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[60]Fullerene derivative modulates adenosine and metabotropic glutamate receptors gene expression: a possible protective effect against hypoxia

Davide Giust^{1,2}, Tatiana Da Ros³, Mairena Martín^{1,4} and José Luis Albasanz^{1,4*}

Abstract

Background: Glutamate, the main excitatory neurotransmitter, is involved in learning and memory processes but at higher concentration results excitotoxic causing degeneration and neuronal death. Adenosine is a nucleoside that exhibit neuroprotective effects by modulating of glutamate release. Hypoxic and related oxidative conditions, in which adenosine and metabotropic glutamate receptors are involved, have been demonstrated to contribute to neurodegenerative processes occurring in certain human pathologies.

Results: Human neuroblastoma cells (SH-SY5Y) were used to evaluate the long time (24, 48 and 72 hours) effects of a [60]fullerene hydrosoluble derivative (t3ss) as potential inhibitor of hypoxic insult. Low oxygen concentration (5% O₂) caused cell death, which was avoided by t3ss exposure in a concentration dependent manner. In addition, gene expression analysis by real time PCR of adenosine A₁, A_{2A} and A_{2B} and metabotropic glutamate 1 and 5 receptors revealed that t3ss significantly increased A₁ and mGlu₁ expression in hypoxic conditions. Moreover, t3ss prevented the hypoxia-induced increase in A_{2A} mRNA expression.

Conclusions: As t3ss causes overexpression of adenosine A₁ and metabotropic glutamate receptors which have been shown to be neuroprotective, our results point to a radical scavenger protective effect of t3ss through the enhancement of these neuroprotective receptors expression. Therefore, the utility of these nanoparticles as therapeutic target to avoid degeneration and cell death of neurodegenerative diseases is suggested.

Keywords: Fullerenes, Adenosine receptors, Metabotropic glutamate receptors, Hypoxia, Neuroprotection

Introduction

Low oxygen availability in neuronal cells is the main cause of cognitive and physical deficiencies in patients who suffered ischemic toxicity in brain. Even though various factors are involved during the partial decrease of oxygen concentration in cells, the activation of specific factors named HIFs (hypoxia inducible-factors), which induce the transcription of specific genes involved

in several transduction pathways, mainly happen at low concentrations of oxygen in cells [1]. The consequent oxidative stress has been related to cell death processes, generally apoptotic, upon the formation of reactive oxygen species (ROS) and subsequently oxidative modifications of lipid, DNA, cell membrane proteins and other target molecules. The oxidative stress has been furthermore related to the development of some neurodegenerative diseases as Alzheimer's and Parkinson's [2]. The oxidative damage by free radicals is known to be originated in mitochondrial respiratory chain by complex I and III as source of superoxide anion (O₂⁻) [3]. However, these organelles have protective antioxidant enzymes that convert reactive species into no toxic ones [4]. Some of these enzymes belong to the family of superoxide dismutase (SOD),

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converting superoxide anion into H_2O_2 ; catalase, converting H_2O_2 into water, and other enzymes like glutathione reductase, whose expression is induced by ROS. Nevertheless some enzymes may change their function from antioxidant to oxidant, mainly due to the increase of iron and copper linked to brain aging [2]. Thus a dramatic increase in ROS has been related to cell death induced by oxidative stress and to neurodegenerative diseases as Alzheimer's [5]. Apoptotic cell death is mainly mediated by an increase in mitochondrial permeability to calcium which promotes cytochrome c release [6]. In that sense, it has been proposed that the first step of Alzheimer's disease is the oxidative damage originated by ROS [7].

Adenosine and metabotropic glutamate receptors (mGluR) have been largely studied for their implication in hypoxic injury. It has been repeatedly shown that adenosine can protect tissues against the negative consequences of hypoxia or ischemia [8,9], mainly by acting on the A_1AR . Hence, survival after a hypoxic challenge may be reduced if A_1AR s are absent or blocked [10]. Because the $A_{2B}AR$ promoter contains a functional binding site for hypoxia-inducible factor, the onset of hypoxia strongly induces its expression [11]. Moreover, a tissue protective role of $A_{2B}AR$ signaling during hypoxic conditions has been reported [12]. On the other hand, it has been described the role of mGluRs during hypoxic conditions. The protective effect of mGluRs of group I and the involvement of protein kinase C during hypoxic conditions have been reported [13]. In addition, Opitz and co-workers described in rat hippocampal slices dissimilar responses to hypoxic conditions depending on different group of mGluRs involved. Thus, the activation of group II was found to decrease cell recovery from hypoxia. However, a beneficial role of pre-activation of group I, as well as their detrimental role if activated during hypoxic conditions, was reported [14].

Fullerene derivatives are effective redox-active compounds towards ROS such as superoxide anion radical (O_2^-), hydroperoxide (ROOH), and hydroxyl radical ($\cdot OH$) produced during oxidative stress and responsible for different type of cell damages [15]. These properties are peculiar of fullerenes because of their general low energy of LUMO (Lowest Unoccupied Molecular Orbital) towards a high HOMO (Highest Occupied Molecular Orbital) level, conferring them radical quenching properties. For example, a polyhydroxylated C_{60} (fullerenol) was reported to have quenching activity for O_2^- [16]. Furthermore, it has been demonstrated that fullerenol presents a decreased ROS quenching activity with respect to the parent C_{60} since conjugated double bonds are widely broken. Thus, experiments with C_{60} -dimalonic acid, having properties rather similar to C_{60} , demonstrated its major efficacy in quenching radical species. This activity seems to be related to anti-aging and neuroprotective effect of fullerene

derivatives [17,18]. Therefore, [60]fullerene may be considered as the ideal candidate for protective properties in living systems subjected to oxidative stress even though the functionalization of [60]fullerene, used to increase its solubility in aqueous systems for better interfacing with biologic systems, is often accomplished with a partial loss of its antioxidant activity. Nevertheless, it was demonstrated that the bis-functionalization of [60]fullerene by means of azomethine ylide 1,3 dipolar cycloaddition leading to bis-pyrrolidine derivatives, confers a certain electronic stability to the fullerene carbon cage without depriving its quenching properties towards radical species [19]. In that sense, a series of highly hydrosoluble [60]fullerene bis-adducts were synthesized and encouraging results on assays in biological substrates have been obtained [20]. The aim of the present work was to study the possible protective effect of the *trans*-3 isomer of [60]fullerene (t3ss) in SH-SY5Y cells subjected to hypoxic conditions and the involvement of adenosine and metabotropic glutamate receptors in this t3ss-mediated effect.

Materials and methods

Materials

The hydrosoluble [60]fullerene bis-adduct *trans*-3 (t3ss) was synthesized as described in Figure 1. All reagents and solvents employed were from Sigma Aldrich (Germany). For an exhaustive reference of the product synthesis see [20].

Cell culture

Human neuroblastoma SH-SY5Y cells (acquired from American Type Cell Collection) were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum, and 1% of mixture antibiotic-antimycotic (Gibco, USA), humidified atmosphere with 5% CO_2 at 37°C. SH-SY5Y cells were subcultured (passages 3–12) on 10 ml Petri dish (Nunc, Denmark). At confluence, they were detached by Trypsin (Tryple Express, Gibco, USA), centrifuged and the cells re-suspended in complete growth medium and plated onto 24- or 96-well dishes (Nunc, Denmark) to have a final density per well of 2×10^5 and 3×10^4 cells, respectively.

MTT reduction assay

Cell viability was determined using an in vitro toxicology assay kit based on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) purchased from Sigma (Madrid, Spain), according to Mosmann [21]. Briefly, SH-SY5Y cells were seeded at 3×10^4 cells per well in 96-well dishes and exposed to hypoxic conditions (95% N_2 , 5% O_2) for desired period (6, 24, 48 and 72 h) and treated with different t3ss concentrations (25–150 μM). At the end of hypoxic insults or fullerene derivative treatment, cells were incubated in culture medium with MTT solution (5 mg/mL) at 37°C for 3 h. After

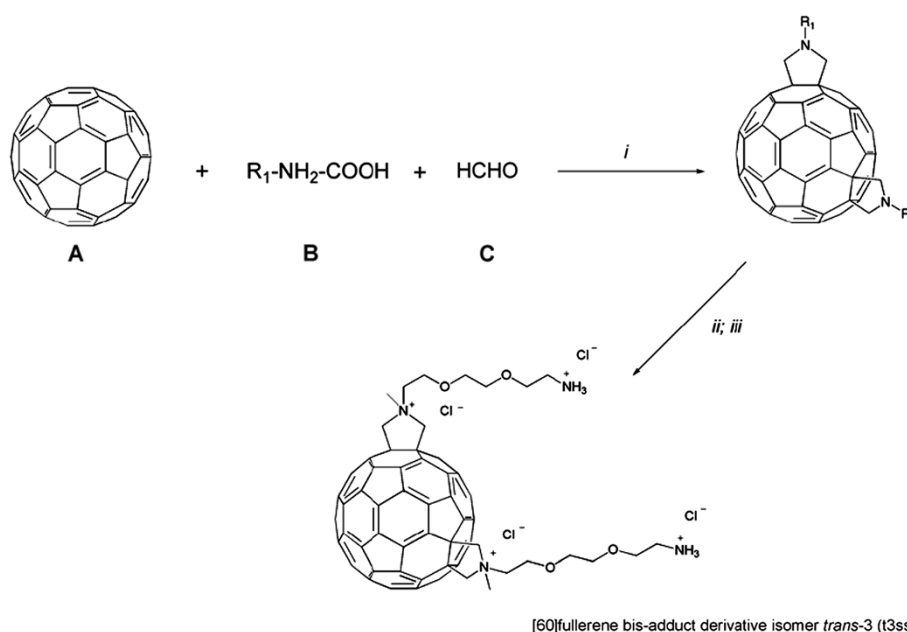


Figure 1 Synthetic scheme for *trans*-3 isomer [60]fullerene bis-adduct derivative (t3ss). R₁ = CH₂CH₂OCH₂CH₂OCH₂CH₂NHBoc. Reagent and conditions: (i) molar ratio A:B:C 1:2:5, toluene, 2 h at reflux, isolation of *trans*-3 isomer among the reaction mixture; (ii) CH₃I, CH₂Cl₂, 24 h at 80°C; (iii) HCl (g), CH₃OH, 20 min at 0°C. For more details and procedures see [20].

incubation, MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to the wells to dissolve formazan crystals. The plates were thoroughly shaken and the absorbance of each well was measured at 570 nm.

Total RNA isolation and preparation of cDNA

Total RNA was extracted from cells using an ABI 6100 Nucleic Acid PrepStation and chemicals according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Ratio of A₂₆₀/A₂₈₀ (RNA purity) was in the range 1.9-2.1. RNA concentrations were determined from the A₂₆₀. Total RNA was isolated and stored at -80°C. One microgram of total RNA was reverse transcribed using Applied Biosystems' High-Capacity cDNA Archive Kit according to manufacturer's protocol.

Quantitative real time RT-PCR analysis

To assess relative gene expression in SH-SY5Y neuroblastoma cells, quantitative real time RT-PCR analysis [22] was performed with an Applied Biosystems Prism 7500 Fast Sequence Detection System, using TaqMan universal PCR master mix according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). The TaqMan probes and primers for A₁ (assay ID: Hs00181231), A_{2A} (assay ID: Hs00386497), A_{2B} (assay ID: Hs00169123), mGlu₁ (assay ID: Hs00168250), mGlu₅ (assay ID: Hs00168275), and β-actin (assay ID: Hs99999903) were assay-on-demand gene expression

products (Applied Biosystems). TaqMan® Gene Expression Assays all have an efficiency of 1.0, which means a doubling of PCR product in every cycle is guaranteed. The TaqMan primer and probe sequences are packaged together in a 20× solution. The sequences are proprietary, so they are not available. The gene-specific probes were labeled using reporter dye FAM. A non-fluorescent quencher and the minor groove binder were linked at the 3' end of probe as quenchers. The thermal cycler conditions were as follows: hold for 20 s at 95°C, followed by two step PCR for 40 cycles of 95°C for 3 s followed by 60°C for 30 s. Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems) according to the 2^{-ΔΔCt} method. Briefly, expression results of a gene were normalized to endogenous control β-actin relative to a calibrator (normoxia sample), consisting of the mean expression level of the receptor gene as follows: 2^{-ΔΔCt} = 2^{-((Ct receptor gene - Ct actin) sample - (Ct receptor gene - Ct actin) calibrator)}. β-actin is an appropriated endogenous control as its expression did not change after t3ss exposure [23,24]. The results from 4-5 independent repeat assays, performed in different plates each using different cDNA's from the cultures analyzed, were averaged to produce a single mean value for each mRNA.

Statistical and data analysis

Data statistical analysis was performed using Student *t*-test and one-way ANOVA test and Dunnett post test

with the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA). Differences between mean values were considered statistically significant at $p < 0.05$.

Results and discussion

MTT assays showed a significant cell death after 6 and 24 h of hypoxic insult (5% O₂). Interestingly, cell viability was strongly recovered when t3ss was present during hypoxia being this effect concentration dependent (Figure 2). This protective effect of t3ss was detected and very similar at both 6 and 24 hours hypoxic insult.

Next step was to test the possible role of t3ss on gene expression modulation of receptors involved in neurodegenerative processes and diseases, such as adenosine (AR) and metabotropic glutamate receptors (mGluR) [25-27]. Even though the treatment of SH-SY5Y cells with 75 μ M t3ss derivative exhibited similar significant protective effect in both the 6 and 24 h period of hypoxia, as observed from MTT assay, in the shorter period of treatment, we did not observe any change in gene expression from qPCR (data not shown). Therefore, we assessed the effect of t3ss (75 μ M) on gene expression for 24 h and longer periods (48 and 72 h) of exposure to hypoxic insult.

Results from qPCR assays revealed a constant gene expression of all the receptors analyzed in the present study during the considered periods of time (24, 48 and 72 h) in normoxia (Figure 3). Only A_{2A} mRNA expression was

significantly enhanced at 24 h and longer time in hypoxia, pointing out to a possible role of A_{2A} in the viability loss detected at 24 h. A slight not significant increase of A_{2B} expression was also detected, while the others genes analyzed were not modified by hypoxia at any time assayed.

Interestingly, although t3ss did not change the expression of the analyzed genes when applied in normoxia, it significantly reduced the hypoxia-induced increase in A_{2A} expression. Moreover, t3ss also increased A₁ and mGlu₁ expression after 24 h in hypoxia, being this effect maintained at 48 and 72 h. However, t3ss did not modify A_{2B} and mGlu₅ gene expression during hypoxia.

These results show that the protective effect of [60]fullerene derivative against hypoxia, with increased effectiveness at higher concentrations, could be attributed, at least in part, to its modulatory effect on target receptor genes.

The neuroprotection induced by adenosine operates two well-known synaptic actions of A₁ARs that also occur under normoxic conditions and are of particular relevance in the case of hypoxia: a decrease of neurotransmitter release via inhibition of presynaptic calcium entry through the blocking of calcium channels [28], and postsynaptically inhibiting calcium entry via inhibition of NMDA (N-methyl-D-aspartate) receptors [29]. In line with this, the observed increase in A₁ARs gene expression by t3ss derivative, as it has been previously reported in SK-N-MC cells [23], could be related to the described protective role exerted by these receptors during hypoxic conditions [30].

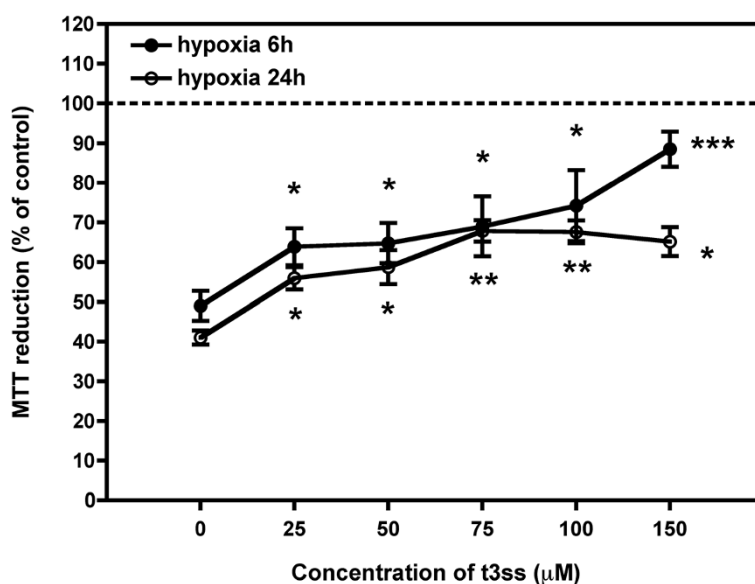
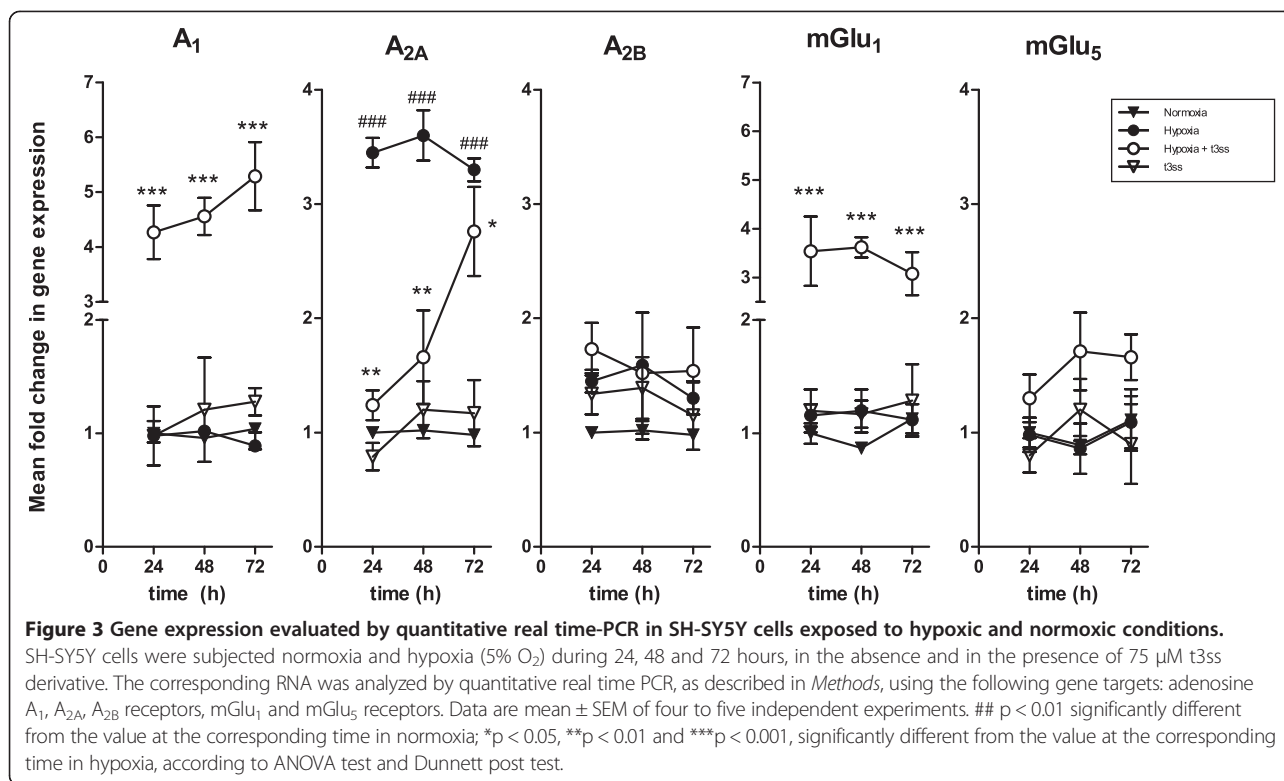


Figure 2 MTT reduction viability assay on SH-SY5Y cells after hypoxic conditions (5% O₂, 95% N₂) in the absence or the presence of t3ss derivative. SH-SY5Y cells were subjected to 6 and 24 h of hypoxic condition in the presence or absence of different concentrations of [60]fullerene hydrosoluble t3ss derivative (t3ss) (25–150 μ M). The obtained values are the average of three independent experiments, collected following MTT reduction assay protocol as described in *Methods*. The dotted line indicates the 100% of cells survival intended as cells growing in normal oxygen conditions (21% O₂). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different from cells survival in the absence of t3ss derivative according to Student *t*-test.



Our group previously demonstrated the implication of A₁ and A_{2A} receptors during hypoxic conditions in rat C6 glioma cells, where hypoxia (5% O₂) caused a significant decrease in A₁ receptors while A_{2A} receptors were significantly increased through a mechanism in which endogenous adenosine and tonic A₁ receptor activation is involved [31]. On the other hand, it has been demonstrated that during oxidative stress and related toxicity, the use of antagonists of adenosine A_{2A} receptors may be considered a valid therapeutic approach [32]. Moreover, it has been reported that A_{2A}ARs modulate glutamate uptake in cultured astrocytes and gliosomes [33] and stimulate glutamate release, thus promoting increased glutamate levels which could be excitotoxic mainly through NMDA receptors overactivation. However, protective effects related to survival of neurons have been attributed to the group I of metabotropic glutamate receptors, mainly mGlu₁ [34].

Fullerene derivatives may act on NMDA receptors and on the variation of Ca²⁺ homeostasis and inhibit the excitotoxic death, as it has been reported in cultured cortical neurons exposed to NMDA agonist [17]. The Ca²⁺ homeostasis has been reported to be also modulated by mGlu₁ receptors as they regulate voltage dependent calcium channels [35]. Thus, the effect of t3ss derivative on the modulation of mGlu₁ gene expression could be hypothetically related to the one reported for mGlu₁ antagonists, which reduce the damage in post ischemic conditions [36]. The

herein proposed possible modulatory effect of t3ss on NMDA receptors, and consequently on Ca²⁺ homeostasis could be related to the modulation of the expression of metabotropic glutamate receptor belonging to group I [37]. Both mechanisms of action of t3ss could thus lead to the protective effect observed during MTT viability assay, acting both synergically or independently one from the other.

In summary, results presented herein show for the first time that the modulation of adenosine and metabotropic glutamate receptors exhibited by a [60]fullerene derivative which could be, at least in part, responsible for its neuroprotective effect against hypoxic insults. Given that hypoxia and subsequent adenosine generation is likely an acute response to numerous injuries, including neurodegenerative diseases as Alzheimer's, the modulation of adenosine and metabotropic glutamate receptors by t3ss during hypoxia could open new lines of research in the field of therapeutics.

Conclusions

In the present paper SH-SY5Y cells were used as a model to hypoxic injury which caused cell death. Hypoxia has been related to neurodegeneration characteristic of many neurodegenerative diseases. Adenosine and metabotropic glutamate receptors have been shown to be impaired in Alzheimer's disease and other neurodegenerative diseases. Results from this work show that a hydrosoluble fullerene derivative (t3ss) is able to avoid the cell death during

hypoxia. In addition, fullerene derivative also increases adenosine A₁ and metabotropic glutamate receptors expression during hypoxia. Therefore, our results suggest fullerenes as neuroprotective molecules and open new therapeutic perspectives to be considered to avoid degeneration and neuronal death which is related to neurodegenerative diseases. However, additional studies will be necessary in order to confirm this hypothesis.

Competing interest

There is no conflict of interest including any financial, personal, or other relationships with other people or organizations.

Authors' contributions

DG carried out all experiments of viability and quantitative real time PCR. TR carried out the synthesis and purification of t3ss. MM and JLA conceived all the study, participated in its design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported in part by grants from the European Union through the Marie-Curie Research Training Network PRAIRIES (contract MRTN-CT-2006-035810) and the Ministerio de Economía y Competitividad from Spain (BFU2011-23034).

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Received: 9 May 2014 Accepted: 23 July 2014

Published: 14 August 2014

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doi:10.1186/s12951-014-0027-7

Cite this article as: Giust et al.: [60] Fullerene derivative modulates adenosine and metabotropic glutamate receptors gene expression: a possible protective effect against hypoxia. *Journal of Nanobiotechnology* 2014 **12**:27.

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