Chapter 2
Adenosine Signaling in Glioma Cells

Stefania Ceruti and Maria P. Abbracchio

Abstract Purines and pyrimidines are fundamental signaling molecules in controlling the survival and proliferation of astrocytes, as well as in mediating cell-to-cell communication between glial cells and neurons in the healthy brain. The malignant transformation of astrocytes towards progressively more aggressive brain tumors (from astrocytoma to anaplastic glioblastoma) leads to modifications in both the survival and cell death pathways which overall confer a growth advantage to malignant cells and resistance to many cytotoxic stimuli. It has been demonstrated, however, that, in astrocytomas, several purinergic (in particular adenosinergic) pathways controlling cell survival and death are still effective and, in some cases, even enhanced, providing invaluable targets for purine-based chemotherapy, that still represents an appropriate pharmacological approach to brain tumors. In this chapter, the current knowledge on both receptor-mediated and receptor-independent adenosine pathways in astrocytomas will be reviewed, with a particular emphasis on the most promising targets which could be translated from *in vitro* studies to *in vivo* pharmacology.

Keywords P1 receptors • A_3 ligands • Astrocytoma • Cladribine • 2-Chloro-adenosine • Caspase-2 • Caspase-9 • p53 mutations • CD39 • CD73 • Matrix metalloproteinases • Equilibrative nucleoside transporters

Abbreviations

ADA	Adenosine deaminase
Ado	Adenosine
AK	Adenosine kinase
APCP	α,β-Methylene ADP
2-CA	2-Chloro-adenosine
2-CdA	2-Chloro-2′-deoxyadenosine
CI-IB-MECA	2-Chloro-N^6-(3-iodobenzyl)-N-methyl-5′-carbamoyladenosine

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CNS	Central Nervous System
8-CPT	8-Cyclo-pentyl-theophylline
DAG	Diacylglycerol
dCyd	2′-Deoxycytidine
ENT	Equilibrative nucleoside transporter
^{18}F-CPFPX	8-Cyclopentyl-3-(3-^{18}F-fluoropropyl)-1-propyl-xanthine
GSK-3β	Glycogen synthase kinase 3β
HIF-1α	Hypoxia-inducible factor 1 α subunit
IFNγ	Interferon-gamma
Ino	Inosine
IP_3	Inositol-1,4,5-trisphosphate
ITub	5-Iodotubercidin
MAP kinases	Mitogen-activated protein kinases
MMP-9	Matrix metalloproteinase-9
MRS1220	N-(9-Chloro-2-furan-2-yl-[1,2,4]triazolo[1,5-c]quinazolin-5-yl)-2-phenylacetamide
MRS1706	N-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy] acetamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NECA	N-Ethyl-carboxamide adenosine
NTPDase	Nucleoside triphosphate diphosphohydrolase
PET	Positron emission tomography
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
SAH	S-Adenosyl-homocysteine
SAM	S-Adenosyl-methionine
TNFα	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
zIETD-fmk	z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone
zLEHD-fmk	z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone
zVAD-fmk	z-Val-Ala-Asp(OMe)-fluoromethylketone
zVDVAD-fmk	z-Val-Asp(OMe)-Val-Ala-Asp(OMe)-fluoromethylketone.

2.1 Introduction

Due to their universal role in cell growth, differentiation and death, purinergic mechanisms are intimately involved in the regulation of tumour growth, with both positive and negative influence on cancer growth, progression and metastatisation depending on the cell type, the involvement of specific extracellular receptors and/or intracellular apoptotic pathways (for a recent commentary, see Tapper et al. (2012). Historically, the first evidences highlighting adenosine (Ado) as a regulator of cancer cell growth dates back to the 1940s, when purine derivatives started to be used as cytotoxic agents in hematologic tumours. It was then hypothesized this action to be due to impaired intracellular synthesis of nucleic acids and it was only relatively recently that the involvement of specific P1 receptors for extracellular Ado was demonstrated. Here, we summarize the available evidence implicating Ado and its receptors in the regulation of brain astrocytomas, with special

emphasis on the translation of these findings to the cure of this form of cancer. It is worth mentioning that, apart from the A_3 receptor subtype, which has been the object of extensive studies as a new target for chemotherapy of brain tumours (see Sect. 2.4.2) and from the studies on the intracellular effects of Ado in astrocytoma cells (see Sect. 2.5), in many papers astrocytoma cell lines have been utilized merely as *in vitro* models of astrocytes to test the presence and the effects of Ado-mediated signaling. This has to be taken into consideration when translating data from these studies to primary normal astrocytes, which can bear significant differences with respect to malignantly transformed cells.

2.2 Adenosine Metabolism and P1 Adenosine Receptors

Ado is considered rather a neuromodulator than a true neurotransmitter, since it is neither stored as such in synaptic vesicles nor released following membrane depolarization (however, see also Melani et al. 2012). It has been estimated that basal extracellular Ado concentration is in the range of 30–200 nM (Fredholm et al. 2011), and can rapidly increase through two different mechanisms: enzymatic hydrolysis of extracellular ATP by the ecto-5′-nucleotidases CD39 and CD73 or the export via membrane bi-directional equilibrative transporters (ENTs) following its intracellular production (see also below, Fig. 2.1). Since ATP is released not only from neurons through synaptic vesicles, but also from various secretory cells (e.g., platelets, macrophages, endocrine cells; Lazarowski et al. 2011) as well as from damaged or dying cells, a delayed increase in Ado concentrations is always detected following ATP release, and, in general, Ado-mediated effects modulate the excitatory ATP effects in a compensatory inhibitory way.

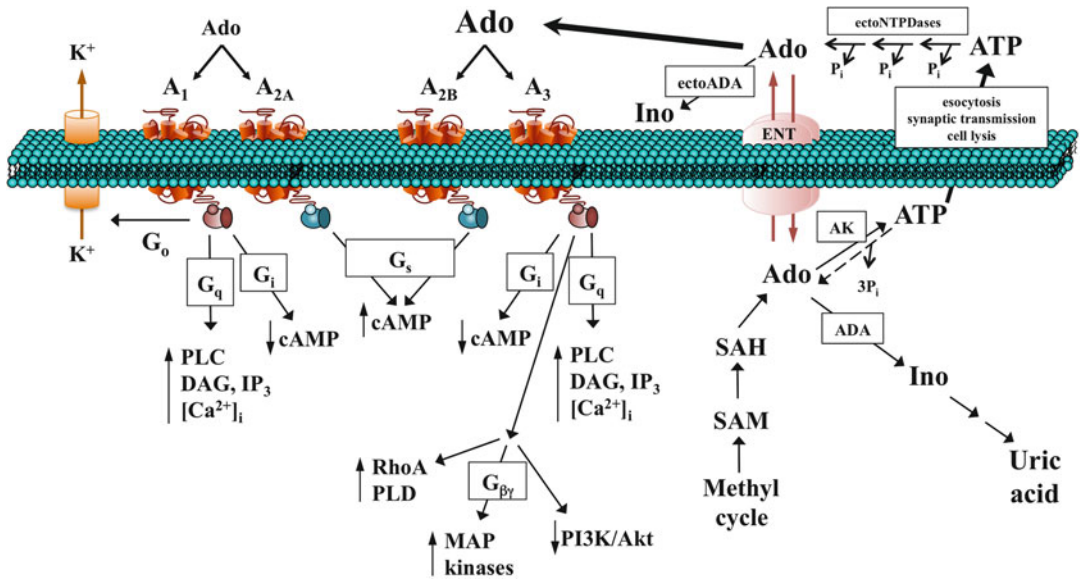

Fig. 2.1 Ado metabolism, P1 Ado receptors and corresponding signaling pathways. Ado can be generated intracellularly after SAH or ATP hydrolysis, and then transported extracellularly through ENT. Extracellular Ado concentrations can also increase thanks to ATP hydrolysis by ectoNTPDases. Low physiological Ado concentrations can activate the A_1AR and A_{2A}AR subtypes, whereas high micromolar concentrations (that can be reached following ischemic or traumatic events) are needed to recruit the A_{2B}AR and A_3AR subtypes. The classical signaling pathways activated by the four P1 receptor subtypes are shown, together with the A_3AR-mediated pathways that have been more recently identified in glioma cells. Ado can also be deaminated by ADA either extracellularly or intracellularly, and finally metabolized to uric acid. See text for details (Sects. 2.2 and 2.4.2)

In the extracellular space, Ado can activate its membrane receptors, collectively referred to as the P1 receptors. Four subtypes of G protein-coupled Ado receptors have been identified, namely the A_1 adenosine receptor (AR), A_{2A}AR, A_{2B}AR and A_3AR subtypes (Fredholm et al. 2011). Ado possesses a high affinity towards the A_1AR and A_{2A}AR subtypes, which can therefore be activated at low physiological Ado concentrations; conversely, only high Ado concentrations can effectively recruit the A_{2B}AR and A_3AR subtypes, which can thus play a key role upon pathological conditions (see also below). The A_1AR and A_3AR subtypes are either coupled to G_i proteins, which negatively regulate adenylyl cyclase activity (Fredholm et al. 2011), or G_q proteins (Linden et al. 1998; Abbracchio et al. 1995a), leading to the hydrolysis of phosphatidylinositol to generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 in turn activates its receptors on the endoplasmic reticulum, and stimulates the release of calcium from the intracellular stores, whereas DAG can activate protein kinase C (PKC) and additional second messenger pathways (Fig. 2.1). Especially in the brain and heart tissues, the A_1 subtype can also directly couple to K^+ channels through the recruitment of a G_o; the opening of these channels leads to the intracellular elevation of K^+ concentrations and to membrane hyperpolarization. This latter action is at the basis of the bradycardic and anti-epileptic actions of Ado (Fredholm 2010). The A_3AR subtype can also couple to various intracellular pathways, which will be discussed in more details in Sect. 2.4.2 below. Conversely, the A_{2A}AR and A_{2B}AR subtypes are coupled to G_s and, consequently, to the activation of adenylyl cyclase and the generation of cAMP (Fredholm et al. 2011). The intracellular positive coupling to adenylyl cyclase is at the basis of the functional antagonism between the A_{2A}AR and the D2 dopamine receptors in the striatum, since the latter inhibits adenylyl cyclase functions through G_i. Alterations in this functional cross-talk between the Ado and dopamine systems plays a crucial role in the development of various neurodegenerative disorders, such as Parkinson's disease, as elegantly reviewed elsewhere (Morelli et al. 2010).

Ado is finally taken up by specific membrane transporters, which can be subdivided into equilibrative (bidirectional) or concentrative (i.e., working against concentration gradient, King et al. 2006), and phosphorylated in the cytosol by adenosine kinase (AK). Since this enzyme can operate only in the presence of high oxygen concentrations, Ado concentrations will remain high upon hypoxic conditions (such as following an ischemic event, at any site of inflammation or within a tumour mass, Vaupel et al. 2001), when no ATP can be generated; therefore, Ado-mediated actions play an important role also upon pathological conditions. Intracellular Ado can also derive from the intracellular hydrolysis of ATP or of S-adenosyl-homocysteine (SAH). The latter represents the final step of the so-called "methyl cycle", which is crucially involved in the synthesis of nucleic acids and in the methylation of proteins (Fig. 2.1). Ado can be also deaminated to inosine (Ino) by Ado deaminase (ADA), and further terminally metabolized to uric acid. Ado deamination and metabolism to uric acid can also occur extracellularly thanks to the presence of ecto-ADA enzymes on the cell surface (Fig. 2.1).

2.3 A Role for Ecto-5′-Nucleotidases CD39 and CD73 in Gliomas?

As mentioned above, Ado can be produced extracellularly from adenine nucleotides by two types of membrane-bound enzymes: CD39, the product of the ectonucleoside triphosphate diphosphohydrolase 1 gene (ENTPD1), which is responsible for the conversion of ATP to ADP and AMP, and CD73, the product of NT5E gene, which converts AMP to adenosine. Several papers have been recently published indicating that scavenging of extracellular ATP and production of adenosine have direct effects on tumour growth, spread of metastases, as well as on the adhesion, migration and homing of both cancer cells and activated immune cells mediating anti-tumour effects (for details, see Tapper et al. (2012).

Recently, Wang et al. have shown that CD73 expression inhibits anti-tumour immune responses and that, conversely, CD73-null mice bearing transplanted non-CNS tumours display a survival benefit

when compared to wild type mice (Wang et al. 2011). Twelve days after tumour inoculation, higher numbers of CD8+ immune T cells are found in CD73-null mice. Authors examined in details the role of CD73 in modulating the interaction of tumour infiltrating regulatory CD4+ T cells (tumour Tregs) that can promote metastatic tumour growth, with effector T cells. Importantly, CD73-null effector T cells did not show any changes in mediating anti-tumour responses. CD4+ tumour Tregs directly inhibited CD8+ T cell functions, such as interferon-gamma (IFN-γ) production, and consequently CD8+ T cell-mediated anti-tumour effects in a CD73-dependent manner. This study therefore shows that both local and systemic production of Ado by CD73 (in tandem with CD39) results in decreased effector functions of CD8+ T cells and in a reduction of their homing to tumours. This occur by several means, including both decreased expression of adhesion molecules, direct in vivo modulation of anti-tumour responses and/or possibly induction of apoptosis (in this respect, see also Sect. 2.4). These novel data suggest that both cancer cells and host-derived cells cooperatively mediate tumour immune evasion, and that this occurs in a CD73-dependent manner.

Highly relevant to the present review, overexpression of CD73 has recently also emerged as a component of glioma cell adhesion and tumour cell-extracellular matrix interactions (Cappellari et al. 2012, see also below). In both C6 and U138MG glioma cells, treatment with 1 μM α,β-methylene ADP (APCP), a competitive CD73 inhibitor, caused a 30 % reduction in glioma cell proliferation. In addition, 100 μM Ado increased cell proliferation by 36 %, and treatment with Ado plus inhibitors of its uptake produced an additional and significant increase in cell proliferation. The inhibitory effect on cell proliferation caused by APCP was reverted by co-treatment with Ado uptake inhibitors. AMP (1 and 3 mM) decreased U138MG glioma cell proliferation by 29 and 42 %, respectively (Bavaresco et al. 2008). Taken together, these results suggest the participation of CD73 in cell proliferation, and that this process is dependent upon (i) the enzyme's production of Ado, acting here as a proliferative factor, and, (ii) removal of AMP, a toxic molecule for gliomas. These findings would therefore suggest CD73 as a new target for anti-glioma therapies. However, they are in contrast with other reports indicating that extracellular adenine nucleotides inhibit C6 glioma cell growth via Ado, which is produced by ecto-nucleotidases including CD73 at the extracellular space and then incorporated into cells by the equilibrative nucleoside transporters ENT2 (Ohkubo et al. 2007). Intracellular AMP accumulation by AK after Ado uptake would then induce C6 cell growth inhibition through pyrimidine starvation. It has to be underlined that these contrasting data have been obtained on cell lines in vitro. Therefore, at variance from other non-CNS tumours (Wang et al. 2011), elucidation of the exact role of CD39 and CD73 in gliomas still awaits direct in vivo evaluation in tumour-bearing animals where factors other than cancer cell proliferation (i.e., tumour cells' adhesion, migration and invasiveness as well as immune cell-mediated responses) can be determined (see also Conclusions).

2.4 Receptor-Mediated Effects of Adenosine on Glioma Cell Growth and Survival

The interest in the role of the purinergic system in general, and of Ado in particular, in modulating the growth and survival of various types of tumour cells has grown since the 1990s, based on the demonstration of high extracellular purine concentrations within the tumour mass. For example, concentration of Ado in the extracellular fluid of glioma tissue has been directly measured in 21 patients undergoing surgical removal of the tumour, and has been reported to be in the low micromolar range (Melani et al. 2003), meaning high enough to stimulate all the four P1 receptor subtypes. All the four Ado receptor subtypes have been identified in C6 glioma cells, functionally coupled to the modulation of adenylate cyclase activity, and quantitative real time PCR allowed to rank the expression of Ado receptors as follows: $A_1AR = A_3AR > A_{2A}AR > A_{2B}AR$ (Castillo et al. 2007). Moreover, functional ENTs (Sinclair et al. 2000) and ecto-5′-nucleotidase/CD73 (Cappellari

et al. 2012) have been also found expressed by glioma cells (see above), thus suggesting that the whole machinery controlling purine metabolism is present and functional in glioma cells, and therefore cell survival and properties could be deeply influenced by intra- and extracellular Ado concentrations. Indeed, in both C6 glioma and U138MG glioblastoma cells the activity of ecto-5′-nucleotidase/CD73 increased in parallel with cell proliferation to a maximum when cells reached confluence (Bavaresco et al. 2008). This means that ATP hydrolysis to Ado will progressively augment with the growing of the tumour mass, and that Ado-mediated effects on cell growth and proliferation might become progressively more important.

Many studies have been performed on in vitro or in vivo models of cancer of different origins (such as melanoma, leukemia, prostate carcinoma etc.), or on P1 receptor-transfected cells, and they have reported different, and often opposite, effects on cell survival exerted by the various Ado receptor subtypes (Merighi et al. 2003). These contradictory effects are probably due to the various experimental models, leading to a consequent coupling of P1 receptor subtypes to different intracellular pathways controlling cell survival and proliferation depending upon the cell type, but also to the different experimental settings (e.g., hypoxic vs normoxic), and to the concentration of the agonists utilized (see Sect. 2.4.2).

The number of studies aimed at directly analyzing the effects of Ado itself or of Ado agonists on the survival of glioma cells are limited (with the exception of the A_3AR subtype; see Sect. 2.4.2), and will be reviewed in the following section.

2.4.1 A_1AR-, A_{2A}AR-, and A_{2B}AR-Mediated Effects on Glioma Cells

Based on studies performed on solid tumour cells or on transfected cells, the A_1AR subtype is generally believed to exert pro-survival and anti-apopotic actions, primarily based on its ability to activate the MAP kinases ERK1/2 and PKB, whereas the A_{2A}AR subtype can both negatively or positively affect cell survival by recruiting different intracellular signaling pathways, and the same holds true for the A_{2B}AR (Merighi et al. 2003). It is interesting to point out that the A_{2A}AR subtype has been crucially involved in the positive regulation of angiogenesis, by promoting the survival and proliferation of endothelial cells in synergy with VEGF (reviewed in Merighi et al. 2003), and its activation could therefore indirectly boost tumour growth by improving the supply of oxygen and nutrients.

At variance from the general anti-apoptotic role for the A_1AR (see above), induction of caspase-dependent cell death by Ado in RCR-1 astrocytoma cells was supposed to involve both intracellular pathways and the activation of this receptor subtype (Sai et al. 2006). Conclusions, however, appear questionable, mostly due to the extremely high Ado concentrations utilized (up to 1 mM), which more likely recall an intracellular site of action, and to the very low reversal of Ado-mediated effects exerted by the A_1AR antagonist 8-CPT.

A significant upregulation of the A_1AR has been identified in the peritumour area in animals injected with F98 or C6 glioma cells by 3D imaging reconstruction of the tumour mass (Dehnhardt et al. 2007). This upregulation was dependent upon the tumour volume. No significant changes, but rather a tendency to decrease, were instead detected within the tumour itself, possibly due to induction of cell death by necrosis. Although the functional significance of this increase in A_1AR is not known, monitoring the A_1AR expression and receptor presence around tumours could represent a valuable diagnostic and prognostic marker for glioblastoma progression. In fact, this receptor system has been studied by monitoring the binding of the radioactive molecule ^{18}F-CPFPX (a selective A_1 antagonist) by positron emission tomography (PET) *in vivo*, and described as being up-regulated during the cerebral response to glioma invasion in primary human *glioblastoma multiforme* (Bauer et al. 2005).

Specific down-regulation of the A_1AR and a parallel up-regulation of the A_{2A}R subtypes have been observed in C6 glioma cells following induction of hypoxia (Castillo et al. 2008). This effect was mimicked by Ado itself, therefore suggesting that high extracellular Ado concentrations that are

generated as a consequence of hypoxic conditions (as observed within a tumour mass; see also below) can auto-regulate Ado-dependent signaling. These data further confirm that the results on receptor recruitment and activation can significantly vary depending upon the experimental setting utilized, and can even become the opposite when shifting from *in vitro* to *in vivo* conditions.

The only paper dealing with a putative role for the A_{2A}AR in controlling glioma survival shows a protective role for this receptor, at least under the experimental conditions utilized. In fact, authors have demonstrated that Ado, at concentrations ranging from 125 to 500 μM, significantly protected C6 glioma cells from NO-dependent cell death induced by the incubation with a mix of cytokines (Isakovic et al. 2008). In this study, the possible involvement of the A_{2A}R (although the Ado concentrations utilized here are extremely high) and of the A_{2B}AR has been only postulated, based on the inability of an inhibitor of ENTs to prevent Ado-mediated effects and on previous data demonstrating that overexpression of the A_{2A}R and inhibition of AK (which in turn increases extracellular Ado concentrations) in C6 glioma cells significantly inhibited NF-κB activation and cytokine-induced iNOS upregulation (Lee et al. 2005; Sands et al. 2004).

Functional A_{2B}AR were identified in human astrocytoma ADF cells, (a cell line directly derived from a cancer patient; Malorni et al. 1994), which underwent desensitization following prolonged agonist exposure due to phophorylation at thereonine residues (Trincavelli et al. 2004). Interestingly, the pro-inflammatory cytokine TNFα potentiated A_{2B}AR coupling and inhibited its desensitization, so that only in its presence could the A_2 receptor agonist NECA promote A_{2A}AR-mediated elongation of numerous thin cellular processes (Fig. 2.2; Trincavelli et al. 2004). These morphological changes generally reflect the commitment of astrocytes towards a cell differentiation program, which could be also accompanied by an increased resistance to cell death (see below, Sect. 2.4.2). It should be noted, however, that in cancer cells any stimulus promoting differentiation would conflict with their intrinsic tendency towards uncontrolled proliferation. These contradictory messages could therefore promote the decision of the cell to undergo apoptotic cell death. Thus, activation of the A_{2B}AR in the presence of pro-inflammatory cytokines could prove useful in glioma therapy.

Moreover, in human U373 *glioblastoma multiforme* cells activation of the A_{2B}AR recruited a series of intracellular pathways, including p38 MAP kinase and PKC delta and epsilon, which in turn promoted IL6 synthesis and release (Fiebich et al. 2005). Since this protein has been associated to the development of a malignant and aggressive phenotype in various types of cancer, such as breast carcinoma, caution should be kept in evaluating the final outcome of A_{2B}AR activation in brain tumours. More experimental data are therefore needed to clearly determine the role of this receptor subtype in modulating the survival and proliferation of glioma cells.

2.4.2 *The A_3 Receptor Subtype as a New Pharmacological Target for Innovative Chemotherapic Approaches to Gliomas*

The A_3AR subtype is the only member of the P1 receptor family that was cloned before its pharmacological characterization. As knowledge on its structure were accumulating, it appeared that this receptor would have been difficult to characterize, mostly based on the low level of interspecies homology, reflecting in highly different pharmacological profiles, especially in terms of antagonist binding and affinities (Gessi et al. 2008). Moreover, also receptor distribution showed significant differences among species, with high receptor expression detected in the lung, spleen, testis, brain, heart and liver (Gessi et al. 2008). Interestingly, a very high levels of A_3AR protein were found in a variety of cancer cell lines, including astrocytoma and glioblastoma, suggesting a role for this receptor as a tumour marker (Gessi et al. 2008) or as a target for new chemotherapeutic approaches (see below).

The analysis of receptor dynamics in ADF astrocytoma cells has led to discover a fast desensitization response to agonist stimulation, followed by agonist-mediated internalization and recycling to the cell surface within 120 min. A more prolonged exposure to a selective A_3 agonist (Cl-IB-MECA) led to a

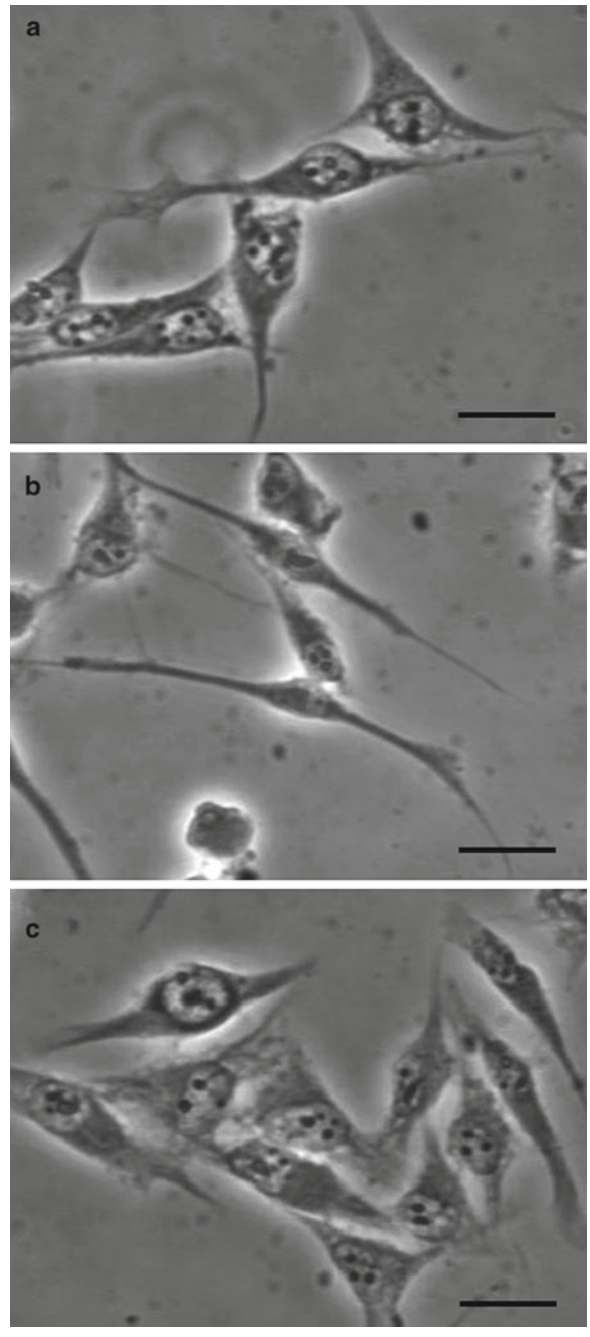

Fig. 2.2 In human astrocytoma ADF cells exposed to TNFα, NECA promotes the emission of long and thin cellular processes through the activation of the A_{2B}AR subtype. (**a**) Typical morphology of ADF cells exposed to TNFα alone for 24 h (control). (**b**) A 30-min incubation of TNFα-treated ADF cells with the non-selective agonist NECA (1 μM) induced clear morphological changes, with the emission of long processes (**c**) The full reversion of NECA-mediated effects was obtained by pre-incubating cells with the A_{2B}AR-selective antagonist MRS1706 (10 nM). Magnification: 32×; scale bars: 30 μm (Reproduced from Trincavelli et al. (2004). With permission from John Wiley and Sons)

significant down-regulation of receptor expression, with slow recovery (Trincavelli et al. 2002). This rapid desensitization kinetics is possibly due to the presence of a higher number of serine and threonine residues at the receptor C-terminal region with respect to A_1AR and A_{2A}AR subtypes (Palmer and Stiles 2000).

Apart from the "classical" second messenger systems (see Sect. 2.2 above), the A_3AR can also couple to the activation of the small G protein RhoA, which in turn stimulates phospholipase D (PLD) (Gessi et al. 2008), and to the MAP kinase signaling pathways through the release of the βγ subunit

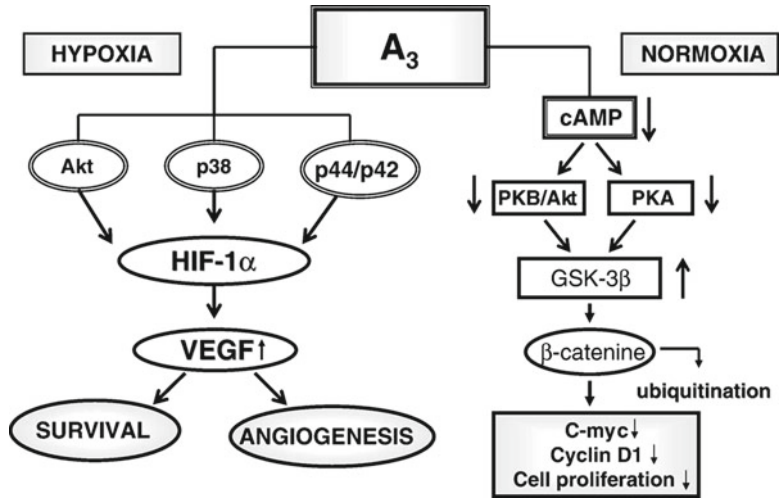

Fig. 2.3 Opposite effects exerted by A_3AR activation in tumour cells upon hypoxic and normoxic conditions. In cancer cells under normoxic conditions, activation of the A_3AR leads to a reduced expression of PKA and PKB, which in turn promotes the activation of GSK-3β, leading to inhibition of cell proliferation. Conversely, upon hypoxic conditions (such as within a solid tumour mass), A_3AR-mediated activation of MAPKinases promotes the synthesis of HIF-1α with a consequent increased cell survival and angiogenesis (Reproduced from Gessi et al. (2008). With permission from Elsevier)

from pertussis toxin-sensitive G_i proteins (Schulte and Fredholm 2000), or to the PI3K/Akt pathway (see Fig. 2.1). Concerning the latter, inhibition of PKA and PKB/Akt was demonstrated in melanoma cells upon activation of the A_3AR; this in turn maintained glycogen synthase kinase 3β (GSK-3β) in its active form, with a consequent deregulation of the Wnt pathway, increase in β-catenin degradation, and finally inhibition of tumour cell proliferation (Fishman et al. 2002). These observations, together with the high expression of the A_3AR in tumour cells and with its pro-apoptotic effects on various lymphoma, melanoma and prostate cell lines in vitro and in mouse xenografts in vivo (Fishman et al. 2002), have led to the hypothesis that A_3AR agonists might be useful as chemotherapic agents. Clinical studies are currently ongoing on the use of CF102 (Cl-IB-MECA) in patients with advanced hepatocellular carcinoma (Fredholm et al. 2011). Taken together, these results suggest an inhibitory action of the A_3AR on cancer cell growth and proliferation.

The opposite, however, seems to hold true for glioblastoma cells. In fact, the selective A_3AR antagonist, MRS1220, blocked Ado-mediated glioma proliferation (Morrone et al. 2003). Upon hypoxic conditions, activation of the A_3AR upregulated the transcription factor HIF-1α, leading to increase in VEGF production, with consequent angiogenesis (Fig. 2.3, Merighi et al. 2006). Hypoxia-induced chemoresistance of human glioblastoma cells was also demonstrated to depend upon the activation of the A_3AR linked to the PKB/Akt pathway, which in turn mediated the phosphorylation and consequent inactivation of the pro-apopototic member of the Bcl2 protein family Bad, leading to cell survival (Merighi et al. 2007). Indeed, upregulation of matrix metalloproteinase-9 (MMP-9) expression and increased tumour migration was also observed upon activation of the A_3AR in human U87MG glioblastoma cells (Gessi et al. 2010). MMPs degrade the extracellular matrix and consequently promote migration of glioma cells towards brain areas protected by an intact blood-brain barrier. Unfortunately, no differences between the migratory biology of transformed and normal brain cells have been identified, which renders this potential target difficult to be exploited (Westphal and Lamszus 2011). Based on the above-mentioned evidence, inhibition of A_3AR functions with selective antagonists could therefore represent an appealing therapeutic approach to solid tumours in general (which are characterized by an hypoxic core) and to gliomas in particular, not only to inhibit MMPs

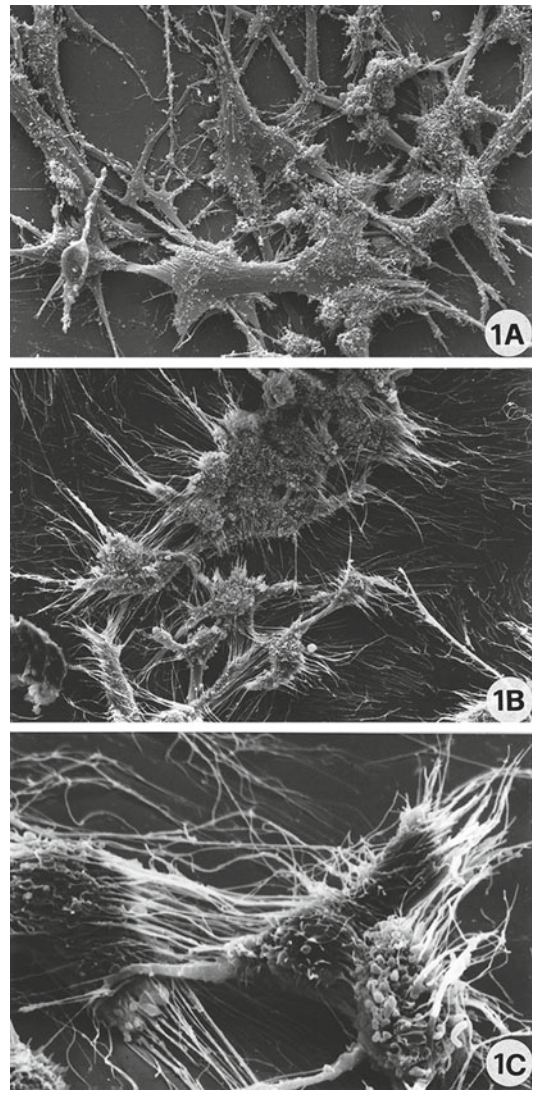

Fig. 2.4 Morphological changes induced by nanomolar concentrations of the A_3AR agonist Cl-IB-MECA in human astrocytoma ADF cells. Scanning electron micrographs of ADF cells grown for 72 h under control conditions (**a**) or in the presence of 100 nM Cl-IB-MECA (**b**, **c**). A typical *bipolar* shape with a relatively low number of cell protrusions was observed in Control cells, whereas exposure to Cl-IB-MECA induced marked morphological changes with an increased of the number and length of cellular processes. Original magnification: **a**, **b** 1000×; **c** 3300× (Reproduced from Abbracchio et al. (1997). With permission from Elsevier)

upregulation, but also to affect cancer cell growth and properties at various molecular levels, thus increasing the possibility of a more effective chemotherapy (Fig. 2.3). In this respect, possible lead compounds could emerge from the development of a series of pyrazol[3,4-d]pyrimidines derivatives with drug-like physicochemical properties, which demonstrated a high affinity and selectivity for the A_3AR and were able to potently inhibit Cl-IB-MECA- and IB-MECA-stimulated glioblastoma cell growth in vitro (Taliani et al. 2010). Taken together, data obtained in various cell cancer models suggest that A_3AR receptor stimulation exerts opposite effects on cancer cell growth and survival when taking place under normoxic or hypoxic situations, with anti-tumour or tumorigenic actions, respectively (Fig. 2.3, Gessi et al. 2008).

In line with the pro-survival role of the A_3AR in glioma cells, our group has demonstrated that in human astrocytoma ADF cells, nanomolar Cl-IB-MECA concentrations produced clear morphological changes (i.e., emission of numerous long filaments, Fig. 2.4), which were accompanied by the appearance of stress fibers, a typical hallmark of cytoskeletal rearrangement, and by the redistribution of the anti-apoptotic protein Bcl-X_L towards cell processes (Abbracchio et al. 1997), through the

specific involvement of the small G protein Rho (Abbracchio et al. 2001). Overall, these changes increased the ability of the cell to adhere to the culture substrate, which in turn reflected in a reduced sensitivity to cell death, thus confirming that the A_3AR could contribute to improve cancer cell survival.

An opposite effect was, however, exerted by high Cl-IB-MECA concentrations, since a significant cytotoxicity, reduction of cell number and induction of apoptosis were detected both in C6 glioma (Appel et al. 2001) and in human astrocytoma ADF cells (Abbracchio et al. 1998) upon treatment with high micromolar concentrations of this selective A_3 agonist. These data were in line with previous results showing a dual opposite effect on ischemia-induced tissue damage exerted by either acute or chronic A_3AR agonist administration (reviewed in Jacobson 1998), suggesting an opposite outcome on cell survival depending upon the "intensity" of receptor activation. Although the selective recruitment of the A_3AR subtype by such high agonist concentrations could be questioned, these apparent discrepancies in A_3AR-mediated effects on cell survival are in line with its enigmatic (and yet-to-be fully clarified) role as "Dr. Jekill and Mr. Hyde" (Gessi et al. 2008).

A further confirmation of the possible anti-proliferative role of the A_3AR in glioma comes from a study showing a significant upregulation of the mRNAs encoding for the A_3AR and for ENTPDase/CD73 upon exposure of C6 and U138-MG glioma cells to indomethacin (Bernardi et al. 2007). Increases in CD73 mRNA was accompanied by a significantly higher ratio of ATP hydrolysis; although actual Ado concentration was not measured in this paper, it can be envisaged that it reaches values high enough to activate the A_3AR subtype. Indomethacin also significantly inhibited glioma cell proliferation, and this effect was reverted by the A_3AR agonist MRS1220, but not by antagonists acting at other P1 receptors (Bernardi et al. 2007), thus suggesting a specific involvement of this receptor subtype in indomethacin-mediated effects.

Finally, it should be also noted that A_3ARs can modulate the immune system by activating NK cells and, possibly, NK-mediated disruption of tumour cells. Conversely, within the environment of a solid tumour Ado has been demonstrated to exert an immunosuppressive role through the A_3AR, which could interfere with the recognition of tumour cells by T killer cells (Gessi et al. 2008, see also above Sect. 2.3). Therefore this double immunomodulatory effect can positively or negatively influence tumour growth, progression, and metastatisation depending upon the cell environment.

2.5 Receptor-Independent Effects of Adenosine Analogues in Glioma Cell Growth and Survival

Apart from the more recently identified receptor-mediated effects, the intracellular cytotoxic actions of purine and pyrimidine derivatives have been known since the 1940s and are at the basis of their use as chemotherapic and anti-metabolites. For example, 2-CdA is utilized as the chemotherapic drug of choice in hairy cell leukemia and in other haematological malignancies, with the name of Cladribine (Golomb 2011). The molecular basis of the effects exerted by nucleoside analogues were initially mainly ascribed to their ability to alter intracellular purine and pyrimidine pools, leading to impaired synthesis of nucleic acids, or to directly interfere with the enzymes involved in DNA synthesis, thanks to their structural similarities with endogenous nucleosides (Dighiero 1996). In the 1990s, however, it was demonstrated that nucleoside analogues could also activate specific pathways of apoptotic cell death. For example, in haemathological disorders, Cladribine was demonstrated to recruit both the extrinsic caspase-8-dependent pathway of cell death (Nomura et al. 2000), and the intrinsic mitochondrial pathway of death, with the involvement of caspase-9 (Genini et al. 2000). Their actions on CNS cancer cells have not, however, been investigated properly.

The first clues of a possible cytotoxic role for Ado analogues on astrocytes came from the demonstration that the exposure of primary rat astrocytes to 2-chloro-adenosine (2-CA), a non-selective

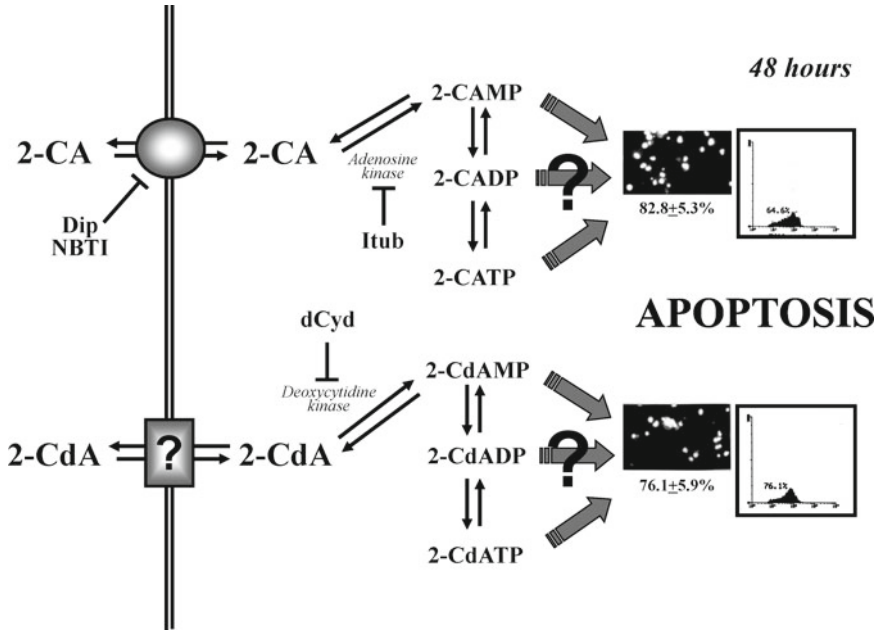

Fig. 2.5 Different intracellular pathways of apoptotic cell death activated by 2-CA and 2-CdA in human astrocytoma ADF cells**.** 2-CA and 2-CdA are prodrugs, since they have to be intracellularly phosphorylated/activated by two distinct kinases to exert their toxic effects. This has been demonstrated by inhibiting 2-CA- and 2-CdA-mediated effects with two specific inhibitors: Itub (acting on adenosine kinase) and dCyd (acting on deoxycytidine kinase), in the case of 2-CA and 2-CdA, respectively. Thus, the active toxic species are represented by the corresponding 2-chloronucleotide or deoxy-nucleotide derivatives, which led to induction of cell death by apoptosis through complex intracellular pathways (see text for details and Fig. 2.6). Apoptosis has been demonstrated by: (1) staining of cells floating in the culture supernatants with the chromatin fluorescent dye Hoechst 33258 (see pictures), and (2) flow-cytometric analysis of PI-stained cultures (see histograms on the *right*). The former technique demonstrated chromatin condensation and nuclear fragmentation in a highly significant percentage of detached cells (see micrographs and numbers *below*), whereas the appearance of hypodiploid DNA after a 48-h exposure to either adenosine analogue was shown by flow-cytometry (see numbers on histograms). Numbers represent the percentage of apoptotic cell death. For more details, see text (Reproduced from Ceruti et al. (2003a). With permission from John Wiley and Sons)

agonist of P1 receptors, induced a dramatic reduction of cell number (Abbracchio et al. 1994), due to the induction of cell death by apopotosis (Abbracchio et al. 1995b; Ceruti et al. 1997). To our surprise, Ado receptor antagonists did not inhibit 2-CA-mediated effects, thus suggesting an intracellular site of action for the Ado analogue. These results prompted us to analyze the possible cytotoxic effects of 2-CA on human astrocytoma cells (ADF cells, see above), in comparison with 2-CdA, in order to verify the possible use of Ado analogues as antitumour agents also in solid brain tumours. First of all, we demonstrated a high cytotoxic activity exerted by the two Ado analogues on ADF cells, due to the induction of apoptosis (Fig. 2.5, Ceruti et al. 2000, 2003a). 2-CdA resulted to be more potent than 2-CA in inducing ADF cell death, with significant effects already detected at 5 μM concentration. Both molecules exerted their effects after their intracellular phosphorylation to the corresponding nucleotide derivatives, which occurred through the recruitment of two independent biochemical pathways, as demonstrated by the complete inhibition of 2-CA-mediated actions by Itub (an inhibitor of AK) and of 2-CdA-induced effects by dCyd, which competes for the active site of deoxycytidine kinase (Fig. 2.5, Ceruti et al. 2000, 2003a). Only in the case of Cladribine, was a cell cycle block demonstrated to precede induction of apoptosis, further confirming that, although closely related chemically, these two compounds indeed recruited diverging pathways of death (Ceruti et al. 2000).

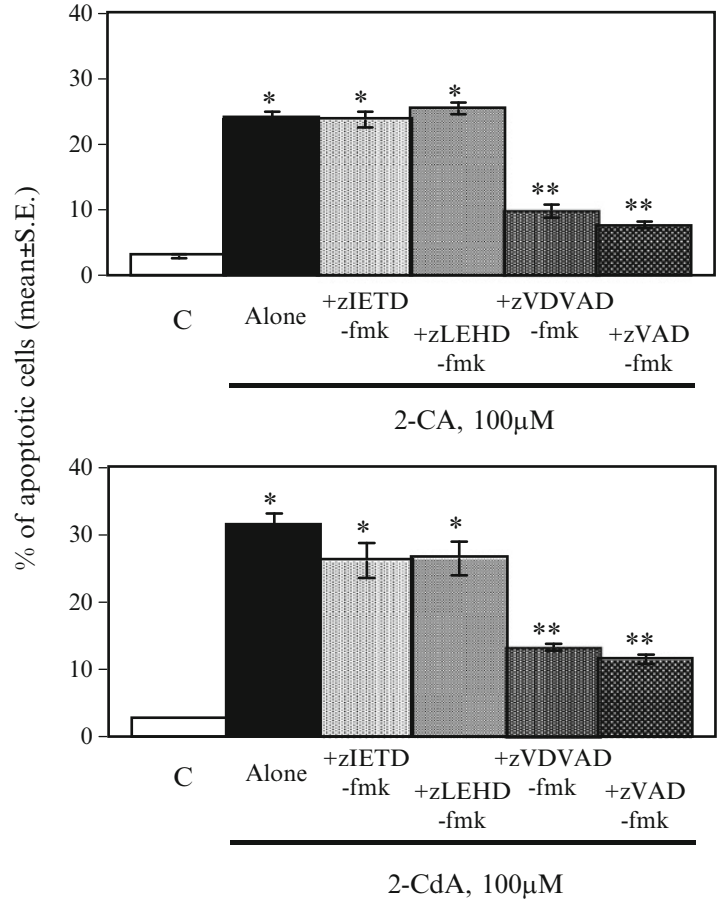

Fig. 2.6 Specific involvement of caspase-2 as the upstream initiator caspase in 2-CA- and 2-CdA-induced cell death of ADF cells. Human astrocytoma ADF cells were exposed for 24 h to either 100 μM 2-CA or 2-CdA, alone or in combination with selective inhibitors of caspase-8 (zIETD-fmk), caspase-9 (zLEHD-fmk), caspase-2 (zVDVAD-fmk) or with the pan-caspase inhibitor zVAD-fmk (all utilized at 3 μM), as indicated. At the end of the incubation, the percentage of apoptotic nuclei was determined by flow cytometric analysis of PI-stained nuclei. Only the selective caspase-2 inhibitors and the pan-caspase inhibitor were able to significantly protect cells from Ado analogue-induced death. See text for details. Data represent the mean ± S.E.M. of three independent experiments. *$p < 0.05$ with respect to control, and **$p < 0.05$ with respect to control and to corresponding Ado analog alone, one-way ANOVA (Scheffe' F test) (Reproduced from Ceruti et al. (2003b). With permission from ASPET)

Nevertheless, the two Ado analogues converged on a common and rather new pathway of apoptosis; in fact, both Ado analogues activated an atypical caspase pathway in ADF cells, with the recruitment of caspase-2 as initiator caspase, followed by the contemporary consequent activation of caspase-8 and caspase-9, and of caspase-3 as the main effector caspase (Fig. 2.6, Ceruti et al. 2003b). This was the first demonstration of the possible activation of a caspase-2-dependent pathway of cell death in astrocytoma cells, which was later demonstrated also following gamma-irradiation and overexpression of the pro-apoptotic molecule Smac/Diablo (Giagkousiklidis et al. 2005). A key contribution of the MAP kinase signaling pathways upon exposure of ADF cells to 2-CA and 2-CdA was also demonstrated (Ceruti et al. manuscript in preparation).

It is well known that tumorigenesis is linked to the acquisition of several genetic defects, which render malignant cells resistant to the normal growth inhibitory and apoptotic signals, with a parallel

deregulation of proliferation leading to out-of-control expansion of damaged cells (Kelly and Strasser 2011). In this respect, we have detected various genetic alterations borne by ADF cells. First of all, ADF cells are insensitive to apoptotic triggers (e.g., betulinic acid, potassium cyanide, and 2-deoxy-ribose) that recruit the "classical" intrinsic pathway of cell death, e.g. the mitochondria/cytochrome C/caspase-9 pathway (Ceruti et al. 2005). This is possibly due to the expression of a mutated form of caspase-9, bearing a single C-to-T nucleotide substitution in position 83 of the coding sequence, leading to a single amino acid substitution (A-to-V) in position 28 of the caspase-9 protein. This mutation occurs in the caspase pro-domain region, which allows the proper assembly of the apoptosome complex (Bratton and Salvesen 2010); it can be anticipated that a highly ramified amino acid as valine substituting for alanine will negatively influence the generation of the proper protein-to-protein interactions. Moreover, ADF cells also express a truncated splice variant of caspase-9, the so-called caspase-9β, which lacks a relevant part of the large subunit of the protein, including the catalytic site (Ceruti et al. 2005). Caspase-9β is therefore inactive and it is generally believed to behave as a dominant negative of the full-length caspase-9. Based on these observations, we therefore postulated that ADF cells couldn't efficiently activate the intrinsic pathway of cell death, which could explain their resistance to several known cytotoxic agents.

We also identified a single mutation in the p53 protein expressed by ADF cells (Ceruti et al. 2006): a single G-to-A nucleotide substitution in position 797 of the coding sequence, with a consequent single amino acid substitution (G-to-E) at position 266 of the p53 protein, belonging to the DNA-binding domain. This mutation leads to an inactive p53 isoform (Ceruti et al. 2006), meaning that ADF cells cannot activate the cell death pathways that depend upon the transcriptional activity of p53.

Despite the presence of the above-mentioned multiple genetic alterations of different cell death pathways, both 2-CA and 2-CdA have proved able to induce massive ADF cell death by recruiting caspase-2 and, in the case of Cladribine, also by interfering with the cell cycle (see above, Ceruti et al. 2000, 2003b). This unveils the cytotoxic potential of these derivatives also in the presence of resistance to various pharmacological approaches, and suggests the use of Ado derivatives as second line drugs in astrocytomas refractory to currently utilized chemotherapic regimens.

It is also important to note that both 2-CA and 2-CdA were ineffective in inducing cell death on primary neuronal cultures when utilized at concentrations, which were already almost maximally effective on ADF cells (Fig. 2.7, Ceruti et al. 2000). Although the mechanism at the basis of this selective sparing of neuronal cells (despite the marked apoptotic effect observed on cancerous glial cells) has not been clarified, it could represent an important additional therapeutic advantage in the case of in vivo administration of these drugs. Moreover, a specific carrier for Ado at the level of the blood-brain barrier has been demonstrated (Pardridge et al. 1994), and since 2-CA and Ado utilize the same ENT to permeate cells, it may be hypothesized that 2-CA may easily cross the blood-brain barrier. Conversely, for 2-CdA, concentrations in cerebrospinal fluid of leukemic patients have been demonstrated to represent 25 % of plasma concentrations (Liliemark 1997), suggesting a high tropism toward nervous tissues. Moreover, an early disruption of the blood-brain barrier is known to occur in several CNS pathologies that would favour accumulation of these antitumour agents in brain tissues.

Despite the above-mentioned encouraging preclinical data, a few unsuccessful clinical studies have been performed on the use of 2-CdA in malignant gliomas. A Phase II study performed on seven patients with recurrent glioma has shown no significant effects by Cladribine (Rajkumar et al. 1999). A Clinical Trial entitled "Chemotherapy Followed by Radiation Therapy in Treating Patients with Malignant Glioma: Cladribine in combination with radiotherapy in patients with high grade glioma" is available on the USA ClinicalTrials.gov website (#NCT00019071). The study has been completed, but neither publications nor results have been posted on the website, thus making it impossible to evaluate its outcome. No data are currently available on the possible effects exerted by Cladribine, which is now considered one of the drugs of choice in multiple sclerosis for its immunomodulatory activities (Killestein et al. 2011), as a second-line drug on refractory gliomas (see above).

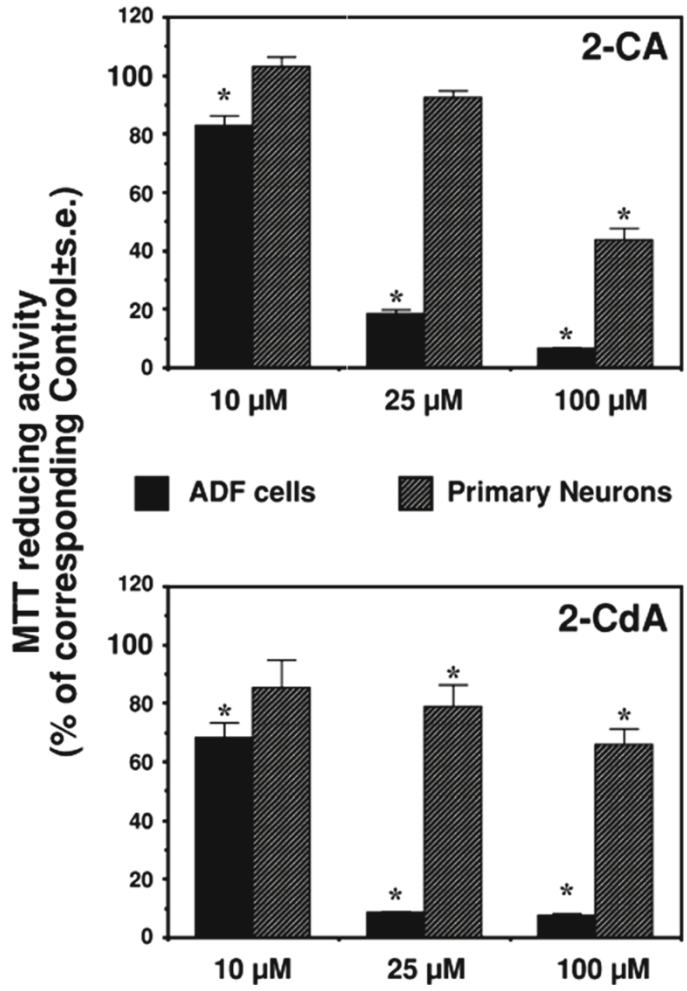

Fig. 2.7 Selective sparing of primary neurons by concentrations of 2-CA and 2-CdA inducing significant cytotoxicity in human astrocytoma ADF cells. ADF cells and primary cortical neurons were exposed to increasing 2-CA (*upper panel*) or 2-CdA concentrations (*lower panel*) for 48 h. At the end of the incubation period the percentage of cell death was evaluated by the ability of cultures to metabolize the MTT dye, a typical feature of healthy cells. Data are expressed as percentage of the MTT metabolizing activity in the corresponding control cultures and represent the mean ± S.E.M. of five independent experiments run in triplicate. *$p < 0.05$ with respect to corresponding control, one way ANOVA (Scheffe's F-test) (Reproduced from Ceruti et al. (2000). With permission from John Wiley and Sons)

More recently, as already mentioned above, Ado itself has been demonstrated to have inhibitory effects on the growth of C6 glioma cells by activating an intracellular pathway involving its uptake through ENT2 followed by phosphorylation by AK (Ohkubo et al. 2007). Authors hypothesize the induction of pyrimidine starvation by excessive intracellular AMP concentrations, since extracellular uridine completely reversed Ado-mediated effects. These results suggest that, in glioma cells, not only can elevated extracellular Ado concentrations activate its membrane receptors (see Sect. 2.4 above), but that the nucleoside can also directly modulate cell proliferation through intracellular signaling pathways.

2.6 Conclusions and Future Perspectives

The ubiquitous distribution and heterogeneity of the various components of Ado-mediated signaling (i.e., metabolizing enzymes, various subtypes of membrane receptors and of membrane transporters, direct actions on intracellular enzymes involved in cell survival, etc.), and the multiplicity of effects described upon its recruitment, with sometimes opposite outcomes, have made it difficult to exploit

this system as a new pharmacological target for brain tumours. Nevertheless, the data collected so far and reviewed in this article strongly suggest that the Ado system plays a fundamental role in controlling the proliferation, migration, and survival of glioma cells, where a marked hypoxic environment is generated. Not only Ado receptors, but also nucleotide metabolizing enzymes could represent important and druggable targets in the fight against brain tumours. Moreover, the ability of Ado analogues to recruit atypical intracellular pathways of death in tumours where the "classical" apoptotic triggers are useless and to selectively spare surrounding healthy cells would represent additional therapeutic advantages, which should be further explored. Contrasting results can often be reconciled by taking into consideration the various experimental settings, the different cell lines, and the agonists' concentrations utilized. While many studies have been addressed to assess the potential of Ado analogs as anti-glioma agents *in vitro*, we feel that an accurate evaluation of these compounds in appropriate *in viv*o models is still missing. We therefore envisage that well-designed *in vivo* studies could shed light on the current inconsistencies between different results, and more clearly highlight the possible advantages of targeting the Ado system for the therapy of glioma.

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