# Adenosine Targeting Strategy for Glioblastoma Aggressiveness

Subjects: Oncology

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Glioblastoma is the most commonly malignant and aggressive brain tumor, with a high mortality rate. The role of the purine nucleotide adenosine and its interaction with its four subtypes receptors coupled to the different G proteins, A1, A2A, A2B, and A3, and its different physiological functions in different systems and organs, depending on the active receptor subtype, has been studied for years. Recently, several works have defined extracellular adenosine as a tumoral protector because of its accumulation in the tumor microenvironment. Its presence is due to both the interaction with the A2A receptor subtype and the increase in CD39 and CD73 gene expression induced by the hypoxic state. This fact has fueled preclinical and clinical research into the development of efficacious molecules acting on the adenosine pathway and blocking its accumulation. Given the success of anti-cancer immunotherapy, the new strategy is to develop selective A2A receptor antagonists that could competitively inhibit binding to its endogenous ligand, making them reliable candidates for the therapeutic management of brain tumors.

glioblastoma adenosine tumor microenvironment

## 1. Adenosine and Adenosine Receptors (ARs)

Adenosine is a small molecule present throughout the human body, capable of performing various physiological functions following interaction with its receptor subtypes (A1, A2A, A2B, and A3). It is present in the cardiovascular system, where it modulates the vasoconstriction and vasodilation of arteries and veins [1]; in the metabolic context, adenosine inhibits lipolysis and induces bronchoconstriction [2][3], and regulates diuresis, muscle tone, and locomotion. At the level of the CNS, it exerts neuroprotective activity against ischemic events [4], hypoxia, and oxidative stress, and modulates the release of neurotransmitters; it is also involved in the regulation of cytokines and the production of T lymphocytes by the immune system [5][6]. There are two forms of adenosine: intracellular and extracellular [7], widely expressed in all tissues, and obtained by the dephosphorylation of its precursors, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), or by hydrolysis of S-adenosylhomocysteine (SAH) [8]. Physiologically, the intracellular concentration of adenosine is regulated by an important enzyme known as adenosine kinase (ADK), and by two transporters: the equilibrative nucleoside transporters (ENT) and the bidirectional passive transporters, which play a critical role, as they allow free movement of adenosine across the cell membrane [9], and nucleoside concentrative transporters (CNTs), Na-

dependent transporters that coordinate the adenosine gradient transport [10]. The direction of this nucleoside, absorbed or released by cells, is determined by the difference in concentration between the two forms, intracellular and extracellular, across the membrane [7]. Adenosine is defined as a “helper” in protecting cells such as neurons and cardiomyocytes against stressful conditions, allowing them to regulate their activity to reduce ATP requirements and ensure cell survival [7]. This is possible because adenosine can be released into the extracellular environment, where it acts as a specific modulator through cell surface receptors [5]; these receptors, called “ado receptors” (Ars), are GPCRs and are classified into four subtypes: A1, A2A, A2B, and A3 [11], which differ in the number of amino acids and in their affinity towards adenosine. In fact, A1 and A2A possess a high affinity for adenosine compared to A2B and A3, which have a low affinity for the nucleoside [12].

Among them, A1A and A3A receptors are coupled to Gi and Go proteins that inhibit adenylate cyclase activity and reduce intracellular cAMP levels. This will result in the activation of phospholipase C(PLC)-β, thereby increasing inositol-1,4,5-triphosphate ($IP_3$) [13] and intracellular calcium levels, which in turn stimulate activation of the Ca-dependent protein kinase (PKC) and all calcium-binding proteins [13]. In the CNS, A1AR is expressed in microglia/macrophages and neurons, and plays a crucial role in their activation [14]; peripherally, it is also highly expressed in cardiac, renal, and adipose tissue. As demonstrated by Synowitz M. et al. in A1AR knockout mice, there is an increase in neuroinflammation and microglia activity [15], and this suggests that, in pathological conditions, A1AR activation produces a neuroprotective effect [16]. In physiological conditions, adenosine, through A1AR, determined a decrease in the proliferation of astrocytes, inducing the release of neurotrophic growth factor (NGF) [17]. A3AR, however, has a low expression in the CNS, but it is highly expressed in immune cells [8], cardiac cells, epithelial cells, colon mucosa, lung parenchyma, and bronchi. It is demonstrated that A3AR is expressed in cells involved in inflammatory processes, suggesting its potential involvement in inflammatory pathologies, such as lung injury, autoimmune diseases, and eye diseases [18]. Moreover, A3AR is present in many types of tumor cells, including astrocytomas, lymphoma, GBM, and other types of cancers [19].

A2AA and A2BA receptors are coupled to the Gs proteins, activating adenylate cyclase and increasing intracellular cAMP levels [20]; moreover, A2AAR activation can promote Protein kinase C (PKC) activation into cyclic AMP-dependent or independent mechanisms [21]. A2BAR activation, however, can stimulate PKC activity by coupling with the Gq protein [22]. They are mainly expressed in the CNS, especially in pre-synaptic regions of the hippocampus, where the release of neurotransmitters such as glutamate, acetylcholine, GABA, and noradrenaline is modulated [23][24], and in post-synaptic regions of the basal ganglia, where they modulate neuronal plasticity. They are also expressed in the astrocytes and oligodendrocytes [25][26] and on the cell surfaces of the immune system [27], such as regulatory T cells, macrophages, and natural killer cells (NKCs) [28], suggesting that they could be valid candidates for cancer immunotherapy. All subtypes of adenosine receptors are expressed on the surface of immune cells, such as macrophages and monocytes, and their expression is regulated by pro-inflammatory cytokines, especially IL-1B and the tumor necrosis factor (TNF) [29], which determined an increase in A2AAR levels on human monocytes [30]. The same pro-inflammatory stimuli regulate the expression of the A2BAR of the macrophages [31]. In physiological conditions, central A2AAR increases NGF and brain-derived neurotrophic factor (BDNF) levels from the hippocampus and cortical neurons [32]. Therefore, given both the prevalence of A2AAR in the CNS and its expression regulated by pro-inflammatory cytokines, this receptor plays a crucial role in

inflammatory processes involving microglia, determining the release of IL-1β and IL-18 [33]. In fact, an antagonistic action against A2AAR prevents hippocampal neuroinflammation and IL-1β-induced exacerbation of neuronal toxicity [34]. Evidence showed that, in spinal intermediate neurons of the striatum, this receptor is related to the dopaminergic D2 receptor, where direct and indirect interactions with cholinergic, GABAergic, dopaminergic, and glutamatergic systems have been described, both in the basal ganglia and in other brain structures [35]. In the periphery, A2AAR is localized in the vascular smooth muscle and, together with A1AR, exerts a vasodilatory action. In this context, at the coronary levels, vasodilation mediated by the activation of A2AAR and A1AR is induced by the endothelial enzyme nitric oxidase synthase [36], producing large quantities of nitric oxide and inducing an increase in coronary flow, thus exerting a cardio-protective role [36], and this action depends on an increase in the intracellular cAMP levels [36]. Depending on the location of its receptors by which it interacts, adenosine exerts multiple physiological actions, including the protection of normal tissues and organs from the autoimmune response of immune cells, following binding with A2AAR [37].

## 2. The Role of Adenosine in Glioblastoma Multiforme

Studies report that extracellular adenosine is an important regulator of several aspects of tumorigenesis, angiogenesis, tumor cell growth, and metastasis [38]. Kezemi et al. provide an interesting research of the expression of adenosine receptors in different tumor cell lines and their effect, including proliferative and tumor-protective expressions, following their activation [39].

It is hypothesized that in the brain, ATP released from the pre- and post-synaptic terminals of neurons and glial cells is the source of extracellular adenosine [40]. In the extracellular area, adenosine is produced from ATP after dephosphorylation by specific ectoenzymes, in this case, CD39 and CD73, expressed in microglial cells [41]. In physiological conditions, CD39 and CD73 exert an important role in the purinergic signals delivered to immune cells through the conversion of ADP/ATP to AMP to adenosine [42]. The CD39/CD73 pathway changes with the pathophysiological context in which it is embedded [43]. It has been demonstrated in vivo study that mice deprived of CD73 presented a lower level of extracellular adenosine, suggesting that ATP degradation is the main source of extracellular adenosine [44]. CD39 is expressed on the surface of the regulatory T cells, and it is the dominant ectoenzyme that controls extracellular nucleoside concentration [41]. Considering that angiogenesis is an important process for the growth of the tumor cells, it has been demonstrated that in mice deprived of CD39, angiogenesis is blocked, causing a slowdown in tumor growth [42].

High concentrations of adenosine and its receptors have also been found in the interstitial fluid tumor, modulating tumor growth [45]. Since the TME contains high levels of extracellular adenosine, it is hypothesized that tumor-derived adenosine is a mechanism by which tumors evade the immune response [46][47]. This evasion strategy is due not so much to the inability of immune cells to recognize the tumor, but the failure of the immune system to activate in the presence of the antigen [48] due to the inhibition of T cells by adenosine itself [48].

It is known that the immune system, through antigen-presenting cells (APC), is able to recognize a specific antigen [49], subsequently allowing the binding with B and T lymphocytes through their receptors, B-cell receptor (BCR) and

T-cell receptor (TCR), respectively, thus initiating the immune response. In this tumor context, the activation of the immune system will lead to the secretion of anti-cancer cytokines [50], such as Interferon-gamma (INF-γ), TNF-α, and IL-6, and cell phagocytosis to eliminate the tumor, thus becoming a tool for the development of new treatments in cancer therapy [51]. Nevertheless, most tumors are able to implement various mechanisms to evade the immune response, such as inhibiting tumor-specific immune cells [52]. As is often the case, a particular tumor may express an antigen that, if presented by resting cells or by unprofessional APCs, recognition of the TCR will not lead to tumor destruction, but to inactivation of the tumor-specific T cell [53].

An important aspect of the mechanism of escape by the tumor from the immune system is the TME [54], which is characterized by a hypoxic state and is rich in inhibitory ligands and cytokines, such as IL-10 and TGF-β, which lead to tolerance by the immune cells towards the tumor [55][56]. These conditions determine the increase in the expression of CD39 and CD73 [43], present on the surface of the tumor by stimulating the production of extracellular adenosine, by activating A2AAR. Moreover, at the same time, there is a reduction in the activity of the adenosine metabolizing enzyme, ADK [57].

In addition, it has been reported that the deletion of functional adenosine receptors, in particular A1AR, results in increased GBM growth [15]. However, subsequent studies have found that the interaction of adenosine with A2AAR induces inhibition of the adaptive immune response, inhibiting the function of $CD4^+$ and $CD8^+$ T cells and NKCs and IL-2/Nkp46-activated NK cells specifically via A2AAR [58], thus promoting tumor escape from the immune system and metastasis [59][60]. Several in vitro and in vivo studies report that genetic deletion of the A2AAR enhances the anti-tumor responses, confirming adenosine's role in evading the tumor from the immune system [61]. Second, adenosine appears to block both the generation and effector phases of anti-tumor responses. In vitro studies have been conducted on GBM cell lines U87MG, U373MG [8], and ASB19, which were subjected to hypoxia [8] for 24 and 72 hrs using ATB702 dichloride hydrate (15uM), an ADK inhibitor, and resulted in an accumulation of adenosine [62]. Subsequently, the cells were treated with TMZ (100 µM), which resulted in a decrease in the vitality of the tumor cells compared with the control GBM cells, thus demonstrating the tumor-protective role of endogenous adenosine against TMZ [63].

It has been shown that extracellular adenosine, defined as an immunosuppressive factor through interaction with its receptor, exploiting the hypoxic condition of the TME [64], is able to lead to an increase in intracellular cAMP, inhibiting lymphocyte-mediated cytolysis and, consequently, functional inhibition of immune cells, thus acting as a protective shield against the tumor, helping it to evade the immune system [47]. Therefore, if GBM cells contribute to immunosuppression, the immune cells recruited into the tumor may also participate in its immune escape [65]. Indeed, most of the anti-tumor immune cells recruited to the TME adopt an immunosuppressive phenotype due to the cytokines secreted by GBM [65].

In this context, a large number of experiments have shown that the concentration of adenosine in the TME is much higher than in normal tissues [66].

Hypoxia and tissue damage are not the only factors determining the release of extracellular adenosine; it is also generated from extracellular nucleotides by ectonucleotidases [42] CD39 and CD73 [43]. Through clinical studies, CD73, rather than CD39, was found to be a critical component in adenosine accumulation and tumor immunosuppression. Indeed, overexpression of CD73 was reported to be a component of glioma cell adhesion and tumor cell–extracellular matrix interactions [67].

Moreover, high adenosine concentrations also induce receptor-independent reactions by reversing the reaction catalyzed by S-adenosylhomocysteine hydrolase (SAH-hydrolase), leading to an accumulation of SAH-inhibiting methyltransferases [68], as was shown in a recent study in which adenosine induced DNA hypomethylation in the brain by inhibiting trans-methylation reactions [69]. This connection between adenosine and methyl group metabolism is important for diagnostic purposes because an alteration in methyl group metabolism has been shown to be a risk factor in brain diseases such as GBM and neurodegenerative diseases [70][71].

In vitro and in vivo studies have shown that the presence of adenosine receptors in microglia is well established [72]. Cell cultures of rat microglia specifically express the A2AAR and were treated with the specific agonist CGS21680, inducing the expression of $K^+$ channels, which are linked to microglia activation [73]. Again, there is conflicting evidence regarding the role of this receptor: stimulation of the A2AAR in rat microglia induces the expression of nerve growth factor and its release, thus exerting a neuroprotective effect [74], and at the same time induces the expression of Cyclooxygenase-2 (COX-2) in rat microglia by releasing prostaglandin [75].

To confirm the involvement of adenosine and its receptors in tumorigenesis and its tumor-protective role, in vivo studies were conducted using the adenosine receptor agonists or antagonists [76]. A2AAR blocking using SCH58261, an A2AAR antagonist, inhibited the tumor growth, reducing $CD4^+$ and regulatory T cells, and improving the anti-tumor response by T cells [76].

# 3. Adenosine Receptor Antagonists

## 3.1. Xanthine Derivates

Compounds belonging to this group result from modifications of the two main alkaloids, caffeine and theophylline [77]. These derivates show a high affinity for all the adenosine receptors, but in the GBM context, receptor affinity must be directed towards A2AAR in order to bind it selectively and competitively, reducing adenylate cyclase activity [78]. The main A2AAR xanthine antagonists are 8-(3-chlorostyryl) caffeine (CSC,7), 1,3-dipropyl-7-methyl-8-(3,4,-dimethoxystyryl)xanthine (KF 17837), 3,7-dimethyl-1-propargylxanthine derivates (DMPX), and Istradefylline (KW-6002), which has a $K_i$ of 2.2 nM, and is an extremely strong, selective, and orally active adenosine A2A receptor antagonist [79]. DMPX was the first selective A2AAR to be detected [80]. Many selective A2AAR antagonists have been obtained, some of which are being used in clinical trials for neurodegenerative diseases such as Parkinson's disease, given the interconnection between dopaminergic D2 receptors and adenosine [81][82].

## 3.2. Polyheterocyclic Nitrogen System

Another group of A2AAR antagonists is represented by the polyheterocyclic nitrogen system, including Preladenant (SCH-420814), Ciforadenant (CPI-444) [83], Taminadenant (NIR178) [84], Imaradenant (AZD4635) [85], SCH442416 [86], and ZM241385 [87], characterized by small molecules that selectively bind to the A2A receptor, competitively inhibiting adenosine binding and signaling [88]. In the GBM context, this compound presents troubles that prevent its use in clinical trials, as it has a high binding affinity for the A2B receptor subtype [83]. With a $K_i$ of 0.048 for human A2AAR, the SCH442416 antagonist is considered a strong, selective, and brain-penetrant antagonist of A2AAR [89]. However, strong evidence has been shown by Ciforadenant being active in multiple preclinical tumor models, both as monotherapy and in combination with PDL1 targets, and it has over 66-fold selectivity over the adenosine A1 receptor [90]. Clinical studies conducted on GBM patients under 1 and 4 months of treatment with Ciforadenant have shown that it possesses immunomodulatory effects [91]. Through in vitro studies, it was possible to characterize adenosine-related gene expression with the production of chemokines and cytokines, including CXCL5, CCl2, IL-8, and CXCL1, of monocytic, CD14+ origin, using the receptor agonist NECA [92], and how Ciforadenant is able to neutralize them [91]. Thus, these reports suggest that adenosine signaling not only directly reduces T lymphocyte immunity but also shifts the balance from T effector responses to both recruitment and myeloid suppressor functions [83].

### 3.3. Enhancement of Immunotherapy Induced by Adenosine Receptor Antagonists

Another therapeutic approach to enhance immunotherapy targets the immune cells in the TME [5]. As previously reported, the A2AAR is expressed on the surface of many cells of the immune system, the activation of which induced an immunosuppressive effect [5]. Consequently, a selective A2AAR antagonist reducing intracellular cAMP levels allows lymphocytes to effectively fight tumor cells. Since A2A and A2B adenosine receptors are coupled to the G proteins, and both increase intracellular cAMP levels [20], the use of A2BAR antagonists leads to a reduction in cAMP by restoring the anti-tumor functions of lymphocytes [91]. It is of interest to note the relationship between A2A and A2B adenosine receptors: A2A is involved in the expression of A2BAR [16]; furthermore, its activity is influenced by the expression of A2BAR, and both proteins can interact to form new functional units [93]. This evidence, therefore, suggests that blocking these receptors may be an effective means of combating cancer [93]. In this regard, clinical trials are already underway in patients with different tumor types where either the use of selective antagonists for individual receptors or dual antagonists is employed [5].

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