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# Guanosine protects human neuroblastoma SH-SY5Y cells against mitochondrial oxidative stress by inducing heme oxigenase-1 via PI3K/Akt/GSK-3β pathway

Tharine Dal-Cim [a,*,1], Simone Molz [a,1], Javier Egea [b], Esther Parada [b], Alejandro Romero [b,c], Josiane Budni [a], Maria D. Martín de Saavedra [b], Laura del Barrio [b], Carla I. Tasca [a,2], Manuela G. López [b,2]

[a] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Trindade, 88040-900 Florianópolis, SC, Brazil
[b] Instituto Teófilo Hernando, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Spain
[c] Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain

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ABSTRACT

Mitochondrial perturbation and oxidative stress are key factors in neuronal vulnerability in several neurodegenerative diseases or during brain ischemia. Here we have investigated the protective mechanism of action of guanosine, the guanine nucleoside, in a human neuroblastoma cell line, SH-SY5Y, subjected to mitochondrial oxidative stress. Blockade of mitochondrial complexes I and V with rotenone plus oligomycin (Rot/oligo) caused a significant decrease in cell viability and an increase in ROS production. Guanosine that the protective effect of guanosine incubated concomitantly with Rot/oligo abolished Rot/oligo-induced cell death and ROS production in a concentration dependent manner; maximum protection was achieved at the concentration of 1 mM. The cytoprotective effect afforded by guanosine was abolished by adenosine $A_1$ or $A_{2A}$ receptor antagonists (DPCPX or ZM241385, respectively), or by a large (big) conductance $Ca^{2+}$-activated $K^+$ channel (BK) blocker (charybdotoxin). Evaluation of signaling pathways showed that the protective effect of guanosine was not abolished by a MEK inhibitor (PD98059), by a $p38^{MAPK}$ inhibitor (SB203580), or by a PKC inhibitor (cheleritrine). However, when blocking the PI3K/Akt pathway with LY294002, the neuroprotective effect of guanosine was abolished. Guanosine increased Akt and p-Ser-9-GSK-3β phosphorylation confirming this pathway plays a key role in guanosine's neuroprotective effect. Guanosine induced the antioxidant enzyme heme oxygenase-1 (HO-1) expression. The protective effects of guanosine were prevented by heme oxygenase-1 inhibitor, SnPP. Moreover, bilirubin, an antioxidant and physiologic product of HO-1, is protective against mitochondrial oxidative stress. In conclusion, our results show that guanosine can afford protection against mitochondrial oxidative stress by a signaling pathway that implicates PI3K/Akt/GSK-3β proteins and induction of the antioxidant enzyme HO-1.



## 1. Introduction

In the central nervous system (CNS) guanosine, the endogenous guanine nucleoside, is available extracellularly through release from glial cells. In astrocytic cell cultures it has been reported that guanine nucleotides and guanosine can be released under basal or toxic conditions (Ciccarelli et al., 1999, 2001). Alternatively, nucleotides such as GTP, GDP and GMP can be metabolized by ecto-nucleotidases to produce extracellular guanosine (Caciagli et al., 2000; Ciccarelli et al., 2001). Guanosine and guanine nucleotides have been implicated in neuroprotection by exerting trophic effects (Ciccarelli et al., 2001; Decker et al., 2007), as well as by counteracting glutamate excitotoxicity *in vitro* (Molz et al., 2005, 2008; Oleskovicz et al., 2008) and *in vivo* (Schmidt et al., 2000, 2005, 2007). Guanosine also protects cultured rat astrocytes from staurosporine-induced apoptosis (Di Iorio et al., 2004) and SH-SY5Y cells from β-amyloid-induced apoptosis (Pettifer et al., 2004). In both cases, the anti-apoptotic effect of guanosine was mediated by stimulation of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) cell survival pathways.

*Abbreviations:* BK, large (big) conductance $Ca^{2+}$-activated $K^+$ channels; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GUO, guanosine; GSK-3β, glycogen synthase kinase 3β; $H_2$DCFDA, 2′,7′-dichlorofluorescein diacetate; HO-1, heme oxygenase-1; LY294002, 2-(4-morpholinyl)-8phenyl-1(4*H*)-benzopyran-4-4hydrochloride; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PKC, protein kinase C; PI3K, phosphatidilinositol-3kinase; ROS, reactive oxygen species; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SnPP, Sn(IV) protoporphyrin IX dichloride; ZM241385, 4-(2-[7-amino-2-{2-furyl} {1,2,4} triazolo {2,3a}{1,3,5}triazin-5-ylamino]ethylphenol.

* Corresponding author. Tel.: +55 48 3721 5046; fax: +55 48 3721 9672.
*E-mail address:* tharinedc@yahoo.com.br (T. Dal-Cim).

[1] These authors contributed equally to this study.
[2] These authors contributed equally to this study.

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Despite several evidences on the protective effect of the exact extracellular site of interaction and mechanisms of action for this nucleoside have not yet been fully characterized. Some studies suggest that guanosine participates in cell proliferation, neurite outgrowth and cellular protection by a mechanism that involves activation of adenosine receptors (Ciccarelli et al., 2000; D'Alimonte et al., 2007; Thauerer et al., 2010). However, we have recently demonstrated that guanosine-induced protection in hippocampal slices subject to oxygen/glucose deprivation depends on large conductance $Ca^{2+}$-activated $K^+$ (BK) channels activation (Dal-Cim et al., 2011).

Oxidative stress is a common mechanism of cell death in distinct cytotoxic models such as glutamate (Parfenova et al., 2006), β-amyloid (Tamagno et al., 2006), $MPP^+$ (Nicotra and Pavrez, 2000), or hydrogen peroxide-induced cytotoxicity (Kim et al., 2005). Oxidative stress has also been related to neurodegenerative diseases like Alzheimer and Parkinson's diseases (Mattson and Magnus, 2006) or stroke (Saito et al., 2005). Overproduction of reactive oxygen species (ROS) leads to damage of both neurons and astrocytes (Lin and Beal, 2006). In this study, we used an oxidative stress model evoked by mitochondrial activity disruption induced by blockade of mitochondrial complexes I and V, by using the combination of rotenone plus oligomycin-A (Rot/oligo) (Egea et al., 2007).

As mentioned above, the detrimental accumulation of ROS plays an important role in multiple pathologies; therefore, cells have developed an antioxidant armamentarium that includes a group of antixenobiotic genes termed phase II detoxification genes (Itoh et al., 1999) to maintain redox homeostasis. Among these genes is heme oxygenase-1 (HO-1), which is the rate-limiting enzyme that degrades the pro-oxidant heme group and produces equimolecular quantities of carbon monoxide (CO), iron, and biliverdin (BV). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. These three by-products have been related to cell protection against oxidative stress in distinct cellular models (Kim et al., 2005; Vitali et al., 2005). HO-1 is induced in response to a great variety of stress-inducing pathological conditions (Keyse and Tyrrell, 1987; Nimura et al., 1996). Moreover, studies in HO-1-deficient mice have confirmed that HO system is indispensable for cell protection against oxidative stress (Poss and Tonegawa, 1997). Furthermore, it has been shown that in the post-mortem brains of Alzheimer's disease patients, there was a HO-1 induction in neurons of the cerebral cortex and hippocampus and HO-1 was co-localized with neurofibrillar tangles (Schipper et al., 1995). Therefore, it is generally accepted that HO-1 represents a physiological protective mechanism against oxidative stress.

The purpose of this study was to evaluate how guanosine, a guanine nucleoside that can be secreted under physiological or pathological conditions, could protect cells against oxidative stress caused by disruption of the mitochondrial respiratory chain. Herein we show that guanosine-induced protective effect depends on activation of adenosine receptors and BK channels. Guanosine can afford cytoprotection under circumstances of cell vulnerability caused by mitochondrial disruption through an intracellular biochemical pathway that implicates the activation of PI3K/Akt leading to inactivation of glycogen synthase kinase-3β (GSK-3β) and induction of the antioxidant enzyme HO-1.

## 2. Materials and methods

### 2.1. Materials

Charybdotoxin, DPCPX (1,3-dipropyl-8-cyclopentylxanthine), F-12 nutrient mixture, Eagle's minimum essential medium (MEM), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), oligomycin A, rotenone, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole] and ZM241385 [4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3a}{1,3,5}triazin-5-ylamino]ethylphenol] were obtained from Sigma (Madrid, Spain). Chelerythrine and PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one] and LY294002 [2-(4-morpholinyl)-8phenyl-1(4*H*)-benzopyran-4-4hydrochloride] were purchased from Tocris (Biogen Científica, Spain). Sn(IV) protoporphyrin-IX dichloride (SnPP) was obtained from Frontier Scientific Europe (Lancashire, UK). Penicillin/streptomycin was purchased from GIBCO (Madrid, Spain). 2′,7′-dichlorofluorescein diacetate ($H_2$DCFDA) was obtained from Molecular Probes (Invitrogen, Madrid, Spain). Pyruvate and heat-inactivated fetal bovine serum (FBS) were purchase from Invitrogen. Bilirubin was purchase from Analisa Gold.

### 2.2. Culture and maintenance of SH-SY5Y cells

The neuroblastoma cell line SH-SY5Y was a kind gift from the Centro de Biología Molecular, Universidad Autonoma de Madrid/Consejo Superior de Investigaciones Cientificas (Madrid, Spain). SH-SY5Y cells were maintained in a 1:1 mixture of F-12 nutrient mixture (Ham 12) and Eagle's MEM supplemented with 15 nonessential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Madrid, Spain). SH-SY5Y cells were seeded into flasks containing supplemented medium, and they were maintained at 37 °C in 5% $CO_2$, humidified air. Stock cultures were passaged 1:3 twice weekly; i.e., one plate was divided (subcultured or split) into three plates. This procedure was performed twice a week. For assays, SH-SY5Y cells were subcultured in 48-well plates at a seeding density of $10^5$ cells per well, or in 96-well plates at a seeding density of $1 \times 10^5$ cells per well (Cañas et al., 2007). Cells were treated with the drugs in MEM supplemented with 1% fetal calf serum. Cells were treated with the drugs before confluence in MEM/F-12 with 1% FBS. Cells were used at a passage below 13.

### 2.3. Induction of cell toxicity and evaluation of cytoprotection induced by guanosine

Cell death was induced by adding a combination of rotenone (30 μM) plus oligomycin-A (10 μM) (Rot/oligo) for 24 h. When present, guanosine was added to SH-SY5Y at the same time as Rot/oligo and remained in the culture medium for the duration of experiments (24 h). Bilirubin (50 nM) was added in medium culture as the same time as Rot/Oligo and remained in culture medium for the duration of experiments (24 h). In experiments where enzyme inhibitors, adenosinergic receptors antagonists or potassium channel blocker were tested, SH-SY5Y cells were pretreated with these agents for 30 min prior to the addition of guanosine, which remained in the incubation medium throughout the duration of the experiment. These treatments included: the potent and selective inhibitor of the PI3K (LY294002, 10 μM); the selective inhibitor of the MAP kinase kinase (MEK) (PD98059, 10 μM); the inhibitor of PKC (cheleritrine, 0,1 μM); the inhibitor of $p38^{MAPK}$ (SB203580, 10 μM); HO-1 inhibitor, Sn(IV) protoporphyrin IX dichloride (SnPP, 3 μM). Adenosinergic antagonists: $A_1$ receptor antagonist (DPCPX, 100 nM); $A_{2A}$ receptor antagonist (ZM 241385, 50 nM); BK channel blocker (charybdotoxin, 100 nM). LY294002, PD98059 and cheleritrine were dissolved in and added to the culture medium at a final concentration of 0.01% dimethyl sulfoxide (DMSO).

### 2.4. Evaluation of cell viability by MTT reduction

SH-SY5Y cell viability was evaluated 24 h after Rot/oligo or Rot/oligo plus guanosine exposure. At the end of each experiment, 3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide (MTT Sigma Aldrich) was added to each well to a final concentration of