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Modulation of Adenosine Receptors by [60]Fullerene Hydrosoluble Derivative in SK-N-MC Cells

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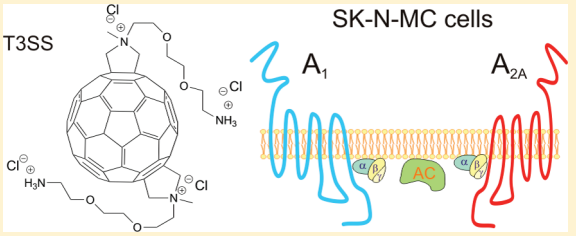
ABSTRACT: The most known fullerenes are spherical carbon compounds composed of 60 carbon atoms. C_{60} fullerenes have shown biochemical and biomedical properties in the last years such as as blockade of apoptosis and neuroprotection. The nucleoside adenosine has a neuroprotective role mainly due to inhibition of glutamate release, which is a neurotransmitter related to excitotoxicity and cell death. In the present work, we have determined the presence of adenosine receptors in SK-N-MC cells, a neuroepithelioma human cell line, and analyzed the effect of fullerenes in these receptors by using radioligand binding, immunoblotting, and quantitative real time PCR assays. Results demonstrated that SK-N-MC cells endogenously express adenosine receptors. Fullerene exposure of these cells did not affect cell viability measured by MTT reduction assay. However, adenosine A_1 and A_{2A} receptors were both increased in SK-N-MC cells after treatment. These results suggest for the first time the modulation of adenosine receptors after C_{60} fullerenes exposure.



KEYWORDS: Adenosine receptor, fullerene, SK-N-MC cells

From its discovery in 1985 by Curl, Kroto, Heath, O'Brien, and Smalley, C_{60} fullerenes have been extensively used in organic chemistry in the recent years, mainly due to their chemical properties and potential applications in many science fields.[1] Furthermore, in the past decade, a lot of experimental evidence has supported the use of C_{60} for biomedical and biochemical purposes.[2] Some biological properties of C_{60} derivatives have been investigated such as studies in photocleavage of DNA, neuroprotection of cells, blockade of programmed cell death,[3,4] and also as radical scavenger in induced oxidative stress.[5,6] In this sense, it has been shown that fullerenes acting as free radical scavengers[7] reduce excitotoxic and apoptotic death of cultured cortical neurons exposed to glutamate, a neurotransmitter which can also acts as an excitotoxin.[8] The greatest difficulty in testing the activity of C_{60} is its poor solubility in aqueous systems, due to apolar characteristics of its carbon cage;[9] to overcome this inconvenience, a new series of hydrosoluble C_{60} bis adducts have been synthesized and tested on the HIV virus with promising results[10] and their hemolysis properties were also tested.[11]

As extensively reported, there is much evidence about the role of adenosine receptors in neurodegenerative process of healthy neurons in the human brain[12] (for a review, see ref 13). Adenosine receptors are G-protein coupled receptors which have been classified in A_1, A_{2A}, A_{2B}, and A_3 receptors. A_1 and A_3 receptors inhibit adenylyl cyclase (AC) through Gi/o proteins, and A_{2A} and A_{2B} receptors activate AC through Gs proteins.[14,15] Adenosine receptors participate in many physiological responses such as as inflammatory processes, ischemia, and hypoxia,[16−18] in which they seem to have a protective role. These receptors are modulated in several neurodegenerative diseases, for instance, the up-regulation of A_1 receptors in Pick's disease[19] and Alzheimer's disease post-mortem brains,[20] where metabotropic glutamate receptors are also impaired.[21,22]

The aim of the present work was to study the activity of the C_{60} fullerene hydrosoluble bis-adduct isomer *trans*-3 (T3SS) on the expression of adenosine receptors in an in vitro model of tumoral SK-N-MC cells derived from human neuroepithelioma and its toxic effect, if any, on these cells.

RESULTS

Synthesis of C_{60} Fullerene Hydrosoluble bis-Adduct Isomer *trans*-3. The *trans*-3 isomer C_{60} fullerene bis-adduct derivative (T3SS) was obtained as described previously.[10] The diagram of the synthesis is represented in Figure 1.

SK-N-MC Cells Endogenously Express Adenosine Receptors. We first evaluated which adenosine receptors are naturally expressed by these cells. Thus, Western blotting assays on SK-N-MC cells using isolated plasma membrane preparations revealed the presence of A_1, A_{2A}, and A_{2B} subtypes of adenosine receptors (Figure 2A). The presence of adenosine A_{2A} receptors was confirmed by immunofluorescence in intact cells (Figure 2B).

In order to determine the relative gene expression of these adenosine receptors, we analyzed the RNA obtained from these

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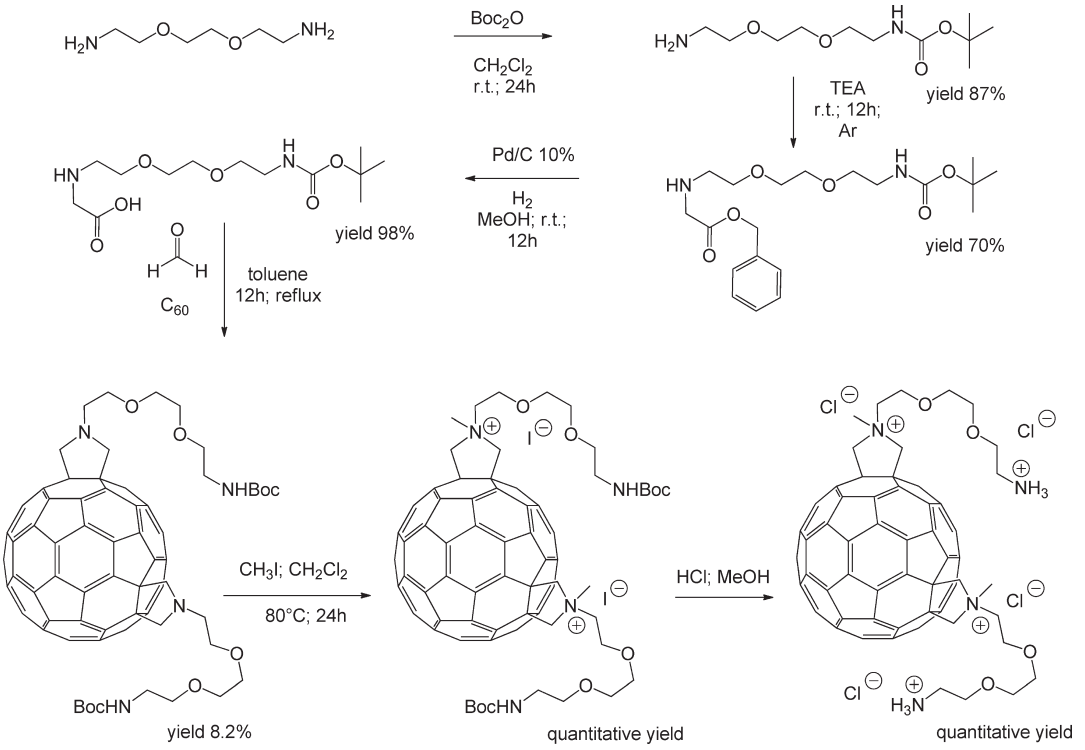

Figure 1. Scheme for the synthesis of *trans*-3 isomer [60]fullerene bis-adduct derivative (for more details and procedures, see ref 10).

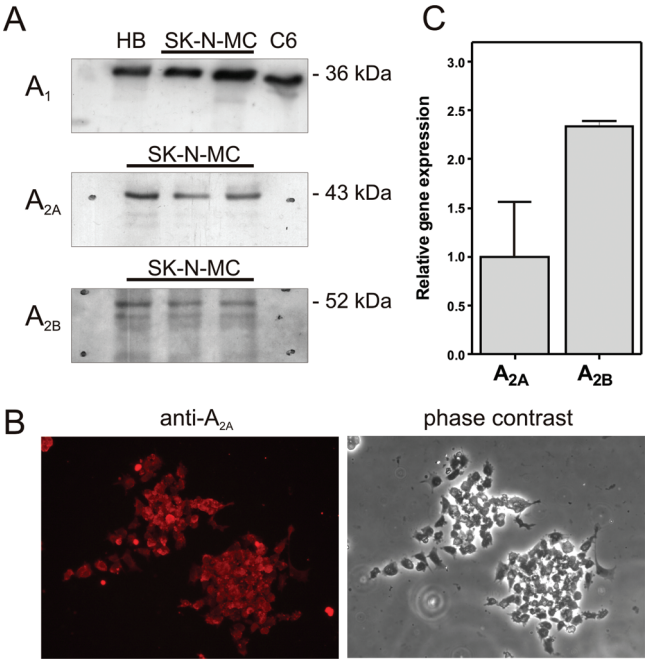

Figure 2. Adenosine receptors presence in SK-N-MC cells. (A) Adenosine receptors A_1, A_{2A}, and A_{2B} were specifically detected by Western-blotting, as stated in the Methods section, in plasma membranes from human brain (HB), C6 glioma cells (C6), and SK-N-MC cells (SK-N-MC). (B) Representative image showing A_{2A} receptors in intact cells by immunocytochemistry. (C) Relative expression levels of A_{2A} and A_{2B} receptors in SK-N-MC cells.

cells by using real time PCR technique, which revealed the presence of the specific gene coding for adenosine receptor types

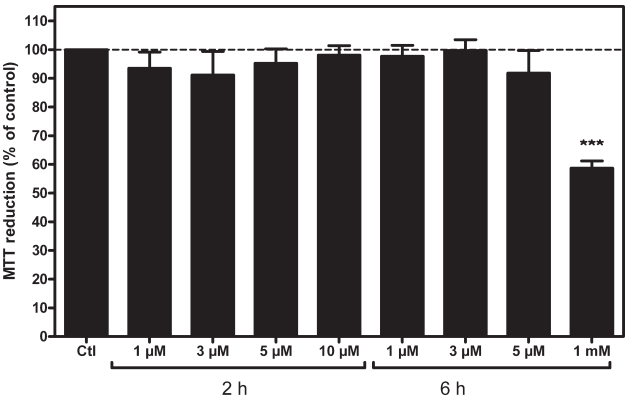

Figure 3. Evaluation of fullerene derivative effect on cell viability. SK-N-MC cells were exposed to fullerene derivative T3SS for the indicated time and dose, and cell viability was measured by the MTT reduction method, as stated in the Methods section. ***, $p < 0.001$ significantly different from control (Ctl) value.

A_{2A} and A_{2B} (Figure 2C). However, we were unable to detect the expression of the gene coding for A_1 receptors in the same conditions, even with different specific oligonucleotides for A_1 mRNA (TaqMan assays Hs00181231_m1 and Hs00379752_m1).

Effect of Fullerene Treatment on SK-N-MC. In order to study the effect that *trans*-3 derivative of C_{60} fullerene (T3SS) may have on these cells, we first evaluated T3SS effect on cell viability at different concentrations and time of exposure (Figure 3). Results show no toxicity for used concentration and observed times of exposure, except at 1 mM for 6 h; this concentration represents only a limit out of the range of acceptable concentrations usually used, and it has been tested only to establish the minimum toxic

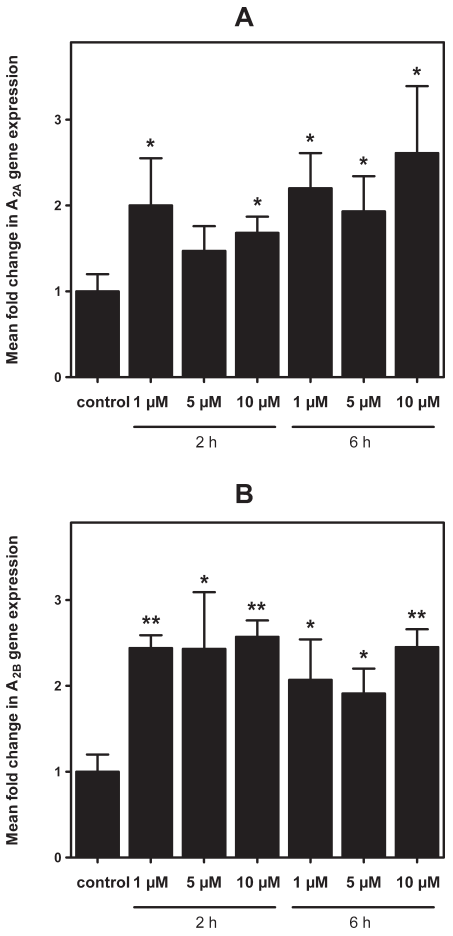

Figure 4. Effect of fullerene derivative on adenosine A_2 gene expression. Real time PCR assays were performed at indicated times and dose of fullerene derivative treatment, using specific oligonucleotides for A_{2A} (panel A) and A_{2B} (panel B) mRNA. Mean fold change calculation as well as specific set of primers are indicated in the Methods section. Data are mean ± SEM values obtained from 3−5 independent experiments performed in duplicate. *, $p < 0.05$ and **, $p < 0.01$ significantly different from control values.

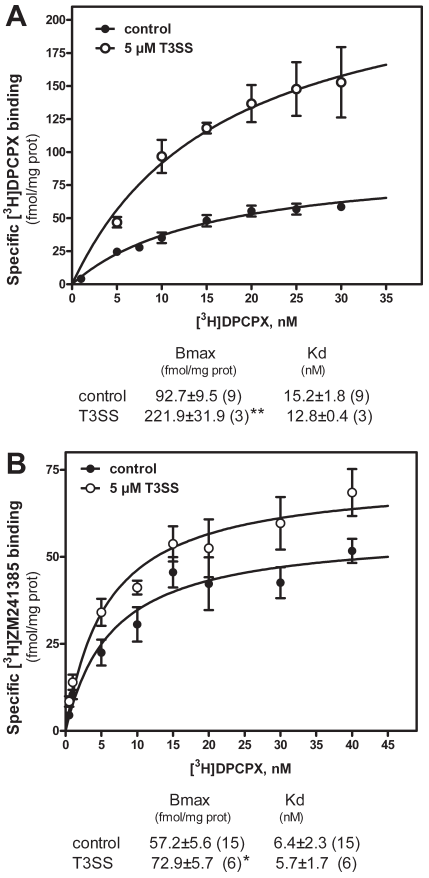

Figure 5. Effect of T3SS treatment on adenosine receptor specific binding. After preincubation with adenosine deaminase in order to remove endogenous adenosine, control and 5 μM T3SS-treated (6 h) SK-N-MC intact cells were incubated with different concentrations of [^{3}H]DPCPX or [^{3}H]ZM241385 to measure specific binding to A_1 (panel A) or A_{2A} (panel B) receptors. Nonspecific binding was determined in the presence of CPA and theophylline, as stated in the Methods section. Data are mean ± SEM values obtained from n (in brackets) separate experiments carried out in triplicate. Inset shows B_{max} and K_d values for control and treated cells. *, $p < 0.05$ and **, $p < 0.01$ significantly different from control values.

dose of T3SS for these cells. Subsequently, it was analyzed the possible variation in the expression of genes coding for adenosine receptors under different concentrations of T3SS (Figure 4). These results show a significant increase in adenosine A_{2A} and A_{2B} receptor gene expression in treated cells. Although we were unable to amplify A_1 receptor gene transcripts in these cells by real time PCR assays, A_1 receptor protein was measured by radioligand binding assay performed in intact cells. As Figure 5 shows, T3SS treatment, at the concentration of 5 μM, promotes an increase in the specific binding to both A_1 (Figure 5A) and A_{2A} (Figure 5B) receptors at the cell surface of intact cells after 6 h of 5 μM T3SS treatment.

DISCUSSION

In the last years, our group has focused on the study of mechanisms of neurodegenerative/neuroprotective processes, from the point of view of neurotransmitter receptors involved, mainly adenosine and glutamate receptors. The aim of the present work was to study the effect of the hydrosoluble T3SS fullerene derivative on cell viability and on adenosine receptors in SK-N-MC cells endogenously expressing these receptors.

A major concern in the potential use of fullerenes is their safety. Nanomaterial cytotoxicity in cells varies with chemical characteristics and surface properties of the molecule, including hydrophobicity, hydrophilicity, and surface area per molecule.[11,23] Mori et al.[24] could not find toxicity in rodents for C_{60} and C_{70} mixtures after oral administration of a dose of 2000 mg/kg BW and did not observe evidence of genotoxic or mutagenic potential in vitro. Moreover, the clearance kinetics of fullerene C_{60} nanoparticles from rat lungs, liver, and brain after intratracheal C_{60} instillation and inhalation C_{60} exposure has been analyzed. Pulmonary C_{60} burden decreased with time and depended on the C_{60} concentration administered, while the concentration of C_{60} in the liver and brain was below 8.9 ng/g tissue, the detection limit of the method employed.[25] A comprehensive review on fullerene toxicity is given by Kolosnjaj et al.[26] These authors review the works on fullerene toxicity and conclude that very little evidence gathered since the discovery of fullerenes indicates that C_{60} is toxic. Furthermore, it has been demonstrated that unmodified

C_{60} fullerenes are not toxic to cells and therefore could be useful for several biological applications.[27] However, the poor solubility of C_{60} fullerene in aqueous systems impedes its analysis for biochemical applications. The most versatile methodology to overcome this problem is based on the chemical modification of the sphere that leads to a wide variety of C_{60} derivatives, possessing different physical and chemical properties.[28] Thus, various functionalizations have been utilized both to increase the hydrophilicity (e.g., −OH, −COOH, $-NH_2$) and to prepare new compounds presenting biological and pharmacological activity; some examples are inhibition of HIV proteases and other enzymes, antimicrobial and antiapoptotic activities, DNA photocleavage, and its role as radical scavenger.[3] A new series of hydrosoluble C_{60} bis-adducted derivatives have been synthesized and tested on HIV virus with promising results.[10] Between them, *trans*-3 isomer (T3SS) has been used in the present work.

As mentioned above, a major concern in the potential use of fullerenes is their safety. Available data shows that pristine C_{60} has no acute or subacute toxicity in a large variety of living organisms, from bacterial and fungal to human leukocytes, and also in drosophila, mice, rats, and guinea pigs. However, some C_{60} derivatives can be highly toxic.[26] No significant cell viability loss has been detected in our SK-N-MC cell cultures at doses equal or less than 10 μM T3SS; even at 1 mM, which largely exceeds any therapeutic dose, viability loss was 40% ($p < 0.001$).

Results reported herein suggest a new approach in the study of biological properties of [60] fullerene derivatives and the investigation of their possible neuroprotective activity. It has been previously described that hydrosoluble fullerenes, such as fullerenols, inhibit ionotropic glutamate receptor binding, lowering the intracellular calcium, acting therefore as neuronal protective molecules.[29] Adenosine A_1 and A_2 receptors modulate neuronal and synaptic function in a range of ways that may make them relevant to the occurrence, development, and treatment of brain ischemic damage and degenerative disorder receptors.[30] These receptors are endogenously expressed in SK-N-MC cells, as here reported, and other proliferating cell lines such as as C6 glioma,[31] 28A,[32] and DDT1MF-2.[33] SK-N-MC cells present RNA transcripts of genes coding for adenosine A_{2A} and A_{2B} receptors subtypes. However, the corresponding mRNA for A_1 was undetectable in our conditions. A very recent study demonstrates that real-time polymerase chain reaction of the plant gene heat shock transcription factor was fully inhibited in the presence of a fullerene derivative $C_{60}(OH)_{20}$ possibly by the clear tendency for hydrogen bonding between the nanoparticle and both the dNTPs and ssDNA components of the polymerase chain reaction.[34] As experimental conditions were the same for both A_2 and A_1 receptor mRNA analysis, a direct effect of nanoparticle presence during PCR reaction can be ruled out. Then one possible explanation could be a very low rate of A_1 gene expression in these cells. In fact, this is, to our knowledge, the first study reporting adenosine receptor presence in SK-N-MC cells. Adenosine A_1 receptors were, at least, detected by radioligand binding assay and Western blotting and found to be abundant on the cell surface, even higher than A_{2A} levels, as B_{max} values from saturation data suggest.

Treatment of SK-N-MC cells using a single nontoxic concentration of C_{60} bis-adduct hydrosoluble derivative (T3SS) led to an increase in A_{2A} and A_{2B} receptor mRNA expression and higher A_1 and A_{2A} protein levels. It is usually considered that prolonged agonist exposure of G-protein coupled receptors results in a progressive loss of receptor (down-regulation), whereas antagonist exposure causes increased receptor level (up-regulation[35]). Most studies of the effects of antagonist treatment report an increase in total receptor number as an adaptive response to high antagonist exposure of receptor. Hydrosoluble fullerenes, such as fullerenols, inhibit binding to ionotropic glutamate receptors. Furthermore, AMPA receptors were found to be more sensitive to fullerenols than NMDA and KA receptors.[29] This antagonist-like role of fullerene could affect the adenosine receptor level in a similar way to that detected after antagonist treatment. In the case of adenosine A_1 receptors, up-regulation after chronic caffeine [36,37] and theophylline[38,39] treatment has been reported. However, previous experiments (data not shown) demonstrated that T3SS is unable to act as an A_1 receptor antagonist in radioligand binding assays, even at a range of high concentrations, including around 10 mM; T3SS cannot substitute CPA in the unspecific binding determination. This result excludes that T3SS may occupy the specific binding site of A_1, suggesting another way to regulate these receptors. In line with this, other C_{60} fullerene reported effects are a reduced contractile response for acetylcholine, histamine, and 5-HT in muscle of guinea pig and rat which may be due to a change in postreceptor processes.[40] Recent studies on the mechanisms involved in the regulation of adenosine receptor expression have shed light on the participation of transcription factors, such as nuclear factor-kappa B (NFκB), hypoxic-inducible factor 1, and cAMP-responsive element binding protein (CREB; for a review see ref 41). Thus, reactive oxygen species (ROS) can increase the expression of the A_1 receptor by activating NF kappa B regulatory site(s) on this gene and thereby enhance the cytoprotective role of adenosine.[42] Interestingly, the protective effect elicited by the hydrophilic fullerene derivative $C_{60}(C(COOH)_2)_2$ against H_2O_2 toxicity in cerebral microvessel endothelial cells (CMECs) seems to be mediated by a decreased phosphorylation of JNK and inhibition of ROS production. Consequently, $C_{60}(C(COOH)_2)_2$ treatment could regulate several downstream signaling events, including reduced activation of transcription factor c-Jun and caspase-3 and inhibition of PARP cleavage and mitochondrial cytochrome c release.[43]

Effector mechanisms other than the classical adenylate cyclase and phospholipase C mediated pathways are associated with the stimulation of adenosine receptors. For example, adenosine action can activate phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), and extracellular receptor signal-induced kinase (ERK).[44] It is well-known the cross-talking between the two types of adenosine receptors studied here, including the formation of heterodimers complexes between A_1 and A_{2A} receptors.[45,46] We have previously reported the modulation of A_1 and A_{2A} receptors during hypoxia in an in vitro model of C6 glioma cells culture, where tonic activation of A_1 by endogenous released adenosine promotes modulation on A_1 receptors but on A_{2A} levels as well.[47] A similar mechanism could take place after fullerene derivative treatment, promoting changes on receptor densities through postreceptor processes.

In summary, the present study provides clear and innovative evidence about the effect of this fullerene derivative on the adenosine receptors, by using SK-N-MC neuroepithelioma cells as a neurodegenerative model. The data show that these cells natively express both A_1 and A_2 receptors, which can be modulated by fullerene derivative exposure without significantly affecting cell viability. This low toxicity in cells even at the high concentration used confirms a good biocompatibility of this fullerene derivative in our in vitro model.

METHODS

Materials. The hydrosoluble [60]fullerene bis-adduct *trans*-3 (T3SS) was synthesized as previously described.[10,11] Cyclopentyl-1,3-dypropylxanthine 8-[dipropy-2,3-^{3}H(N)] ([^{3}H] DPCPX 120 Ci/mmol) was purchased from Amersham. [2-^{3}H](4-(2-[7-Amino-2-(2-fury1)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol ([^{3}H]ZM241385 27.4 Ci/mmol) was from Tocris. N^6-Cyclopentyladenosine (CPA), N^6-cyclohexyladenosine (CHA), and calf intestine adenosine deaminase (ADA) were obtained from Sigma. All other products were of analytical grade.

Cell Culture. SK-N-MC neuroepithelioma cells (purchased from ATCC, HTB-10)[48] were grown in modified Eagle's medium (MEM, Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM pyruvate, 1% nonessential amino acids, and antibiotics in a humidified atmosphere with 5% CO_2 at 37 °C, as previously reported.[49] Cells were subcultured on 10 mL Petri dishes and when at confluence were detached by using 4 mL of Trypsin (Tryple Express, Gibco) and centrifuged, and the pellet was resuspended in complete medium and cells seeded in 24- or 96-well plates and allowed to grow for 24 h before experiment, to have a final density per well of 2×10^5 cells and 3×10^4 cells, respectively.

Detection of Adenosine A_1 and A_{2A} Receptors in Intact Cells by Radioligand Binding Assay. Radioligand binding assays using intact cells were performed in 24-well plates at 80−90% of confluence (2×10^5 cells/well) as previously described[50] with some modifications. Briefly, cells were washed with serum-free MEM pH 7.4 and preincubated with 2 U/mL adenosine deaminase at 37 °C for 30 min to remove endogenous adenosine. After incubation, in order to measure adenosine A_1 receptors, the indicated concentrations of [^{3}H]DPCPX (1−30 nM) were added in the absence or the presence of 1 mM nonlabeled CPA to obtain nonspecific binding. Adenosine A_{2A} receptors were quantified by using the radioligand [^{3}H]ZM241385 (0.1−40 nM) and 3 mM theophylline to obtain nonspecific binding. After incubation at 25 °C for 2 h in a final volume of 250 μL, cells were washed with 500 μL of ice-cold medium and disrupted with 0.1% sodium dodecyl sulfate (SDS). Well contents were then transferred to vials and scintillation liquid mixture was added in order to measure radioactivity. At least two wells from each plate were reserved for protein concentration measurement.

MTT Reduction Assay. Cell viability was determined using an in vitro toxicology assay kit based on the reduction of 3-[4,5-dimetylthiazol-2-yl]-2,5-dipheniltetrazolium bromide (MTT) purchased from Sigma, according to Mosmann.[51] Briefly, SK-N-MC neuroepithelioma cells were seeded at 3×10^4 cells per well in 96-well plates. At the end of fullerene derivative treatment, SK-N-MC cells were incubated in culture medium with MTT solution (5 mg/mL) at 37 °C for 3 h. After 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.

Plasma Membrane Isolation. The isolation of plasma membranes was performed as previously described.[52] Cells were homogenized in isolation buffer (50 mM Tris-HCl pH 7.4, containing 10 mM $MgCl_2$ and protease inhibitors) and centrifuged for 5 min at 1000*g* in a Beckman JA 21 centrifuge. The supernatant was centrifuged for 20 min at 27 000*g*, and the pellet was resuspended in isolation buffer. The concentration of protein was measured by the method of Lowry, using bovine serum albumin as the standard.

Western Blotting Assay. Western blotting assay was performed as previously described[53] by using 50 μg of protein. After transfer to nitrocellulose membranes, proteins were incubated with rabbit polyclonal anti-A_1R (Calbiochem), anti-A_{2A}R (Upstate), and anti-A_{2B}R (Santa Cruz Biotechnologies) antibodies, all at dilution of 1:1000. The incubation with the secondary anti-rabbit IgG antibody (Dako) was carried out at a dilution of 1:5000. The monoclonal anti-β-actin antibody (Sigma) was used as a gel loading control (1:5000).

Total RNA isolation and Preparation of cDNA. Total RNA was extracted using an ABI 6100 Nucleic Acid PrepStation according to the manufacturer's protocol. All chemicals for the ABI 6100 were purchased from Applied Biosystems. Total RNA from cells was isolated and stored at −80 °C. The ratio of A_{260}/A_{280} (purity of RNA) was in the range 1.9−2.1. RNA concentrations were determined from A_{260}. One microgram of total RNA was reverse transcribed using the Applied Biosystems High-Capacity cDNA Archive Kit according to manufacturer's protocol.

Quantitative Real Time RT-PCR Analysis. To assess relative gene expression in SK-N-MC neuroepithelioma cells quantitative real time RT-PCR analysis[54] 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 Inc.) for the A_1, A_{2A}, A_{2B}, and β-actin genes for which validated TaqMan gene expression assays are available. The TaqMan probes and primers for A_1 (assay ID: Hs 00181231), A_{2A} (assay ID: Hs 00386497), A_{2B} (assay ID: Hs 00169123), and β-actin (assay ID: Hs 99999903) were assay-on-demand gene expression products (AppliedBiosystems). 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 nonfluorescent 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 $2^{-\Delta\Delta Ct}$ method. Briefly, expression results of a gene were normalized to internal control β-actin relative to a calibrator, consisting of the mean expression level of the receptor gene as follows: $2^{-\Delta\Delta Ct} = 2^{-((Ct\ receptor\ gene - Ct\ actin)sample - (Ct\ receptor\ gene - Ct\ actin)calibrator)}$. The results from 4−5 independent repeat assays, performed in different plates each using different cDNAs from the cultures analyzed, were averaged to produce a single mean quantity value for each mRNA.

Fluorescence Microscopy. Images for labeled fluorescent antibody of receptors A_{2A} have been obtained with a digital camera Leica DFC350FX, attached to a Leica DMI6000B (Leica Microsystem) fluorescent inverted microscope. A 20× HCX PL FLUOTAR objective and the LAS AF Lite software were used. The protocol for the fluorescent immune assay was performed as follows: SK-N-MC cells were grown in complete medium for 24 h before assay. After this time, complete medium was replaced with phosphate saline buffer (PBS), allowed to sit for 5 min, and repeated for three times, and the cells were fixed in 4% paraformaldehyde in phosphate buffer for 15 min. Cells were then incubated with bovine serum albumin (3% BSA) and Triton TX-100 (0.1% in PBS) at room temperature for 10 min and then incubated with primary antibody goat anti-mouse A_{2A} (Santa Cruz) diluted 1:100 in PBS/3% BSA for 3 h at 37 °C. Cells were then washed three times with PBS at 37 °C and incubated with secondary IgG anti-goat antibody labeled with fluorescein isothiocyanate (FITC, Invitrogen)) at dilution of 1:500 in PBS/% BSA for 30 min. Cells were then washed to remove nonfixed fluorescent antibody.

Protein Determination. Protein concentration for radioligand binding assay and Western blotting was measured by the method of Lowry, using bovine serum albumin as standard.

Statistical and Data Analysis. Data statistical analysis was performed using the Student's *t* test. Differences between mean values were considered statistically significant at $p < 0.05$. Saturation (B_{max}, K_d) binding curves were analyzed by performing Scatchard and nonlinear regression

analysis of binding data with the GraphPad Prism 5 program (GraphPad Software).

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Author Contributions

D.G. performed experimental procedures here reported and wrote sections. D.L. and I.B.-Y. researched the scientific literature. T.R. performed synthetic chemistry work. J.L.A. and M.M. oversaw and designed all experiments. J.L.A. compiled, reviewed, and edited the collated manuscript.

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Notes

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

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