Adenosine inhibits human astrocyte proliferation independently of adenosine receptor activation

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Title

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Abstract

In resting conditions brain adenosine concentrations are in nanomolar range, but can reach micromolar concentrations in stressful situations such as stroke, neurodegenerative diseases or hypoxic regions of brain tumours. Adenosine usually produces its cellular effects by activating surface receptors but can also act by reversing the reaction catalysed by S-adenosylhomocysteine (SAH) hydrolase, leading to SAH accumulation and inhibition of S-adenosylmethionine (SAM)-dependent methyltransferases. Astrocytes are essential in maintaining brain homeostasis but their pathological activation and uncontrolled proliferation plays a role in neurodegeneration and glioma. Adenosine can affect cell proliferation, but the effect of increased adenosine concentration on proliferation of astrocytes is not clarified and was addressed in present work.

Human astrocytes (HA) were treated for 3 days with test drugs. 30μM-Adenosine caused a 40%±3% ($P<0.05$, $n=5$) reduction in cell proliferation/viability, an effect reversed by 2U/ml-adenosine deaminase, but unchanged in the presence of antagonists of any of the adenosine receptors, suggesting an receptor-independent mechanism of action. Adenosine alone did not induce cell death, assessed by lactate dehydrogenase release and αII-Spectrin cleavage. 100μM-Homocysteine alone caused 16%±3% ($P<0.05$) decrease in HA proliferation. Combined action of adenosine and homocysteine decreased HA proliferation by 76%±4%, an effect higher ($P<0.05$) than the sum of the effect of adenosine and homocysteine alone (56%±5%). The inhibitory effect of adenosine on HA proliferation/viability was mimicked by two adenosine kinase inhibitors and attenuated in the presence of folate (100μM) or SAM (50-100μM). The results suggest that adenosine reduces HA proliferation by a receptor-independent mechanism probably involving reversal of SAH hydrolase-catalysed reaction.

1. Introduction.

Adenosine is generated in response to cell stress and cell injury and its concentration increases during episodes of hypoxia and inflammation (Lopes et al., 2011), as those occurring upon stroke or in brain tumours. Adenosine plays a regulatory role in the nervous system by decreasing neurotransmitter release and synaptic transmission, including excitatory synaptic transmission (Dunwiddie and Hoffer, 1980; Serpa et al., 2009; Dias et al., 2013; Pinto et al., 2016), protecting against neurotoxic insults (Ribeiro et al., 2003; Serpa et al., 2015) and modulating synaptic plasticity (Santschi et al., 2006; Dias et al., 2013). Most of these adenosine actions are mediated by activation of G protein-coupled adenosine receptors located at the extracellular membrane, specifically A_1, A_{2A}, A_{2B} and A_3 receptors (Dias et al., 2013; Serpa et al. 2015, 2014). However, adenosine may play relevant adenosine receptor-independent functions, such as modulation of epigenetic processes (Boison et al., 2013; Williams-Karnesky et al., 2013).

Adenosine can be formed in the extracellular milieu from adenine nucleotides, by the action of ecto-nucleotidases, and then enter the cell trough nucleoside transporters, or can be formed inside the cell by the action of 5'-nucleotidase, which hydrolyses AMP into adenosine, and by the action of the S-adenosylhomocysteine (SAH) hydrolase, which converts SAH into adenosine and homocysteine (for review see Fredholm et al., 2001). SAH is the final product of the S-adenosylmethionine (SAM)-dependent methyltransferases-catalysed reactions, in which SAM is the methyl group donor. The reaction catalysed by SAH hydrolase is near-equilibrium, so if intracellular levels of adenosine or homocysteine increase, SAH levels rise; SAH is a potent inhibitor of SAM-dependent methyltransferases (for review see Stipanuk, 2004). Intracellular adenosine is metabolized by the actions of adenosine kinase (ADK) and adenosine deaminase (ADA), which catalyse the phosphorylation and deamination of adenosine into AMP and inosine, respectively (Pignataro et al., 2008).

A recent study has shown that adenosine induces DNA hypomethylation in the brain through inhibition of transmethylation reactions (Williams-Karnesky et al., 2013). This link between adenosine and methyl group metabolism is relevant since impairment of methyl group metabolism is a risk factor for several brain pathologies, including

Alzheimer Disease and glioblastoma (Cascalheira et al. 2009, 2015; Semmler et al. 2006).

Astrocytes are essential for maintaining brain homeostasis, by supporting neuronal development and survival, regulating extracellular ions concentration, uptaking neurotransmitters and adenosine, regulating the CNS immune system and modulating neurotransmission (see Mederos et al., 2018; Guttenplan and Liddelow, 2018). Another role of astrocytes is preservation of tissue integrity after injury, which involves their activation into so called reactive astrocytes. Reactive gliosis is characterized by phenotypic alterations, increased size and number of astrocytes (Pekny and Nilsson, 2005). Although astrocytes have all these protective actions, their pathological activation and proliferation, may also play a role in neurodegeneration and tumorigenesis. Astrocytes in the adult brain do not proliferate and rest in the quiescent state of the cell cycle (the G0 phase), unless damage occurs to nerve tissues either from trauma or hypoxia as in stroke. In both cases, astrocyte proliferation produce glial scar formation by mechanisms still poorly understood (Koyama 2014). In other instances abnormal astrocyte proliferation may derange in the growth of astrocytomas, such as glioblastoma, or support migration and invasion of brain tumours (Matias et al. 2018). It is thus of outmost importance to deeply understand the regulation of the astrocyte cell cycle.

In stressful conditions, found in brain injury, after stroke, in neurodegenerative diseases or in hypoxic regions of brain tumours, adenosine can reach micromolar concentrations (Latini et al., 1999; Fredholm et al., 2001). These elevated concentrations of adenosine modulate astrocyte functions. Previous reports have shown that adenosine, by activating adenosine A_{2A} receptors, increases reactive gliosis, while activation of adenosine A_1 receptors decreases it (see Daré et al., 2007; Boison, 2008 for review). Activation of adenosine receptors also regulates glutamate uptake and the release of immune mediators by astrocytes (Daré et al., 2007). However, the effect of adenosine in astrocyte proliferation and viability is not fully clarified. Previous studies reported conflicting results. In rat mesencephalic astrocyte/neurons co-cultures, micromolar adenosine concentrations decreased the number of GFAP positive astrocytes while increasing the number of dopaminergic neurons (Michel et al.

1999), probably by activating A_{2B} adenosine receptors. On the other hand, Ciccarelli et al. (1994), reported an adenosine A_1 receptor-mediated decrease in rat astrocyte proliferation, while A_2 adenosine receptors agonists elicited an increase in astrocyte proliferation. However, the possibility of receptor-independent effects of increased levels of adenosine on human astrocytes proliferation and apoptosis, is not clarified and was investigated in the present work.

2. Materials and Methods.

2.1. *Cells and materials*.

Cerebral cortex human astrocytes (HA; passage one; Ref. 1800) and astrocyte medium were purchased from Sciencell. The drugs deoxycoformycin, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH 58261), *N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS 1754), *N*-[2-(2-Furanyl)-8-propyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl]-*N*'-(4-methoxyphenyl)urea (MRE 3008F20) and 4-Amino-5-(3-bromophenyl)-7-(6-morpholinopyridin-3-yl)pyrido[2,3-d]pyrimidine (ABT 702) were purchased from Tocris (Bristol, UK). Adenosine deaminase, L-homocysteine, S-(5'-Adenosyl)-L-methionine (SAM), folic acid and adenosine were from Sigma. 5-iodotubercidin, the anti-αII-spectrin mouse monoclonal antibody (1:1000), raised against human αII-spectrin (C-3; aa. 2368–2472; RRID: AB_2194351), were from Santa Cruz Biotechnology and the horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody was from Bio-Rad. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from VWR. The anti-GAPDH (6C5; RRID: AB_2536381) mouse monoclonal antibody (1:5000) was purchased from Thermo Fisher Scientific. Stock solutions of DPCPX (2 mM), SCH 58261 (5.1 mM), MRS 1754 (5 mM), MRE 3008F20 (50 mM), 5-iodotubercidin (32 mM), ABT 702 (100 mM) were prepared in dimethyl sulfoxide (DMSO) while adenosine (15 mM), deoxycoformycin (10 µM), SAM (109 mM) and homocysteine (370 mM) stock solutions were prepared in water. Stock solution of folic acid (113 mM) was prepared in 1M-NaOH.

2.2. *HA cultures and drugs incubation conditions*:

HA were plated at a 6500 cell/cm^2 density, on Poly-L-Lysine (1 mg/ml) coated plates, and grown at 37ºC, in a humidified atmosphere of 5% CO_2 and 95% atmospheric air, in astrocyte medium (AM) consisting of basal medium supplemented with 2% foetal bovine serum (FBS), 1% of astrocyte growth supplement (AGS) and 1% of penicillin (10,000 units/mL)/streptomycin (10,000 µg/mL) solution (all from ScienCell). For the MTT and LDH assays (see below) cells were grown in 96 wells plates, while for cell counting and Spectrin breakdown assays cells were grown in 6 wells plates. Two days after plating, cells were treated for 3 days with 30µM-adenosine, 100µM-homocysteine or both. 30µM adenosine concentration was chosen because this concentration of adenosine can be reached in pathophysiologic situation like ischemia (Latini et al., 1999), while adenosine concentrations higher than 30µM (e.g. Sai et al., 2006) are not physiological and its use has been criticised (see Ceruti and Abbracchio, 2013). Since adenosine and homocysteine are rapidly metabolised intracellularly (Di Iorio et al., 2002; Han et al., 2014), adenosine (30µM final concentration) and homocysteine (100µM final concentration) were reapplied every 8h throughout the 3 days incubation period, accordingly to Han et al. (2014) protocol. In experiments where selective adenosine receptor antagonists were used, they were applied to cells 30 min before the first adenosine application. In some experiments cells were also incubated, from the beginning of the 3 days incubation period, in the presence of exogenous adenosine deaminase (ADA, 2 U/ml), inhibitors of adenosine kinase (25 µM-5-idotubercidin or 15 µM-ABT 702), folate (100 µM) or SAM (50-100 µM). When present, SAM (50-100 µM final concentration) was reapplied 48h after the first application of SAM. When testing the effect of drug(s), a parallel control assay was performed, where the same volume of vehicle replaced the volume of drug(s) solution added to the well.

2.3. *Cell proliferation and cytotoxicity assays*:

MTT assay: Viability of HA cells was assessed by the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide. After incubation with drugs, culture medium was refreshed, MTT (0.5 mg/ml final concentration) was added and cells incubated for 3h at 37ºC. After this period, medium was discarded and the

formazan crystals, formed from MTT reduction catalysed by cell dehydrogenases, were solubilized with dimethyl sulfoxide (DMSO, Merck) and absorbance of the resulting solution was measured at 570 nm. The effect of a drug on cell viability was assessed as the absorbance at 570 nm corresponding for the drug assay, expressed as the percentage of the absorbance at 570 corresponding to the control assay. Since the final absorbance at 570 nm increases proportionally with the number of cells present in each well, the MTT assay is also an indirect measure of cell proliferation.

Cell counting: In some experiments cell proliferation was directly evaluated by counting cells with a Neubauer chamber. Briefly, after incubation with drugs, cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS), and treated with 0.2 ml of trypsin/EDTA solution (ScienCell) in 1ml of DPBS for 1-2 min to detach cells. Trypsinization was stopped by adding FBS (20% final concentration), cells were collected, centrifuged at 1000 rpm for 5min, resuspended in DPBS and counted with a Neubauer chamber after adding an equal volume of 0.4% trypan blue dye. In experiments were released LDH was quantified, prior to trypsinization of adherent cells, the culture medium in each well was collected, centrifuged at 1000 rpm for 5min, and non-adherent cells were counted after being resuspended in DPBS. Total cells, adherent + non-adherent, were then quantified.

Cytotoxicity LDH assay: After incubation with drugs, aliquots of culture medium were collected to assess lactate dehydrogenase (LDH) activity. LDH activity was measured at room temperature using a commercial kit (LDH cytotoxicity assay kit, Cayman Chemical, Ann Arbor, USA). Released LDH activity was expressed as µUnits/1000 total cells.

2.4. *Evaluation of apoptosis by western blot analysis of Spectrin Breakdown*:

As described previously (Jerónimo-Santos et al., 2015), after incubating with drugs, HA cells were washed with ice cold DPBS and lysed with lysis buffer containing: 1% NP-40, 50mM TrisHCl (pH 7.5), 150mM NaCl, 5mM ethylenediamine tetra-acetic acid (EDTA), 2mM dithiothreitol (DTT) and protease inhibitors cocktail (Roche). Cell lysates were centrifuged (16,000 g, 10 min) and the protein concentration in the supernatant was

measured by Bio-Rad DC reagent. All Samples (30µg of total protein) were separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (GE Healthcare). Membranes were stained with Ponceau S solution to evaluate for protein transference efficacy. Membranes were first blocked with a 3% BSA solution in TBS-T (137 mM NaCl, 20 mM Tris base and 0.1% Tween-20; pH 7.5), and then incubated overnight at 4 ºC with the primary antibodies (anti-αII-spectrin or GAPDH mouse monoclonal antibodies). Finally, membranes were incubated for 1 h at room temperature with the goat anti-mouse IgG secondary antibody. Immunoreactivity was visualized using an ECL chemiluminescence detection system (Amersham-ECL Western Blotting Detection Reagents from GE Healthcare). Bands intensities were quantified using ImageJ 1.45 software. The band intensities were normalized to the correspondent GAPDH bands (loading control). Extension of activation of cell death pathways in astrocytes was assessed by measuring the αII-spectrin breakdown, a cytoskeletal protein that is cleaved by both calpains, producing an 145 and an 150 kDa spectrin breakdown products (SBDPs), and caspase-3, forming an 150 and an 120 kDa SBDPs (see Zhang et al. 2009). The effect of the tested drugs on the 120 SBDP/αII-spectrin and 145-150 SBDP/αII-spectrin ratios was evaluated.

2.5. *Statistical Analysis*:

The values are expressed as mean ± S.E.M. from n independent cell cultures. Normality of data was assessed by the Kolmogorov-Smirnov and Shapiro-Wilk tests. All studied variables did not deviate from Normal distribution. The significance of the differences between the means obtained in two different conditions, or when comparing means with a fixed value, was evaluated by Student's t-test, where the paired Student's t-test was used whenever evaluating the significance of differences between two conditions tested in a paired way in the same experiment. When more than two different conditions were simultaneously considered, the one-way or two-way (when two factor were analysed) ANOVA were used, followed by the Tukey HSD post hoc test. Statistical significance was considered for $P<0.05$. The maximal effect (Emax) and the concentration of agonist producing half-Emax (EC_{50}) were calculated by fitting the agonist concentration-response curve data to a sigmoidal curve equation, through

non-linear regression analysis. Statistical analysis was performed using the SPSS Statistics for Windows program version 22.

3. Results.

3.1. *Adenosine decreases astrocyte proliferation/viability independently of receptor activation*.

Adenosine (30 μM) when applied for 3 days, caused a 40%±3% decrease on HA cell proliferation/viability (n=5; $P<0.05$ when compared with control, ANOVA followed by Tukey HSD test) as assessed by the MTT assay (Fig. 1). We then tested if this adenosine effect was receptor-mediated. For that purpose, the effect of Adenosine (30 μM) on HA proliferation/viability was assessed in the absence and in the presence of adenosine receptors selective antagonists. The following antagonists concentrations were used: 15μM for DPCPX, 10μM for SCH 58261, 0.1μM for MRS 1754 and 1.5μM for MRE 3008F20; these concentrations are high enough to block effectively the 30μM-adenosine activation of human A_1, A_{2a}, A_{2b} and A_3 adenosine receptors, respectively (see Fredholm et al., 2001). As shown in Figure 1A, the decrease on HA proliferation/viability produced by 30μM-adenosine was not modified by the presence of selective antagonist of any of the adenosine receptors, alone or in combination, (n=4; $P>0.05$ when comparing the effect of adenosine alone with the effect of adenosine in the presence of any of the antagonists or in the presence of all antagonists, ANOVA followed by Tukey HSD test) indicating that the effect of adenosine is not receptor-mediated.

Since adenosine phosphorylation into AMP by the action of ADK is the main route of intracellular adenosine removal in astrocytes (see Boison, 2013), we tested if inhibiting ADK, and consequently increasing endogenous adenosine intracellular concentration, would mimicked the effect of exogenously applied adenosine. Indeed 5-iodotubercidin (ITU, 25 μM), a high potency ADK inhibitor, decrease HA proliferation/viability by 82%±3% (n=3, see fig. 1A). Since ITU, although highly potent at inhibiting ADK, might also inhibit other kinases (e.g. Massillon et al., 1994), we also tested the effect of a

more selective although less potent ADK inhibitor, ABT 702 (Jarvis et al., 2000), on HA proliferation/viability. Performing a concentration-response curve for ABT 702 (0.5-15 µM), a maximal inhibition of 74%±19% and an EC_{50} of 12±5µM were obtained. ABT 702 at a concentration of 15 µM produced a 41%±3% (n=3) inhibition of the HA proliferation/viability (see fig. 1A), which was nearly identical to the inhibitory effect elicited by 30 µM-adenosine. The observation that inhibition of ADK mimicked the inhibitory effect elicited by exogenous adenosine on HA proliferation/viability, also indicates that the adenosine effect does not require its phosphorylation into AMP.

Since the hydrolysis of adenosine into inosine, by the action of ADA, is one of the catabolic reactions involved in adenosine metabolism, we investigated if the adenosine effect on HA proliferation/viability requires its conversion into inosine. For that purpose, the effect of adenosine was assessed in the absence and in the presence of exogenously added ADA (2U/ml). As shown in fig. 1B, when present, ADA completely reverted the effect 30 µM-adenosine (n=3; $P<0.05$ when comparing adenosine alone with adenosine in the presence of ADA, ANOVA followed by Tukey HSD test), while ADA alone did not modify HA proliferation/viability (n=3; $P>0.05$ when compared with control, ANOVA followed by Tukey HSD test). These results indicate that the effect of adenosine on HA proliferation/viability is not mediated by its conversion into inosine. On the other hand, when we tried to increase endogenous adenosine by inhibiting its degradation by endogenous ADA using deoxycoformycin, a potent and selective ADA inhibitor, we didn't observe modification of HA proliferation/viability. The HA proliferation/viability obtained in the presence of 1nM-deoxycoformycin – a concentration which fully inhibits ADA (Klohs and Kraker, 1992) – was 106%±11% (n=3) of the control, which was not significantly different from the control proliferation/viability ($P>0.05$, Student's t-test). This result suggests that ADA might not play a major role on adenosine catabolism in HA, in contrast to ADK (see Boison 2013).

3.2. *The inhibitory effect of adenosine on astrocyte proliferation/viability is potentiated by homocysteine and attenuated by methyl-group donors*.

Since the effect of adenosine on HA proliferation/viability was not adenosine receptor-mediated, the possibility that the adenosine effect might involve reversal of the near-equilibrium reaction catalysed by SAH hydrolase, was investigated. The effect of adenosine (30 μM) on HA proliferation/viability was assessed in the absence and in the presence of homocysteine (100 μM). As shown in fig. 2A, the inhibitory effect of combined adenosine and homocysteine on HA proliferation/viability was 76%±4% (n=5), which was higher than the sum of the effects of adenosine and homocysteine alone (56%±5% inhibition, n=5; $P<0.05$ when compared with the % inhibition produced by combined adenosine and homocysteine, ANOVA followed by Tukey HSD post hoc test), indicating a potentiation of the adenosine effect by homocysteine. This result suggests that the adenosine effect on HA proliferation/viability involves its combination with homocysteine to produce SAH, a reaction catalysed by SAH hydrolase; since this reaction is near equilibrium (see Stipanuk, 2004) an increase in both the adenosine and homocysteine concentrations would produce a multiplicative, rather than additive, increase on SAH intracellular concentration.

If the adenosine inhibition of HA proliferation/viability involves reversal of the SAH hydrolase catalysed reaction, leading to SAH accumulation and consequent inhibition of SAM-dependent methyltransferases, then it would be expected that increasing methyl-group donors concentration would reverse or attenuate the inhibitory effect of adenosine. We started by studying the influence of increasing folate concentration on the inhibitory effect of adenosine on HA proliferation/viability. In the cell, folate can be converted to N^5-methyltetrahydrofolate, a coenzyme for the methionine synthase, leading to an increased remethylation of homocysteine to methionine, the precursor of SAM (see Stipanuk, 2004). As shown in fig. 2B, addition of folate (100 μM) produced a decrease, from 41.2%±4.8% in the absence to 16%±10% in the presence of added folate, of the inhibitory effect of adenosine (30 μM) on HA proliferation/viability ($P<0.05$; ANOVA followed by Tukey post hoc test). In the presence of added folate (100 μM), adenosine (30 μM) did not significantly modify HA proliferation/viability; added folate (100 μM) alone had no effect on HA proliferation/viability (see fig. 2B). In another set of experiments, we investigated if increasing SAM concentration, the co-substrate of methyltransferases, would affect the inhibitory effect of adenosine on HA

proliferation/viability. As shown in fig. 2C, the inhibitory effect of adenosine (30 μM) on HA proliferation/viability was decreased from 33.2%±4.2%, in the absence, to 18.2%±4.6% or 17.4%±4.2%, in the presence of respectively 50 μM or 100 μM SAM ($P<0.05$; n=7-8, ANOVA followed by Tukey post hoc test). The HA proliferation/viability obtained in the presence of 50 μM or 100 μM SAM alone was 88%±9% or 77%±8% of control, respectively, which was not significantly different from the control HA proliferation/viability ($P>0.05$; n=7-8, ANOVA followed by Tukey post hoc test).

3.3. *Adenosine decreases HA proliferation but does not increase cell death*.

Although adenosine has shown to decrease HA proliferation/viability, as assessed by the MTT assay, the above data do not allow to discriminate between an inhibitory action of adenosine upon cell proliferation and an enhancement of cell death. To answer this question we assessed cell proliferation, by counting the number of cells, and cell death, by measuring released LDH activity. In figure 3A are represented the number of cells/cm^2, obtained after incubating HA for 3 days with adenosine (30 μM), homocysteine (100 μM), adenosine plus homocysteine, or the ADK inhibitor ABT 702 (15 μM). Adenosine, homocysteine and ABT 702 all produced a decrease in the number of cells, which was similar to the decrease produced by each of these molecules in cell proliferation/viability assessed by the MTT assay (compare fig. 3A with fig. 2A). On the other hand, as shown in figure 3B in another set of experiments, neither adenosine (30 μM) alone nor the ADK inhibitor ITU (25 μM) affected cell death assessed by released LDH activity. However, homocysteine (100 μM) alone produced a marked increase in LDH release, which was potentiated by the presence of adenosine (fig. 3B). These results suggest that the decrease in cell number produced by adenosine alone is consequence of a decrease in cell proliferation, while the decrease on cell number produced by homocysteine is due, at least in part, to increased cell death.

Since LDH release does not discriminate cell death by necrosis or late apoptosis, the effect of adenosine (30 μM), homocysteine (100 μM), adenosine plus homocysteine and ABT 702 (15 μM) on αII-Spectrin breakdown was investigated in HA cells (Fig. 4). Similar to what was observed for LDH release, neither adenosine alone nor the ADK

inhibitor ABT 702 modify the SBDP120/αII-Spectrin or the SBDP145-150/αII-Spectrin ratios, suggesting that increased levels of adenosine did not *per se* induce HA apoptosis or necrosis (Fig. 4B and 4C). Homocysteine, both alone and in the presence of adenosine, increased the SBDP120/αII-Spectrin ratio, indicating increased caspase-3 activation and apoptosis (Fig. 4B). A significant increase in the SBDP145-150/αII-Spectrin ratio was only observed when homocysteine and adenosine were both present (Fig. 4C).

4. Discussion.

The present work shows, for the first time, an inhibition of human astrocyte proliferation by increased adenosine concentrations that is not mediated by activation of adenosine receptors. The effect of adenosine on astrocyte proliferation was potentiated by homocysteine and attenuated by the methyl group donors folate and S-adenosylmethionine. Adenosine alone did not induce cytotoxicity, but potentiated homocysteine cytotoxic effect on astrocytes.

Previous reports of the effect of adenosine on astrocyte proliferation present conflicting results. In rat mesencephalic astrocyte/neurons co-cultures, micromolar adenosine concentrations decreased the number of GFAP positive astrocytes while increasing the number of dopaminergic neurons (Michel et al. 1999), probably by activating A_{2B} adenosine receptors. On the other hand, Ciccarelli et al. (1994), reported an adenosine A_1 receptor-mediated decrease in rat astrocyte proliferation, while A_2 adenosine receptors agonists elicited an increase in astrocyte proliferation. Rathbone et al. (1992) also described an adenosine A_2 receptor-mediated increase in chick astrocytes proliferation. The differences among the results obtained by the different groups in these previous reports and between these previous studies and the results obtained in the present work, might be due to the use of astrocytes from different species and/or different astrocyte culture conditions. Since all previous studies of the adenosine effect on astrocyte proliferation only focus on adenosine receptors-mediated effects, the inhibition of HA proliferation by adenosine described in the present work, independent of receptor activation, constitute a novel mechanism by which elevated concentrations of adenosine regulate astrocyte proliferation.

The results obtained in the present study, strongly suggest that adenosine inhibits astrocyte proliferation by a receptor-independent mechanism, probably mediated by reversal of the SAH hydrolase-catalysed reaction, leading to accumulation of SAH and consequent inhibition of SAM-dependent methyltransferases. The proposed mechanism is depicted in figure 5. The results which support this conclusion are:

i) The effect of adenosine is not modified in the presence of antagonists of any of the adenosine receptors, alone or in combination.

ii) The effect of combined adenosine and homocysteine on astrocyte proliferation/viability is higher than the sum of the individual effects of adenosine and homocysteine. This suggests that the adenosine effect involves its combination with homocysteine to produce SAH, by the action of SAH hydrolase, which catalysed a near equilibrium reaction (Stipanuk, 2004), and therefore an increase in both the adenosine and homocysteine concentrations would produce a multiplicative increase on SAH intracellular concentration, rather than an additive increase which would be the case if the effects of both molecules were independent.

iii) The adenosine-induced inhibition of HA proliferation was attenuated by the presence of exogenous folate and SAM. Folate, after being converted to N^5-methyltetrahydrofolate, acts as coenzyme for the methionine synthase, leading to an increased remethylation of homocysteine to methionine, the precursor of SAM (see Stipanuk, 2004). SAM is the methyl group donor for the methyltransferases-catalysed reactions.

A previous study performed in the RCR-1 rat astrocytoma cell line (Sai et al. 2006), reported an adenosine-induced decrease on cell viability by two independent mechanisms: activation of adenosine A_1 receptors and activation of AMPK after intracellular adenosine phosphorylation into AMP by ADK. However the conclusions made in this study had been questioned by others (see Ceruti and Abbracchio 2013), since Sai et al. (2006) used extremely high adenosine concentrations (1mM) and obtained a very low reversal of the adenosine-mediated effect by both A_1 receptor antagonist (8-CPT) and an ADK inhibitor (AMDA), which leaves the possibility that the reported adenosine effect might also involve reversal of SAH hydrolase-catalysed reaction. In the present study a 30 µM adenosine concentration was used, since this concentration has been reported to be reached under pathophysiological situations such as ischemia (Latini et al. 1999; Fredholm et al. 2001). On the other hand, the observation in the present study, that two selective ADK inhibitors (5-iodotubercidin and ABT 702), in concentrations able to elicit endogenous adenosine intracellular accumulation (Boison 2013; Jarvis et al. 2000), mimicked the inhibitory effect of

exogenously added adenosine on astrocyte proliferation, indicates that adenosine does not requires ADK-catalysed phosphorylation into AMP to produces its inhibitory effect on astrocyte proliferation.

The observation in the present study that adenosine alone did not modify cell death, indicates that the decrease in cell number induced by adenosine reflects a decrease in cell proliferation rather than an increase in cell death. This contrast to the observed effect of homocysteine, which, although produced a much smaller reduction in HA cell number than adenosine, was able to increase cell death. Cell death was evaluated by measuring released LDH activity – which reflects cell necrosis and late apoptosis (Parhamifar et al. 2013) – and increase in αII-Spectrin breakdown – which measures both cell apoptosis and necrosis (Zhang et al. 2009). The increase in SBDP120/αII-Spectrin ratio by homocysteine reflects an increase in caspase 3-mediated apoptosis, while the increase in SBDP145-150/αII-Spectrin ratio and in LDH release suggests that an increased cell necrosis might also be involved in the homocysteine effect. Curiously, the cytotoxic effect of homocysteine in HA was potentiated by adenosine. Although both adenosine and homocysteine may decrease HA proliferation by reversal of SAH hydrolase-catalysed reaction, the results suggest that part of the effect of homocysteine in decreasing HA number involves increased cell death, which is not unexpected since increased homocysteine levels also increase oxidative stress (see Mattson and Shea 2003). Contrasting with the results obtained in the present study, Di Iorio et al. (2002) reported an increase of apoptosis by micromolar adenosine concentrations in rat astrocytes, an effect partially mediated by adenosine A_3 receptors and in part mediated by an intracellular mechanism probably involving accumulation of SAH and reduction of the SAM/SAH ratio. Again, the difference between the results obtained by Di Iorio et al. (2002) and the results obtained in the present work, may reflect the use of astrocytes from different species and/or different astrocyte culture conditions.

The inhibition of HA proliferation by adenosine observed in the present study, suggests that adenosine may play a relevant role in situations of elevated adenosine concentrations, such as in hypoxia, inflammation or brain injury, preventing uncontrolled astrocyte proliferation that might occur in neurodegenerative diseases

and brain tumours. Receptor-mediated actions of adenosine have been described in glioblastoma, the most aggressive astrocyte-derived brain tumour, but the results are conflicting. Activation of A_1 and A_{2B} adenosine receptors decreased proliferation and induced apoptosis of glioblastoma cancer stem cells (CSC) while in non-CSC glioblastoma cell lines activation of either A_1, A_{2B} or A_3 adenosine receptors increased cell proliferation (Daniele et al. 2014; Rathbone et al. 1992). On the other hand, in glioblastoma CSC under hypoxic conditions, Liu et al. (2014) reported a pro-proliferative action of adenosine, trough A_{2B} receptor activation.

The results obtained in the present study, support the link between adenosine and methyl group metabolism, which is relevant since impairment of methyl group metabolism is a risk factor for several brain pathologies, including Alzheimer Disease and glioblastoma (Cascalheira et al., 2009, 2015; Semmler et al., 2006). Previous studies have shown that adenosine can regulate cell functions by interfering with the transmethylation pathway. In the brain, adenosine was able to reduced epileptogenesis by inducing DNA hypomethylation through inhibition of transmethylation reactions (Williams-Karnesky et al., 2013). Adenosine, by increasing SAH accumulation and inhibition of SAM-dependent methtyltransferases, was also able to induced tumour cells cytotoxicity and DNA hypomethylation in a mouse lymphoma cell line (Kredich and Martin, 1977) and induced apoptosis in a hepatoma cell line (Hermes et al. 2007; Liu et al. 2016). SAM-dependent methyltransferases mediate epigenetic modifications by methylation of DNA, histones, mRNA and tRNA, besides regulating catecholamines concentration and phospholipid turnover (Stipanuk, 2004; Struck et al., 2012). Since these enzymes have such diverse targets, the actual mechanism(s) by which adenosine regulate HA proliferation, described in the present study, is unknown but deserves future investigation.

In conclusion, the results obtained in the present study show that increased adenosine concentrations inhibit human astrocyte cell proliferation by an adenosine receptor-independent mechanism, without affecting cell death providing that homocysteine levels stay low. The results also strongly suggest that this effect is a consequence of reversal of the near-equilibrium SAH hydrolase-catalysed reaction, and subsequent inhibition of SAM-dependent methyltransferases. This study thus opens a

new avenue towards the design of novel strategies to fight dysfunctional astrocytic proliferation.

Acknowledgements

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Figure 1

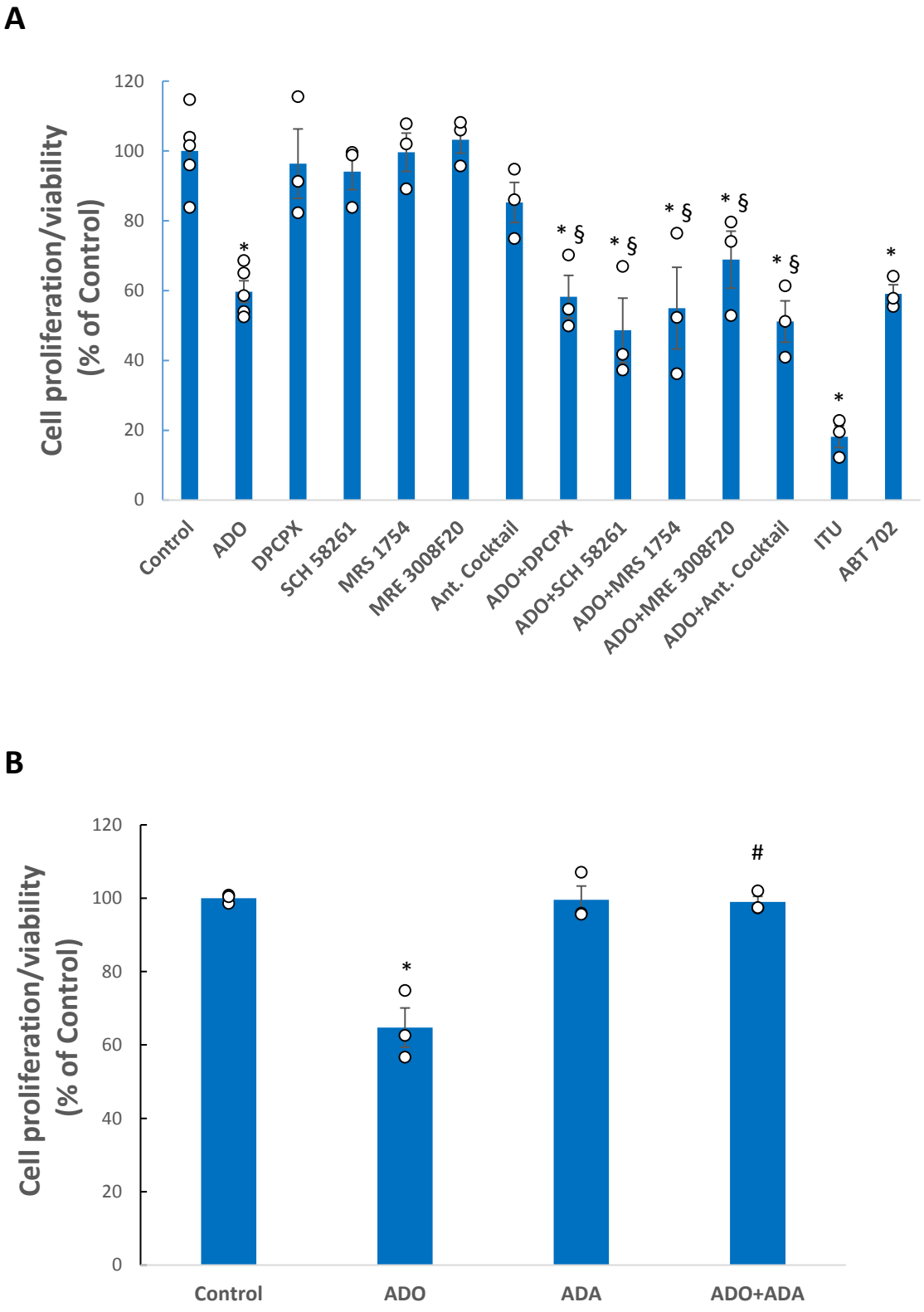
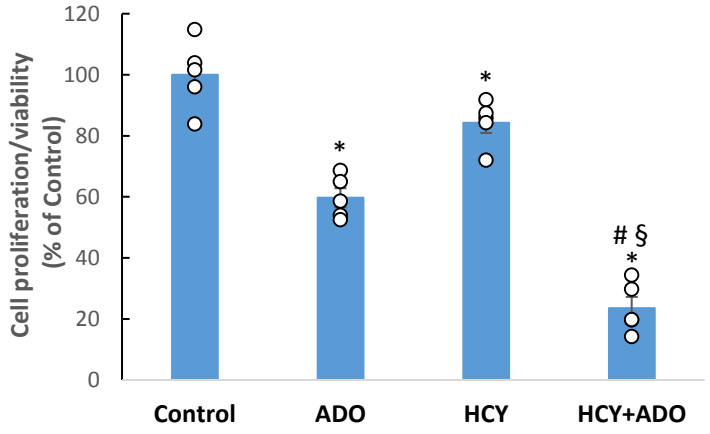
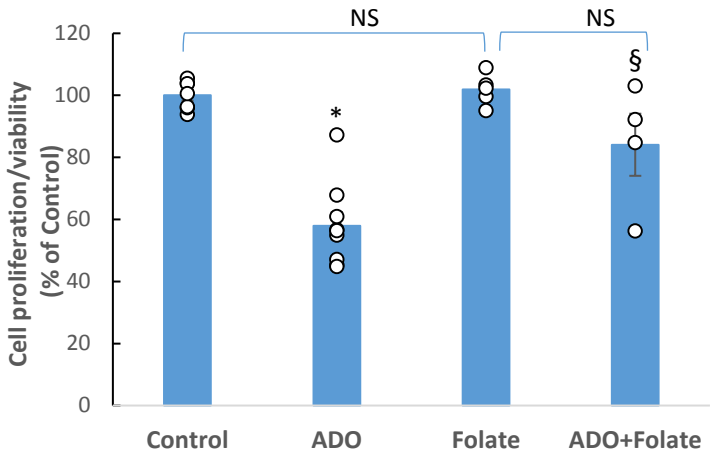

A
Cell proliferation/viability (% of Control)
120
100
80
60
40
20
0
Control
ADO
DPCPX
SCH 58261
MRS 1754
MRE 3008F20
Ant. Cocktail
ADO+DPCPX
ADO+SCH 58261
ADO+MRS 1754
ADO+MRE 3008F20
ADO+Ant. Cocktail
ITU
ABT 702
B
Cell proliferation/viability (% of Control)
120
100
80
60
40
20
0
Control
ADO
ADA
ADO+ADA

Figure 2

A


Cell proliferation/viability (% of Control)
120
100
80
60
40
20
0
*
*
*
§
Control
ADO
HCY
HCY+ADO

B


Cell proliferation/viability (% of Control)
120
100
80
60
40
20
0
NS
NS
*
§
Control
ADO
Folate
ADO+Folate

C

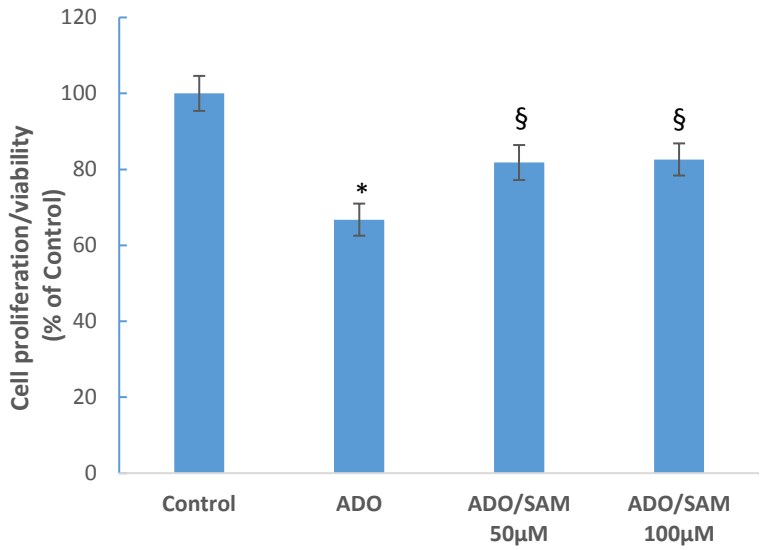
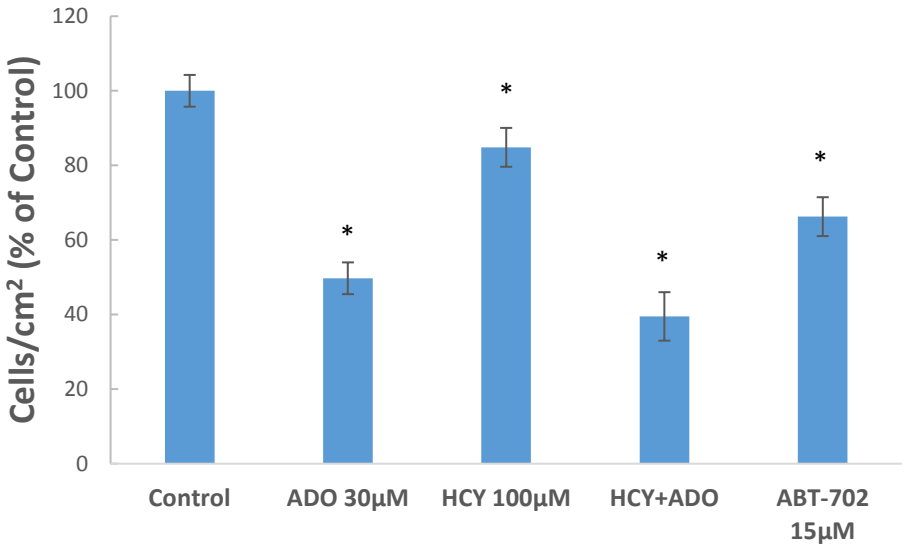

Cell proliferation/viability (% of Control)
120
100
80
60
40
20
0
*
§
§
Control
ADO
ADO/SAM 50µM
ADO/SAM 100µM

Figure 3

A


120
100
80
60
40
20
0
Cells/cm² (% of Control)
*
*
*
*
Control
ADO 30µM
HCY 100µM
HCY+ADO
ABT-702 15µM

B

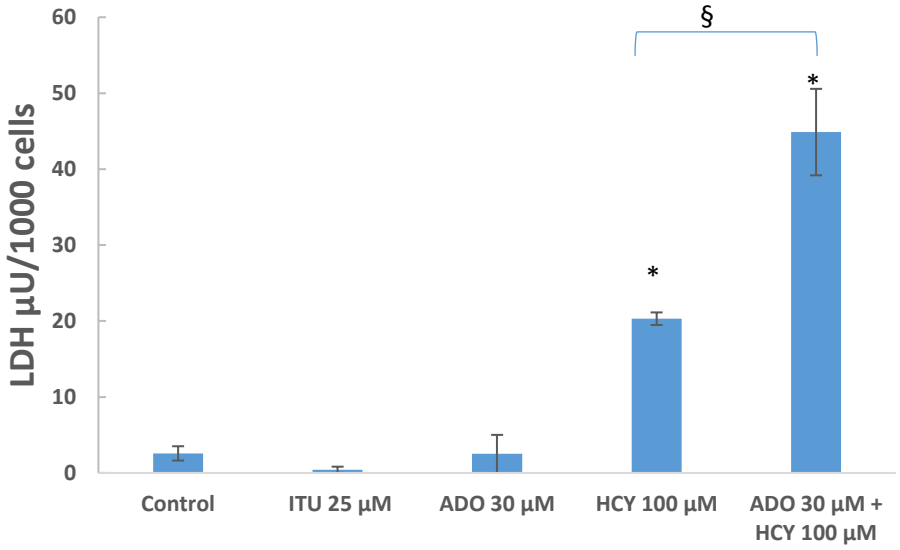
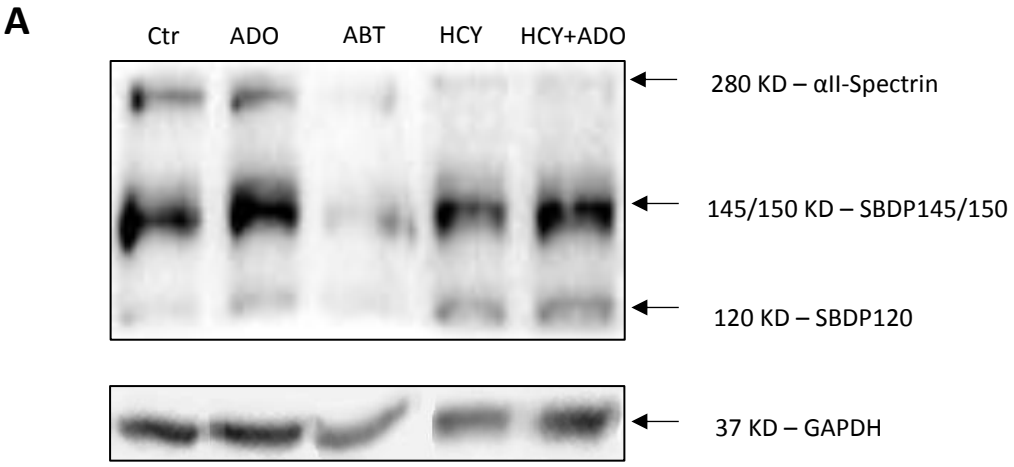
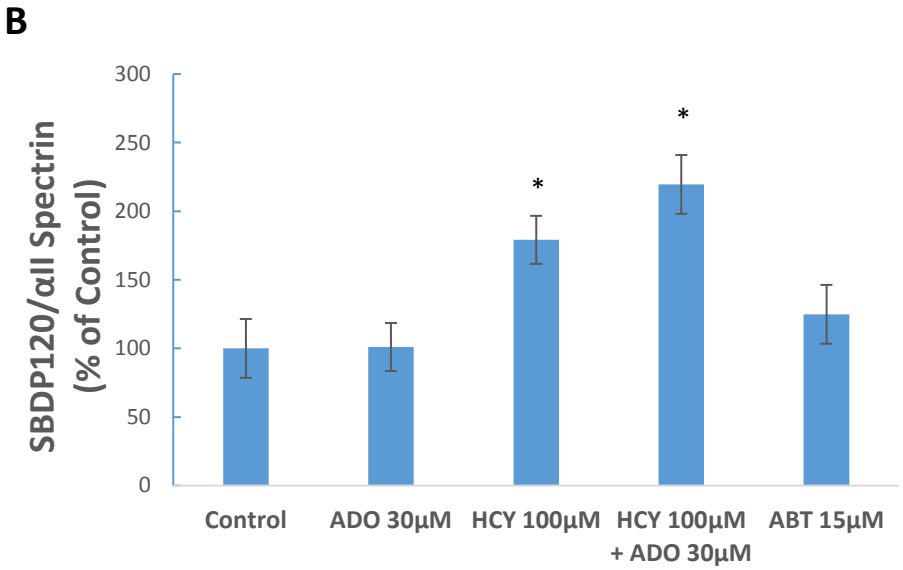

60
50
40
30
20
10
0
LDH µU/1000 cells
§
*
*
Control
ITU 25 µM
ADO 30 µM
HCY 100 µM
ADO 30 µM + HCY 100 µM

Figure 4


A
Ctr
ADO
ABT
HCY
HCY+ADO
280 KD – αII-Spectrin
145/150 KD – SBDP145/150
120 KD – SBDP120
37 KD – GAPDH


B
SBDP120/αII Spectrin
(% of Control)
300
250
200
150
100
50
0
*
*
Control
ADO 30μM
HCY 100μM
HCY 100μM
+ ADO 30μM
ABT 15μM

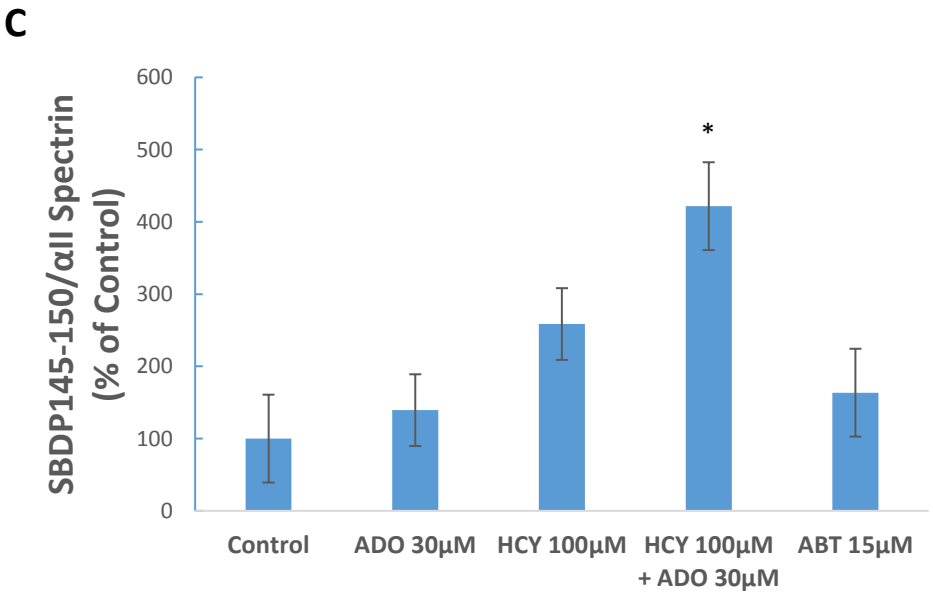

C
SBDP145-150/αII Spectrin
(% of Control)
600
500
400
300
200
100
0
*
Control
ADO 30μM
HCY 100μM
HCY 100μM
+ ADO 30μM
ABT 15μM

Figure 5

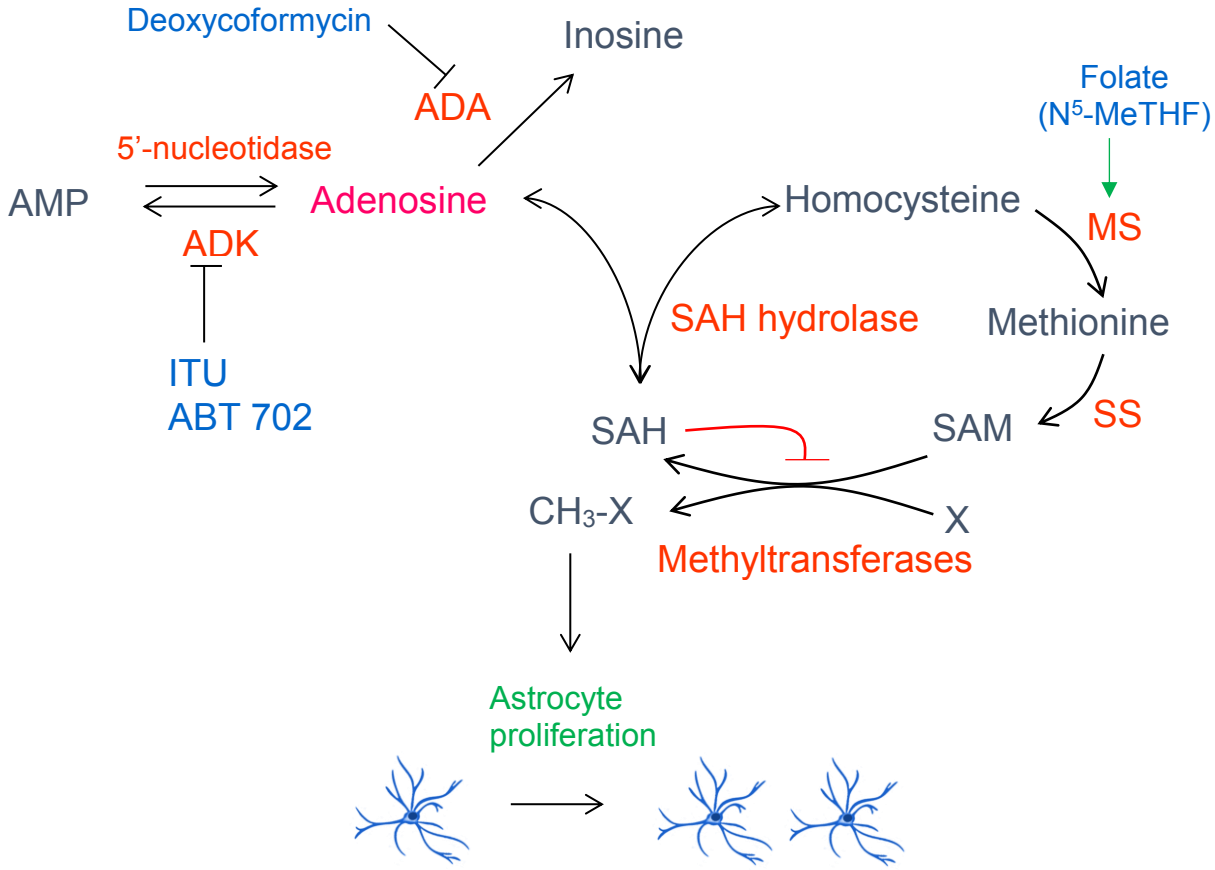

Deoxycoformycin
Inosine
ADA
Folate
(N5-MeTHF)
5'-nucleotidase
AMP
Adenosine
Homocysteine
MS
ADK
SAH hydrolase
Methionine
ITU
ABT 702
SAH
SAM
SS
CH3-X
X
Methyltransferases
Astrocyte
proliferation

Figure 1. **Effect of adenosine, adenosine receptors antagonists and adenosine kinase inhibitors on human astrocyte proliferation/viability.** (A) Cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30µM-adenosine, with or without selective adenosine receptor antagonists, or in the presence of 25 µM-5-iodotubercidin (ITU) or 15 µM-ABT 702. After this period, medium was replaced by fresh medium and cell proliferation/viability was assessed by the MTT assay. The following antagonists were used: 15µM-DPCPX for A_1, 10µM-SCH 58261 for A_{2a}, 0.1µM-MRS 1754 for A_{2b} and 1.5µM-MRE 3008F20 for A_3 adenosine receptors. Results are mean ± SEM of % of control cell proliferation/viability of 3-4 independent experiments run in sextuplicate. (B) HA cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30µM-adenosine, with or without adenosine deaminase (2 U/ml). After this period, medium was replaced by fresh medium and cell proliferation/viability was assessed by the MTT assay. Results are mean ± SEM of % of control cell proliferation/viability of 3 independent experiments run in sextuplicate. ADO- adenosine; Ant. Cocktail – cocktail of all antagonist; ADA- adenosine deaminase. (*), (§), (#) Statistically different from control, from the corresponding antagonist(s) alone or from adenosine alone, respectively ($P<0.05$, One-Way ANOVA followed by Tukey HSD post hoc test). Individual points are represented by open circles.

Figure 2. **The inhibitory effect of adenosine on astrocyte proliferation/viability is potentiated by homocysteine and attenuated by Folate and SAM.** (A) HA cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30 µM-adenosine (ADO), 100 µM-homocysteine (HCY) or 30 µM-adenosine+100 µM-homocysteine (HCY+ADO). After this period, medium was replaced by fresh medium and cell proliferation/viability was assessed by the MTT assay. Results are mean ± SEM of % of control cell proliferation/viability of 5 independent experiments run in sextuplicate. (B) HA cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30 µM-adenosine (ADO), with or without folate (100 µM). After this period, medium was replaced by fresh medium and cell proliferation/viability was assessed by the MTT assay. Results are mean ± SEM of % of control cell proliferation/viability of 4-9 independent experiments run in sextuplicate. (C) Cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30 µM-adenosine (ADO), with or without S-adenosyl-L-methionine (SAM, 50-100 µM). After this period, medium was replaced by fresh medium and cell proliferation/viability was assessed by the MTT assay. Results are mean ±SEM of % of control proliferation/viability of 7-8

independent experiments run in sextuplicate; for calculating the effect of adenosine alone the control was vehicle while for calculating the effect of adenosine in the presence of SAM (50-100 μM) the control was SAM (50 -100 μM) alone. ADO/SAM 50 μM, ADO/SAM 100 μM – Effect of adenosine on the presence of SAM 50 μM or SAM 100 μM, respectively. (*), (§), (#) Statistically different from control (vehicle), from adenosine or from HCY alone, respectively ($P<0.05$, ANOVA followed by Tukey HSD post hoc test). NS- non-significantly different, $P>0.05$. Individual points are represented by open circles.

Figure 3. **Effect of adenosine on cell proliferation and adenosine cytotoxicity in HA.** Cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30 μM-adenosine (ADO), 100 μM-homocysteine (HCY), 30 μM-adenosine+100 μM-homocysteine (HCY+ADO) and 15 μM-ABT 702 (A) or 25 μM-5-iodotubercidin (ITU, B). After this period, cells and medium were collected for further analysis. (A) Cell proliferation was assessed by cell counting. Results are mean ± SEM of % of control cell number of 3-4 independent experiments run in quadruplicate. (B) Cell death was evaluated by quantifying the released LDH activity in the cell medium. Results are mean ± SEM of released LDH activity per 1000 cells of 3 independent experiments run in quadruplicate. (*), (§) Statistically different from control or from homocysteine alone, respectively ($P<0.05$, ANOVA followed by Tukey HSD post hoc test). Individual points are represented by open circles.

Figure 4. **Effect of adenosine on αII-Spectrin breakdown.** Cells were incubated for 3 days in the presence of vehicle (control) or in the presence of 30 μM-adenosine (ADO), 100 μM-homocysteine (HCY), 30 μM-adenosine+100 μM-homocysteine (HCY+ADO) or 15 μM-ABT 702 (ABT). After this period cells were lysed and cell lysates were analysed by Western-Blot using an anti-αII-Spectrin antibody. The areas corresponding to αII-Spectrin and to the SBDP120 and SBDP145-150 breakdown products were quantified. (A) Representative Wester-blots, showing the protein levels of αII-Spectrin, SBDP145-150 and SBDP120 obtained in the different conditions. GAPDH was used as loading control. (B) and (C) SBDP120/αII-Spectrin and SBDP145-150/αII-Spectrin ratios, respectively, obtained in the different conditions. Results are mean ± SEM of % of control SBDP120/αII-Spectrin or SBDP145-150/αII-Spectrin ratios, corresponding to 3 independent experiments run in quadruplicate. (*) Statistically different from control ($P<0.05$, ANOVA followed by Tukey HSD post hoc test).

Figure 5. **Proposed mechanism of adenosine-induced inhibition of human astrocyte proliferation.** Under increased intracellular concentrations, adenosine combines with homocysteine to produce SAH, by reversal of the near-equilibrium SAH hydrolase-catalysed reaction, leading to SAH accumulation and consequent inhibition of SAM-dependent methyltransferases. Decreased methyltransferases activity would consequently decrease astrocyte proliferation. ADA- Adenosine deaminase; ADK- adenosine kinase; SAH- S-adenosylhomocysteine; ITU- 5-iodotubercidin; MS- methionine synthase; SS- SAM synthetase; X- methyltransferase substrate; CH_3-X- methylated substrate; N^5-MeTHF- N^5-methyltetrahydrofolate.