA fragmentation is already in progress. Thus, these findings reveal a unique

neuroprotective effect of guanosine with a potential for effective pharmacological intervention in PD.

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