Pharmacology and Therapeutic Applications of A_3 Receptor Subtype

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Abstract: The present study summarizes the biological effects elicit upon A_3 adenosine receptor (A_3AR) activation in normal and tumor cells. Anti-inflamatory response is mediated upon A_3AR activation in neutrophils, eosinophils and macrophages via direct effect on cell degranulation or the production of anti-inflamatory cytokines. In basophils, which highly express A_3AR, degranulation and mediator release upon receptor activation lead to pro-inflammatory effects resulting in bronchospasm and asthma. In other normal cells such as cardiomyocytes, neuronal cells and bone marrow cells A_3AR activation induces cytoprotective effects *in vitro*. *In vivo*, A_3AR agonists act as cardio- and neuroprotective agents and attenuate ischemic damage. Furthermore, agonists to A_3AR induce granulocyte colony stimulating factor (G-CSF) production and myeloprotective effect in chemotherapy treated mice. Interestingly, A_3AR agonists inhibit tumor cell growth both *in vitro* and *in vivo* through a cytostatic effect mediated via the de-regulation of the Wnt signaling pathway.

The variety of activities elicit by A_3AR agonists suggest their potential use as therapeutic agents in inflammation, brain/cardiac ischemia and cancer. Antagonists to A_3AR may be implemented to the therapy of asthma and additional allergic conditions.

Key Words: Adenosine, A_3 adenosine receptor, cancer therapy, myeloprotection, cardioprotection, neuroprotection, anti-inflammatory, asthma.

INTRODUCTION

The A_3AR was the last to be cloned [1,2]. It belongs to the family of the Gi protein associated cell surface receptors containing seven α-helical membrane spanning domains [3]. Its unique feature is the high serine and threonine residues in the carboxyl terminus tail which are rapidly phosphorylated resulting in receptor desensitization [4,5]. The synthesis of specific agonists and antagonists to A_3AR enable the study of the biological effects and the mechanisms involved upon A_3AR activation.

IB-MECA (N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide) and Cl-IB-MECA (2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide), synthesized by Jacobson *et al.*, [6] are the most potent and specific A_3AR agonists and have been widely used in a variety of studies.

A_3AR expression level was found to be low in most body tissues other than testis, eosinophils, basophils and neutrophils which all demonstrated massive expression [7-11]. Zhao *et al.* reported that during normal embryo development, no expression of A_3AR was found, except in the aorta and heart. When the A_3AR gene was overexpressed in smooth, cardiac and skeletal muscle lineages during early embryogenesis in knockout or wild-type mice, it was lethal to the embryos [12]. Thus, most of the studies which describe A_3AR characteristics were carried out with transfected rat or sheep cells [13-15]. On the other hand, other studies showed that tumor cells such as human A375 melanoma, human Jurkat T cell lymphoma and murine pineal tumor cells, significantly express A_3AR [16-18]. Receptor exhibition and spread is not the only factor determining cell response to a specific ligand. An additional parameter is the exhibition of A_{2A} and A_{2B} adenosine cell surface receptors, known to elicit opposite effects to that of A_3AR. A_3AR agonists, at high concentrations, may also activate A_{2A} and A_{2B} adenosine receptors, affecting the balance of the response [19,20]. Interestingly, low concentrations of A_3AR agonists, activating only A_3AR, induce beneficial responses in various cell types such as cardiomyocytes, neuronal cells (cytoprotection), G-CSF producing cells (activation) and tumor cells (cell proliferation inhibition) [21-25]. However, in other organ systems, A_3AR activation leads to the development of negative responses such as degranulation in basophils and mast cells [26] as well as pro-inflammatory effects [27].

Activation of A_3AR evokes different downstream signal transduction pathways which are cell type dependent and may attribute to the diverse responses described above. Upon A_3AR activation, adenylyl cyclase activity and cAMP formation are inhibited leading to decreased level of the

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1568-0266/03 $41.00+.00

effector PKA [28]. Additional signaling pathway which may be up or downregulated upon A_3AR activation (and is cell type specific) is the PI3K-PKB/Akt which induces the formation of phosphatydil inositides known to phosphorylate and activate the kinase PKB/Akt [29-31]. The latter acts as a cell survival factor by modulating caspases' level, the expression of anti-apoptotic genes and the level of GSK-3β, a key protein in the Wnt signaling pathway [32,33]. In addition, activation of phospholipase C and D and mobilization of Ca++ from intracellular and extracellular sources were reported following receptor activation leading to additional signaling pathways [28].

This review will summarize the *in vitro* and *in vivo* biological activities, which take place upon activation of A_3AR in various cells, with an emphasis on the differential effect on tumor and normal cells and the mechanistic pathways involved.

BASOPHILS/MAST CELLS, EOSINOPHILS AND NEUTROPHILS: A_3AR EXPRESSION AND ITS INVOLVEMENT IN THE PATHOPHYSIOLOGY OF ASTHMA AND ADDITIONAL INFLAMMATORY CONDITIONS

The accumulation of adenosine in the extracellular matrix, following its release from inflamed, necrotic or ischemic tissues, has been reported as being part of the pathogenesis of asthma and additional inflammatory diseases [34,35]. The effect of adenosine was found to be mediated via A_3AR activation in neutrophils, basophils/mast cells and eosinophils, inducing degranulation and mediator release. Moreover, adenosine's capability to cause cell migration into inflamed airways and to produce inflammatory cytokines is also mediated by A_3AR [36]. Neutrophils, basophils and eosinophils are the main players in the inflammatory response. Interestingly, these three subtypes, belonging to the myeloid-hematopoietic system, massively express A_3AR [8-11]. There is a debate in the literature regarding the pro- or anti-inflammatory response mediated via A_3AR activation in these cells. Some reports recommend activating A_3AR to block the inflammatory response while others favour the implementation of A_3AR antagonists for the very same purpose.

Bronchospasm induced in asthmatic subjects is attributed to activation of A_3AR on basophils and mast cells which elicit degranulation, leading to the release of histamine, leukotrienes and additional mediators [34]. The latter cause the contraction of smooth muscle, thereby inducing airway bronchospasm [37]. In mast cells and basophils A_3AR activation results in the production of inositol 1,4,5-triphosphate, leading to an increase in the level of intracellular Ca^{2+}, which subsequently induces exocytosis [38]. Recently, Gao *et al.* [31] showed that an additional mechanism includes PKB activation upon the exposure of rat basophilic leukemia cells to IB-MECA, thus prolonging cell survival and maintaining the accumulation of those cells in inflamed tissues. Based on these studies, a therapeutic approach aimed at the activation of A_{2A} receptor by specific agonists, eliciting responses opposing those of A_3AR, showed efficacy *in vivo* [39].

Activation of A_3AR on eosinophils, neutrophils and monocytes/macrophages resulted in the following responses which led to anti-inflammatory effects:

1. Eosinophils: inhibition of cell migration, platelet activating factor, eosinophil chemotaxis and generation of free radicals by human peripheral blood eosinophils [9,40-43]. A good example is the anti-inflammatory effect of theophylline which has been attributed to activation of A_3AR on eosinophils [44].

2. Neutrophils: inhibition of oxidative burst and degranulation. [11,45].

3. Monocytes: inhibition of superoxide anion generation [46].

Modulation of cytokine production also contributes to the anti-inflammatory effect mediated via A_3AR activation. Inhibition of the inflammatory cytokines TNF, interleukin-12 and interferon-γ was noted in macrophages upon A_3AR activation, while the anti-inflammatory cytokine, interleukin-10 was upregulated [47-51]. This was further demonstrated in mice treated with A_3AR agonists where prevention of anti-inflammatory effects such as endotoxemia were observed [27].

Taken together, A_3AR seems to play a positive role in counteracting inflammatory responses upon its activation in eosinophils, neutrophils, monocytes and macrophages, while mediating pro-inflammatory responses in basophils/mast cells.

CARDIO-, NEURO- AND MYELO-PROTECTIVE EFFECTS OF A3AR AGONISTS

Cardioprotection

In stress conditions, such as hypoxia or ischemia, adenosine concentration in the extracellular fluid is raised due to ATP metabolization. This leads to a cardioprotective effect in chronic heart failure by the reduction in the severity of ischemia and reperfusion injury [52]. A_3AR agonists were shown to contribute to this effect, whereas A_3AR antagonists neutralized it. Moreover cardioprotection against doxorubicin-induced cytotoxicity was also found to be mediated via A_3AR activation [53].

The expression of A_3AR in cardiac cells was found to be directly related to the degree of protection evoked. Indeed, increased protection against damage induced by ischemia has been demonstrated in A_3AR transfected cardiac myocytes and in cardiac ventricular cells which highly express the receptor [54]. However, cardiac arterial cells exhibiting low A_3AR are less protective. [55]. These findings are supported by the *in vivo* studies showing that low doses of A_3AR agonists elicit optimal protective effects in mice, rabbits and chicken cardiomyocytes [56].

The mechanism underlying the cardioprotective effect mediated via A_3AR includes the opening of K_{ATP} channels. This can be induced either by PLC/PLD-DAG-PKC

activation and translocation of the latter to the mitochondrial membrane or by p38-MAPK activation [14,57-62]. The mechanism of cardioprotection against doxorubicin-induced cytotoxicity included a decrease in intracellular Ca^{2+}, reduction of free radical generation, moderation of mitochondrial damage and attenuation of the decrease in ATP production [63].

Neuroprotection

Adenosine's neuro-protective effect has been demonstrated in epilepsy, trauma and brain ischemia [64]. It was shown to attenuate glutamate level known to induce neuro-cytotoxicity [65]. Several lines of evidence support the involvement of A_3AR in neuroprotection. *In vitro*, nanomolar concentrations of A_3AR agonists enhance astrocyte proliferation [19]; reorganization of the cytoskeleton, accompanied by the induction of Rho expression and changes in the intracellular distribution of the antiapoptotic protein Bcl-XL in human astrocytoma ADF cells [66]. Those effects were associated with a reduction in the degree of spontaneous apoptotic cell death. A_3AR antagonist prevented the antiapoptotic effect, demonstrating the specificity of this response [67,68].

In vivo, chronic treatment with IB-MECA administered 10 or 20 min prior to forebrain ischemia in gerbils, improved postischemic cerebral blood circulation, reduced histopathological damage in the hippocampus and enhanced neural preservation and survival [69]. The mechanism involved was the preservation of ischemia-sensitive microtubule-associated protein 2 (MAP-2), enhancement of the expression of glial fibrillary acidic protein (GFAP) and a very intense depression of nitric oxide synthase [70]. Moreover, chronic administration of IB-MECA protects against chemically induced seizures in mice, as measured by neurological impairment [71]. In addition, postischemic treatment (20 min) of transient cerebral ischemia with IB-MECA decreased the intensity of reactive gliolsis, reduced microglial infiltration, preserved neurons and significantly decreased infarct volume [72].

However, the protective effect depends on a low agonist concentration and continuation of treatment [19].

Myeloprotection

Myelotoxicity is a severe, dose limiting complication of chemotherapy which limits the administration of larger potentially more effective doses of cytotoxic drugs to cancer patients. Recently, we showed that adenosine acts as a chemoprotective agent due to its capability to stimulate the production of G-CSF and the induction of myeloid bone marrow cell proliferation and differentiation [24]. Adenosine, when given subcutaneously to C57BL/6J mice pretreated with the chemotherapeutic agent, cyclophosphamide, demonstrated a myeloprotective effect. It restored the number of white blood cells and the percentage of neutrophils to normal values. Pharmacological studies demonstrated that the effect of adenosine is mediated via A_3AR. Further studies, utilizing nanomolar concentrations of IB-MECA and Cl-IB-MECA, resulted in a stimulatory effect on the myeloid system via the induction of G-CSF production. Experiments in which anti-G-CSF antibodies blocked the proliferation of bone marrow cells in the presence of Cl-IB-MECA, provided evidence of A_3AR agonists' effect on G-CSF production [73]. *In vivo* studies in naïve mice revealed an increased serum G-CSF level which was followed by increase in white blood cell and neutrophil counts [74]. When administered orally to mice pretreated with cyclophosphamide, IB-MECA and Cl-IB-MECA accelerated the recovery of these hematological parameters (73,75).

A_3AR AS A TARGET FOR TUMOR GROWTH INHIBITION

Adenosine, at nanomolar concentrations, was shown to inhibit the *in vitro* proliferation of various tumor cell types, including melanoma, lymphoma and colon carcinoma. We observed that this effect was mediated via A_3AR activation [82]. Indeed, at nanomolar concentrations, IB-MECA and Cl-IB-MECA induced an inhibitory effect on the growth of Nb2-11C lymphoma, B16-F10 melanoma and HCT-116 colon carcinoma. [81,25,76]. These two agonists were found to act specifically *via* A_3AR activation, as confirmed by the reversal of tumor growth inhibition in the presence of an antagonist to the receptor. During the past 5 years, the Wnt signaling pathway has emerged as an important player in embryogenesis and tumorigenesis. This pathway controls the growth of a number of neoplasia, particularly colon carcinoma and melanoma, via the regulation of Wnt-responsive genes that participate in cell cycle progression [77]. Wnts are paracrine and autocrine factors that regulate cell growth and cell fate. Signaling is initiated when Wnt ligands bind to transmembrane receptors of the Frizzled family. Frizzleds signal through Dishevelled to inhibit the kinase activity of a complex containing glycogen synthase kinase 3β (GSK-3β), APC, axin and other proteins. The complex targets β-catenin and phosphorylates the threonine and serine residues of exon 3. The phosphorylated β-catenin is rapidly degraded by the ubiquitin-proteasome pathway. In colon carcinoma and melanoma, upregulation of the Wnt pathway leads to β-catenin hypophosphorylation resulting in its accumulation in the cells. It then translocates to the nucleus where it binds to the Lef/Tcf complex of transcription factors and upregulates the expression of cyclin D1 and c-myc. This chain of events leads to cell cycle progression. Interestingly, IB-MECA was shown to de-regulate the Wnt signaling pathway in melanoma cells via the inhibition of adenylyl cyclase, cAMP and its effectors PKA and PKB. Consequently, the GSK-3β level increased, followed by destabilization of β-catenin and subsequent suppression of cyclin D1 and c-myc. The specificity of this response was demonstrated by the A_3AR antagonist, MRS-1523, which reversed the increase in PKA and GSK-3β level, counteracting IB-MECA's effect on melanoma cell growth [33]. Further studies revealed that de-regulation of the Wnt pathway led to cell cycle arrest in the G0/G1 phase and inhibition of telomerase activity in the melanoma cells [78].

Moreover, inhibition of tumor cell growth *in vivo* has also been observed in melanoma and colon carcinoma

murine models. Oral administration of Cl-IB-MECA to melanoma-bearing mice markedly inhibited the development of metastatic lung foci. In combination with cyclophosphamide, Cl-IB-MECA synergized with cyclophosphamide to enhance the chemotherapeutic index. The efficacy of IB-MECA (given orally) in preventing the development of human colon carcinoma xenografts in nude mice has also been demonstrated [78].

In summary, the inhibition of tumor cell cycle progression by A_3AR agonists, entails crosstalk between A_3AR and the Wnt pathway. A_3AR agonists, which are orally bioavailable small molecules, have potential to be developed as anti-cancer agents.

CONCLUDING REMARKS

Taken together, A_3AR seems to play a positive role in counteracting inflammatory responses in eosinophils, neutrophils, monocytes and macrophages, while mediating pro-inflammatory responses in basophils/mast cells. A_3AR agonists produce cardio-, neuro and myeloprotective effects while inhibiting tumor growth at nanomolar concentrations *in vitro* and at μg dosages *in vivo*. This unique characteristic differential effect on tumor and normal cells suggest the application of synthetic A_3AR agonists as therapeutic agents.

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