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A2A Adenosine Receptor antagonists to weaken the hypoxia-HIF-1α driven immunosuppression and improve immunotherapies of cancer

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Abstract

Hypoxic and adenosine rich tumor microenvironments represent an important barrier that must be overcome to enable T and NK cells to reject tumors. The A2A adenosine receptor (A2AR) on activated immune cells was identified as a critical and non-redundant mediator of physiological immunosuppression. Observations showing that tumor-protecting A2AR also suppress and redirect the anti-tumor immune response pointed to the importance of inhibiting this pathway to improve cancer immunotherapy. We advocated i) blocking immunosuppressive Adenosine-A2AR-cAMP–mediated intracellular signaling by A2AR antagonists and ii) weakening Hypoxia-HIF-1α-mediated accumulation of extracellular adenosine by oxygenation agents that also inhibit CD39/CD73 adenosine-generating enzymes. In view of commencing clinical trials of synthetic A2AR antagonists in combination with cancer immunotherapies, we discuss their promise and exclusion criteria.

Introduction

At the center of the current excitement surrounding cancer immunotherapy are spectacular examples of tumor rejection in some patients by T cell-based immunotherapies and immune-checkpoint inhibitors [1]. The introduction of CTLA-4 and PD-1 blocking antibodies as FDA-approved drugs that target not the tumor, but cells of the immune system, represents a new approach in the development of cancer therapies.

However, there is room to further improve clinical outcomes. The hypoxic and adenosine rich “Hypoxia-A2-Adenosinergic” tumor microenvironment (TME) (Fig. 1) is now considered an important barrier that must be overcome in order to enable tumor-reactive T cells and Natural Killer (NK) cells to infiltrate and kill tumors. This is because anti-tumor T cells are still inhibited by other immunosuppressive mechanisms even after blockade of

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CTLA-4 and PD1. Currently, several pharmaceutical companies, including Novartis, AstraZeneca and others are preparing for clinical trials where immunotherapeutic drugs such as anti-PD1 monoclonal antibody (mAb), would be combined with synthetic A2A adenosine receptor (A2AR) antagonists to weaken the Hypoxia-A2-Adenosinergic immunosuppression.

This clinical focus on A2AR puts a premium on a better understanding of how A2AR functions in the regulation of the immune response, including the anti-tumor immune response. We will summarize the studies of the anti-hypoxia A2-adenosinergic co-adjuvants (Fig. 2), which target both anti-tumor immune cells and the TME. Blockade of this pathway can prevent the inhibition of anti-tumor T and NK cells by the weakening of the Adenosine-A2AR signaling (Fig. 1 and Fig. 2).

Investigations of blocking A2AR to improve immunotherapy complemented the long-term studies and important advances of Bruce Cronstein and co-authors. These studies were motivated by the opposite aim; to decrease the inflammatory damage to normal tissues by pharmacologically recruiting A2AR and A2BR on overactive myeloid cells (reviewed in [2]). The field of anti-Hypoxia-A2-Adenosinergic treatments (“Co-adjuvants”) to improve cancer immunotherapy began following the genetic *in vivo* evidence that A2AR on T cells and myeloid cells are negative physiological regulators of virtually all types of tested effector functions [6–8]. Similarly, HIF-1α was found to be inhibitory in cells of the adaptive immune system [6–8].

It is now well established that hypoxic and adenosine-rich TMEs strongly inhibit anti-tumor T and NK cells. Our initial studies provided the proof-of principle to combine the immunotherapies of cancer with synthetic or natural antagonists of A2AR [9]. We also demonstrated the feasibility of inhibiting the accumulation of extracellular adenosine in inflamed tissues by oxygenation agents that reprogram the TME away from immunosuppression and toward an immunopermissive phenotype [8].

These original studies were reviewed and interpreted in [10–12] and they provided the necessary justification for other scientists to invest in the further development of the anti-hypoxia-A2-adenosinergic drugs by focusing on CD39/CD73–Adenosine-A2AR axis[13–19].

Discovery of A2A-adenosinergic protection of normal and cancerous tissues from immune cells

The long-term interest in understanding the biochemical mechanisms of cancerous tissue protection was triggered by the “Hellstom paradox”, describing the coexistence of tumors and anti-tumor lymphocytes in the same cancer patients [20].

We started with the consideration to target intracellular cAMP, based on established evidence that cAMP was inhibitory to lymphocytes (reviewed in [21]). We hypothesized and then demonstrated [12][22–26] that increases in intracellular cAMP may explain the

Hellstrom Paradox. The most important remaining question was regarding the identification of a cAMP elevating G-protein coupled receptor (GPCR) and its ligand [21].

The initial studies of the pharmacological effects of the cAMP-elevating G-protein coupled high affinity A2AR on T cells supported the view that A2AR could be among many other GPCR candidates that could serve as physiological negative regulators of the immune response (reviewed in [2, 12, 27]). However, only *in vivo* genetic studies in animals with A2AR gene-deletion could establish whether A2AR was indeed inhibiting activated immune cells at physiological and pathophysiological levels of extracellular adenosine. These studies conclusively demonstrated the critical and non-redundant role of A2AR in the protection of normal tissues from collateral damage during the anti-pathogen immune response [5].

Importantly, genetic targeting of A2AR was also recapitulated by pharmacological treatments with synthetic (ZM 241385) and natural (caffeine) A2AR antagonists. These studies were the first to suggest and provide the proof-of-principle for targeting the Hypoxia → Adenosine → A2AR pathway as a strategy to prevent the inhibition of anti-tumor T cells in the TME [5].

The available data regarding the inhibitory role of A2AR in activated immune cells *in vivo* suggested that all activated immune cells–including anti-tumor immune cells–might be under inhibitory influence of adenosine-A2AR axis *in vivo*. However, additional studies were required to establish the crucial role of A2AR in the protection of cancerous tissues during the anti-tumor immune response [9,12].

In support of our original hypothesis, it was found that genetic deletion of A2AR resulted in rejection of established tumors in approximately 60% of A2AR-deficient mice by unleashing the otherwise inhibited anti-tumor immune cells. These data positioned the A2AR as promising therapeutic targets to improve the immunotherapy of cancer, even though the immunosuppressive role of A2AR and A2BR in anti-tumor killer cells had been explicitly excluded from considerations by pharmacological studies of others [29].

Using antagonists of A2AR to prevent the inhibition of anti-tumor T and NK cells

Anti-A2-adenosinergic pre-clinical studies provided proof of principle for the use of A2AR antagonists, which recapitulated–although not as strongly–genetic deletion of A2AR [9]. It was found that targeting of A2AR by antagonists de-inhibits $CD8^+$ T cells, facilitating their antitumor effector functions. This was due to the prevention of the adenosine-triggered cAMP elevation and reversal of the adenosine-mediated inhibition of activated T cells and other immune cells *in vivo.*

Enhanced tumor rejection by T cells with A2AR-targeted siRNA suggested that the effects of A2AR are T cell-autonomous [9]. In these studies, ZM241385 (synthetic antagonist of A2AR/A2BR) and the natural antagonist 1,3,7 trimethylxanthine (caffeine) were effective in i) promoting T cell-mediated tumor regression ii) increasing tumor cell apoptosis and iii) preventing tumor neo-angiogenesis by enhancing IFN-γ production. This suggested that

A2AR antagonists not only unleash perforin and Fas ligand-mediated cytotoxicity, but that increased IFN-γ production may also starve tumors by denying nutritional supply [9].

The limitation of these early studies of A2AR in preclinical models of cancer immunotherapy was the lack of availability of a selective synthetic A2AR antagonist with a long *in vivo* half-life. Thus, survival was not previously reported with short-lived A2AR antagonists, in contrast to the improved survival shown in tumor-bearing mice with genetic deletion of A2AR [9].

The recent commercial availability of the long-lived A2AR antagonists Preladenant, or SCH58261, allowed others to confirm and extend our studies demonstrating the original proof-of principle. Several groups of scientists supported the targeting of A2AR by demonstrating that blocking A2AR genetically or by synthetic A2AR antagonists, resulted in powerful inhibition of tumor metastasis. This was due to the unleashing of the anti-tumor immune cells from tolerization by A2AR. In their studies, Powell and co-authors [13] showed that A2AR promotes peripheral tolerance and that deletion of A2AR improves immunotherapy-enabled tumor rejection in mice [14]. The case for translating A2AR antagonists into clinical trials of cancer immunotherapy was also greatly strengthened by cancer immunologists in Australia, Canada, and the USA [16, 19, 30–34]

More advanced synthetic A2AR antagonists for cancer immunotherapy now exist since their development was motivated by observations of A2AR involvement in the neurobiology and pathogenesis of Parkinson's disease [35]. These A2AR antagonists were demonstrated to be safe and well tolerated in extensive clinical trials, including phase III. While not approved by the FDA in the US, the A2AR antagonist KW6002 was approved in Japan.

A2AR antagonists improve anti-tumor effects if combined with blockade of immunological negative regulators CTLA-4/PD1

Current interest of Pharma was strengthened greatly by the studies of mechanisms of immunosuppression of human cancers that are resistant to chemotherapy [30]. Authors demonstrated that tumors with high expression of adenosine-generating CD73 are resistant to chemotherapy and immunotherapy. However, these tumors can be rejected by T and NK cells if chemotherapy or immunotherapy is combined with antagonists of A2AR [30].

In these studies, authors demonstrate that A2AR/A2BR antagonists were effective in reducing metastasis of tumors expressing CD73. Additionally, A2AR inhibitors unleashed anti-tumor effector functions and promoted NK cell function by increasing Perforin-mediated cytotoxicity and increasing the expression of Granzyme B in NK cells *in vivo* [30]. This suggests the therapeutic potential of an A2AR/A2BR blockade strategy for the treatment of CD73(+) metastatic tumors [30].

Directly feeding into current clinical trials, A2AR antagonists were also shown to improve the effects of blockade of CTLA-4/PD-1 [33, 36]. These anti-tumor effects were strongly dependent on NK cells and IFNγ, although $CD8^+$ T cells and perforin also played a role [31, 36]. Overall, these results provide strong rationale for the use A2AR with immunological

checkpoint inhibitors for the treatment of residual and metastatic disease. Importantly, these studies also offered a novel approach and biomarkers to stratify patients for this immunotherapy by selecting only patients with tumors expressing high levels of adenosine-generating CD73 [32, 36].

Considerations of important properties of A2AR antagonists

The studies presented here strongly support the feasibility and promise of the weakening of hypoxia-adenosinergic signaling as a way to prevent tumor resistance to tumor-reactive T cells. The advantage of existing A2AR antagonists [35, 37, 38] is demonstrated in their safety profile in healthy volunteers and patients with Parkinson's disease. The efficacy in cancer immunotherapy can be predicted from sophisticated imaging studies of the occupation of A2AR binding sites by synthetic A2AR antagonists in human tissues *in vivo*.

Among the most important properties of an antagonist is the requirement for the high level of occupation of the A2AR at safe and well-tolerated doses. This requirement should be met during the evaluation of new synthetic A2AR antagonists prior to human clinical trials. This is important to emphasize due to the associated risk with rushing A2AR antagonists into clinical development prematurely with under-developed and poorly characterized drugs.

Future directions in the development of A2AR antagonists for cancer therapies may address the need in blood brain barrier-impermeable drugs since existing A2AR antagonists have been developed specifically to act in the brain of Parkinson's disease patients [37]. This may prevent the potential neurological side effects in cancer patients with tumors in anatomical locations other than the brain, and increase the number of patients eligible for this therapy.

Also to be addressed is the poor aqueous solubility and photoisomerization, a known limitation of existing A2AR antagonists of this class. One way to solve this problem was offered by an one-pot route to 8-substituted xanthines from 5,6-diaminouracils and carboxaldehydes with good yields [39]. As an example, these limitations were addressed in the modification of the drug approved in Japan, KW-6002, which was converted to a PEG derivative. Importantly, it was shown to be a functional derivative in *in vitro* bioassays used to confirm efficacy. It was also observed that the PEGylated version had much better aqueous solubility and was inert to photoisomerization [39].

Facilitating competitive A2AR antagonism by lowering adenosine in TME

The promise of A2AR antagonists as anti-tumor treatments attracted attention to the upstream events of the hypoxia-adenosinergic pathway, the generation of extracellular adenosine by the tandem of ecto-enzymes CD39-CD73 (Figure 1).

Thus, the targeting of the upstream hypoxia-HIF-1 [8] and CD39/CD73-adenosine stages of this pathway are the subject of many ongoing preclinical and clinical investigations. The adenosine-generating ecto-enzymes CD39/CD73 as drug targets have been extensively reviewed by leading scientists in this field [15–19]. Increased levels of CD39 [15, 18, 47] and CD73-generated tumor-protecting extracellular adenosine [15, 16, 30, 32, 48–51] may

signal through A2AR/A2BR to induce suppression of anti-tumor immune cells [5, 8, 9, 12, 19, 31].

A very important advance from fundamental biochemical and pre-clinical studies toward human cancer was in the uncovering of the connection between the adenosine-rich TME and immunosuppression in human cancer patients. This was done by extensive bioinformatics analysis of more than 6000 data sets from individual triple negative breast cancers (TNBC), which are also resistant to chemotherapies [19, 32, 53]. These studies have demonstrated the predictive power of CD73 expression as being predominantly associated with poor prognosis, emphasizing the correlation between tumor protection from chemotherapy and the overproduction of extracellular adenosine by the high levels of expression of these ecto-enzymes.

Several major studies have demonstrated that the inhibition of adenosine generation by CD73 mAbs or by small molecule drugs (APCP) significantly enhances the anti-tumor activity of T cells and NK cells [15,16]. Indeed, these data have stimulated AstraZeneca to prepare clinical trials with MEDI9447, a mAb against CD73, alone and in combination with MEDI4736 (durvalumab), a monoclonal antibody directed against PD-L1, in advanced solid tumors (ClinicalTrials.gov Identifier: NCT02503774).

Taken together, these data support and extend the implications of the reported anti-tumor effects of A2AR antagonists [9], strongly suggesting that the combination of CD39/CD73 inhibitors with A2AR or A2BR antagonists should be tested during the immunotherapy of cancer.

Considerations of the A2B Adenosine Receptor

A2BR was known to be immunosuppressive after pharmacological activation and after stimulation by pathophysiological levels of adenosine in inflamed tissues [41]. A2BR has also been implicated in the regulation of myeloid cells, including myeloid derived suppressor cells (MDSC) [42, 43, 54]. A2BR has been shown to promote the development of MDSC, which can be a source of the CD73-generated immunosuppressive adenosine [44, 45]. It is expected that A2BR may also contribute to the inhibition of human T cells, which do express both A2AR and A2BR. Studies by Morello’s group indicated that A2BR had a significant role in tumor progression [44, 45]. Similar studies by Ryzhov and colleagues [46] explored the important role of A2BR in tumor development in studies of Lewis lung carcinoma in A2BR-deficient mice.

Concluding remarks

There is an important advantage of synthetic small molecule inhibitors of anti-Hypoxia-A2-Adenosinergic drugs over mAbs that block immunological negative regulators. In the case of side effects, it is impossible to remove antibodies from patients due to their long persistence. In contrast, synthetic A2AR antagonists can be used as a once-a-day small molecule, in which treatment can be immediately ceased to avoid adverse side effects.

It is important to note, there could be potential side effects of A2AR antagonists as was described in “Caveats and cautions for the therapeutic targeting of the anti-inflammatory A2 adenosine receptors” [58]. These drugs must be used carefully since unleashing anti-tumor immune cells may be accompanied by increased auto-immunity in cancer patients if this treatment coincides with simultaneous acute inflammation or sub-threshold auto-immunity. The observations of autoimmunity during melanoma rejection in A2AR-deficient mice [15] suggest that A2AR in T cells is also important in preventing autoimmunity. Since unleashing the anti-tumor immune cells may be accompanied by an increased auto-immunity, we propose that episodes of even sub-threshold levels of ongoing inflammation should be considered among the exclusion criteria [58].

It would be interesting to test whether drugs that act as inhibitors of adenine nucleotide release and reuptake might also affect anti-tumor activity. Specifically, adenosine reuptake inhibitors (AdoRI) that block the action of one or more of the equilibrative nucleoside transporters (ENTs), may lead to increased extracellular concentrations of adenosine and immunosuppressive effects. These drugs should therefore be excluded in patients who are treated with A2AR antagonists. Future studies are needed to determine the effects of such drugs on the anti-tumor immune response.

There is a paucity of well-characterized synthetic A2AR antagonists and need in being careful in the pharmaceutical development, preclinical studies, and clinical trials of novel drugs [55–56]. Additionally, there is a need in the development and characterization of A2AR antagonists with a long *in vivo* half-life. The development of new A2AR antagonists is facilitated by important advances in structure-based A2AR antagonist design that are taking advantage of the recently revealed molecular basis of GPCR-ligand binding and activation [54].

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Highlights

- A2AR and Hypoxia in TME are important barriers that must be overcome in cancer immunotherapy
- A2AR on immune cells are critical and non-redundant mediators of physiological immunosuppression
- Blocking immunosuppressive A2AR-mediated signaling may improve current cancer immunotherapy

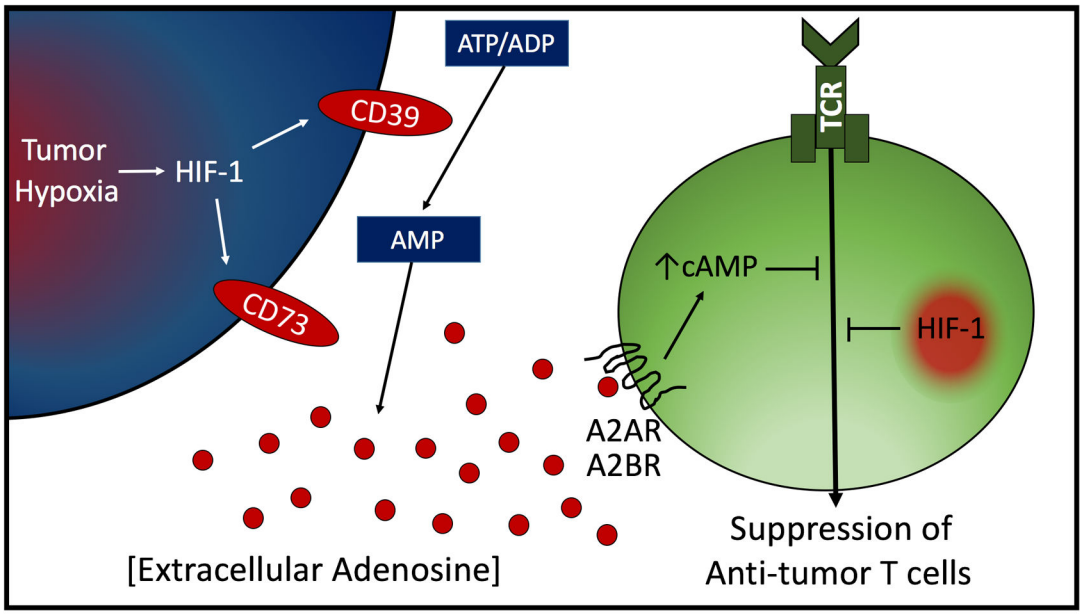

Figure 1. Intratumoral Hypoxia→HIF-1α driven and A2A/A2B Adenosine Receptor-mediated suppression of anti-tumor T cells

Shown are the HIF-1α regulated ecto-enzymes CD39/CD73 which act in tandem to generate extracellular adenosine. Adenosine triggers the accumulation of immunosuppressive intracellular cAMP by signaling through high affinity A2AR and low affinity A2BR. HIF-1α is also shown to suppress cells of the adaptive immune system [20].

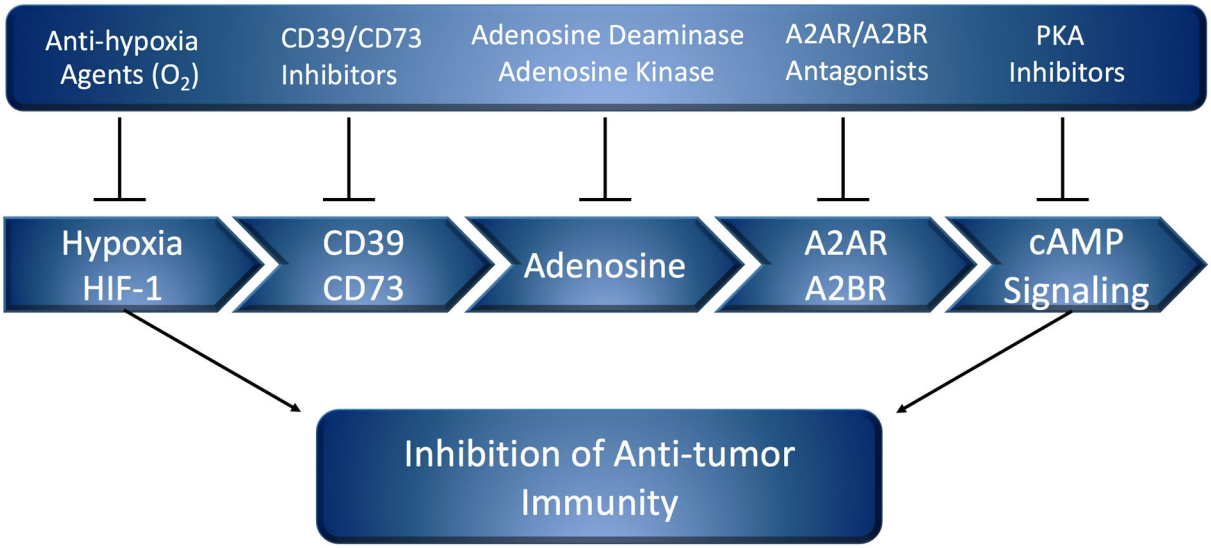

Figure 2. Anti-hypoxia A2-adenosinergic coadjuvants to enable the effector functions of anti-tumor T cells

Shown are the individual classes of drugs that inhibit the upstream and downstream stages of Hypoxia-HIF-1α driven and A2A/A2B Adenosine Receptor-mediated suppression of anti-tumor T cells. Under consideration for clinical trials are i) anti-hypoxia treatments such as oxygenation agents ii) inhibitors of CD39 and/or CD73 to prevent the generation of extracellular adenosine iii) enzymes that degrade extracellular adenosine and iv) A2AR antagonists.