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Novel selective antagonist radioligands for the pharmacological study of A_{2B} adenosine receptors

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Abstract The adenosine A_{2B} receptor is the least well characterized of the four adenosine subtypes due to the lack of potent and selective agonists and antagonists. Despite the widespread distribution of A_{2B} receptor mRNA, little information is available with regard to their function. The characterization of A_{2B} receptors, through radioligand binding studies, has been performed, until now, by using low-affinity and non-selective antagonists like 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX),(4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol ([^{3}H]ZM 241385) and 3-(3,4-aminobenzyl)-8-(4-oxyacetate)phenyl-1-propyl-xanthine ([^{125}I]ABOPX). Recently, high-affinity radioligands for A_{2B} receptors, [N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy] acetamide ([^{3}H]MRS 1754), N-(2-(2-Phenyl-6-[4-(2,2,3,3-tetratritrio-3-phenylpropyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino)-ethyl)-acetamide ([^{3}H] OSIP339391) and N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide] ([^{3}H]MRE 2029F20), have been introduced. This minireview offers an overview of these recently developed radioligands and the most important applications of drugs towards A_{2B} receptors.

Key words A_{2B} adenosine receptors · novel and selective antagonist radioligands · pharmacological studies

Abbreviations

ABOPX	3-(3,4-aminobenzyl)-8-(4-oxyacetate)phenyl-1-propyl-xanthine
DPCPX	1,3-dipropyl-8-cyclopentyl-xanthine
IB-MECA	N^6-(3-iodo-benzyl) adenosine-5′-N-methyluronamide
IPDX	3-isobutyl-8-pyrrolidinoxanthine
MRE 3008F20	5-N-(4-methoxyphenyl-carbamoyl)amino-8-prpyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [1,5-c]pyrimidine
MRE 2029-F20	N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide]
MRS 1754	[N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide
NECA	5′-N-ethylcarboxamidoadenosine
OSIP339391	N-(2-(2-Phenyl-6-[4-(2,2,3,3-tetratritrio-3-phenylpropyl)-piperazine-1-carbonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino)-ethyl)-acetamide

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SCH 58261	7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]-pyrimidine
hA_{2B}HEK 293 cells	Human embryonic kidney-293 cells transfected with human adenosine A_{2B} receptor
PLC	Phospholipase C
ZM 241385	(4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino] ethyl)-phenol

Introduction

Adenosine, an ubiquitous nucleoside released from metabolically active or stressed cells, is known to act as an important regulatory molecule through activation of cell surface receptors named A_1, A_{2A}, A_{2B} and A_3, all of which belong to the G protein-coupled superfamily of receptors [1]. In particular A_1 and A_3 inhibit, through G_i proteins, adenylyl cyclase activity, whereas A_{2A} and A_{2B} stimulate, via G_s proteins, this enzyme. Collectively, these receptors are widespread on virtually every organ and tissue and represent promising drug targets for the pharmacological intervention in many pathophysiological conditions that are believed to be associated with changes of adenosine levels such as asthma, neurodegenerative disorders, chronic inflammatory diseases and cancer [2, 3]. It has been suggested that adenosine receptors act as "sensors" and that extracellular adenosine acts as a "reporter" of metabolic changes in the local tissue environment [4, 5]. Therefore, under normal conditions adenosine, which is continuously produced intracellularly and extracellularly and maintained at low intracellular levels (about 100 nM) by adenosine kinase and adenosine deaminase, interacts with the high-affinity A_1 and A_{2A} receptor subtypes. In hypoxic, ischaemic or inflamed conditions the intracellular production of adenosine is increased to very high micromolar concentrations and transported across cell membranes by specific agents finally leading to the stepwise activation of all adenosine receptors, including the low-affinity A_{2B} and A_3 subtypes [6].

Whilst for A_1, A_{2A} and A_3 adenosine subtypes good agonists and antagonists have been synthesized allowing a plethora of binding and functional studies, no high-affinity analogues have been identified for the A_{2B} receptors until a couple of years ago, and neither high-affinity nor selective antagonists had been introduced until 2001 [3, 7, 8]. Therefore studies concerning A_{2B} receptors rely on mRNA distribution, cloning and functional assays performed with not very selective molecules [9]. However considering the potential therapeutic applications of A_{2B} ligands, many efforts have been made in the last few years to search for potent and selective antagonists versus this receptor subtype [10] and the recent discovery of novel radiolabelled A_{2B} antagonists will be useful to the scientific community for the exploration and characterization of the pathophysiological role of A_{2B} receptors.

A_{2B} adenosine receptors

A_{2B} receptors were distinguished from A_{2A} in 1983 [11] due to the high and low affinity of adenosine in the stimulation of cAMP levels in rat striatum and were cloned for the first time in 1992 from human hippocampus [12] and rat brain [13, 14] and subsequently from mouse bone marrow-derived mast cells [15]. The distribution of A_{2B} receptors has been detected on practically every cell in different species and relies on the reverse transcription polymerase chain reaction technique. A_{2B} messenger RNA has been found at low levels in all rat brain regions evaluated, aorta, stomach, testis, skeletal muscle, jejunum, kidney, heart, skin, spleen and liver whilst high levels have been detected in the proximal colon [6]. A_{2B} are coupled to different signalling pathways such as stimulation of adenylate cyclase through G_s proteins and accumulation of intracellular calcium. This pathway is related to different mechanisms such as potentiation of a P-type calcium current in hippocampal neurons [16], activation of a calcium channel, G_s protein-coupled, in human erythroleukemia cells [17] or involvement of phospholipase C in HMC1 cells [18]. More recently, it has also been suggested that they are involved in activation of extracellular regulated kinases 1/2 (ERK1/2) at physiologically normal concentrations [19, 20]. Functional studies have suggested the presence of A_{2B} receptors in airway smooth muscle, fibroblasts, vasculature, mast cells, macrophages, endothelium, haematopoietic cells and intestinal epithelial cells. In particular, they have been reported to be involved in stimulation of cell proliferation, differentiation and migration of retinal endothelial cells, where A_{2B} antagonists may offer a way to inhibit retinal angiogenesis and provide a novel therapeutic approach for treatment of diseases associated with aberrant neovascularization, such as diabetic retinopathy [21, 22]. In macrophages it has been reported that their up-regulation induced by interferon gamma (IFN-γ) would lead to the cAMP-mediated down-regulation of MHC class II molecules and other macrophage activities. This may constitute an important mechanism of cell deactivation at the inflammatory foci [23]. In human mast cells 5′-N-ethyl-carboxamidoadenosine (NECA) induced interleukin-8 secretion through the involvement of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase and this effect was blocked by 3-isobutyl-8-pyrrolidinoxanthine (IPDX) suggesting a basis for the development of new antiasthmatic drugs [24–26]. The recent discovery that A_{2B} receptors are also functionally active on human airway smooth muscle

cells to enhance cytokine and chemokine release [27] and on lung fibroblasts where they promote differentiation to a myofibroblast phenotype [28] supports the view that this subtype may be involved in airway wall remodelling and in asthma [29]. Interestingly a role of A_{2B} receptors has also been observed in the analgesic effects of caffeine in an acute animal model of nociception, suggesting that specific A_{2B} antagonists might be valuable adjuvant drugs for opioid analgesia with minimal side effects [30]. The high expression of A_{2B} receptors in different parts of the intestinal tract raised great interest in defining their function. It has been suggested that they play a role in epithelial secretion with potential relevance to diarrhoeal processes [31]. Moreover A_{2B} receptors are involved in coronary flow regulation [32], stimulation of proangiogenic factors [33–35] and they have been proposed as targets to control cell growth and proliferation in a human breast cancer cell line [36]. All together these effects suggest a possible role of the A_{2B} subtype in the modulation of inflammatory processes involved in asthma, tumour growth, tissue injury, ischaemia and pain [37, 38]. Targeting of the A_{2B} receptor protein to specific cells or tissues is crucial in order to understand their role in pathophysiological conditions. Unfortunately, the lack of selective agonists and antagonists has hampered the pharmacological characterization of this receptor system. Only recently Ijzerman's group developed new, high-potency A_{2B} agonists with an improved selectivity profile compared to the reference agonist NECA, and the discovery of the first A_{2B} partial agonist that appears very promising for future functional studies [39]. The characterization of A_{2B} receptors, therefore, often was based on the lack of effectiveness of compounds that are potent and selective agonists of other receptor subtypes. The agonist CGS 21680, for example, has been useful in differentiating between A_{2A} and A_{2B} receptors [40]. However, pharmacological characterization of receptors based on apparent agonist potencies is far from ideal, because it depends not only on agonist binding to the receptor but also on multiple processes involved in signal transduction. Recently, based on functional assays using novel A_{2B} selective antagonists, significant progress has been made in the understanding of the molecular pharmacology and physiology of A_{2B} adenosine receptors and novel progress in this field is supposed to be increased by the introduction of the tritiated radiolabelled form of some new A_{2B} antagonists named [^{3}H]MRS 1754, [^{3}H]OSIP339391 and [^{3}H]MRE 2029-F20 [41–43].

Novel antagonist radioligands to A_{2B} adenosine subtype

The characterization of A_{2B} receptors, through radioligand binding studies, has been performed, until now, by using low-affinity and non-selective antagonists like [^{3}H]DPCPX, [^{3}H]ZM 241385 and [^{125}I]ABOPX, which, as a consequence of their low affinity, display a rapid dissociation rate from the receptor [44]. In addition, since these ligands are non-selective their utility in native systems is hampered as many tissues and cell lines express several adenosine subtypes.

Xanthines, including the natural derivatives theophylline and caffeine, are the natural non-selective antagonists of adenosine and they are able to bind to all four adenosine receptor subtypes A_1, A_{2A}, A_{2B} and A_3 [6]. Therefore the xanthine core has been maintained and modified in order to develop new A_{2B} drugs. This approach led to the discovery of new molecules introduced as high-affinity radioligands for A_{2B} receptors named [^{3}H]MRS 1754, [^{3}H]OSIP339391 and [^{3}H]MRE 2029-F20 [41–43].

MRS1754, which belongs to a series of amides derived from the 8-phenyl xanthine molecules [45], was the first antagonist proposed in radiolabelled form for the labelling of A_{2B} receptors [41]. MRS1754 was shown to be a potent and selective antagonist. Radioligand binding studies showed that it binds to recombinant A_{2B} receptors in membranes of stably transfected HEK 293 cells. Specific binding was saturable, competitive, and followed a one-site model, with a K_D value of 1.13 nM and a B_{max} value of 10.9 pmol/mg protein. Specific binding of [^{3}H]MRS 1754, at the K_D value, was >70% of total binding. The affinity calculated from association and dissociation binding constants was 1.22 nM. Binding to membranes expressing rat and human A_1 and A_3 adenosine receptors was not significant, and binding in membranes of HEK 293 cells expressing human A_{2A} receptors was of low affinity (K_D > 50 nM). The pharmacological profile in competition experiments with [^{3}H]MRS 1754 was consistent with the structure-activity relationship for agonists and antagonists at A_{2B} receptors. In spite of the high affinity and selectivity, this radioligand has not been used in cells and tissues endogenously expressing A_{2B} receptors.

Some years later a pyrrolopyrimidine compound [^{3}H] OSIP339391 was introduced as a high-affinity and selective radioligand to A_{2B} receptors [42]. OSIP339391 had a selectivity of greater than 70-fold for A_{2B} receptors over other human adenosine receptor subtypes. Using membranes from HEK 293 cells expressing the human recombinant A_{2B} receptor, [^{3}H]OSIP339391 was characterized in kinetic, saturation and competition binding experiments. From the association and dissociation rate studies, the affinity was 0.41 nM and in close agreement with that found in saturation binding experiments (0.17 nM). In competition binding studies of [^{3}H]OSIP339391, the affinity of a range of agonists and antagonists was consistent with previously reported data. However, also in this case, its suitability to label endogenous A_{2B} receptors

cannot be assessed due to the lack of binding studies in cells or tissues.

Our research group has identified a series of 8-pyrazole xanthine derivatives as potent and selective human A_{2B} adenosine antagonists [46], and a radiolabelled form of one compound of this series, [^{3}H]MRE 2029-F20 [47], was used as new pharmacological tool to describe the comparison between human recombinant A_{2B} receptors stably transfected in HEK 293 cells and endogenous receptors present in human neutrophils and lymphocytes [43]. [^{3}H]MRE 2029-F20 showed high affinity and selectivity for hA_{2B} versus hA_1, hA_{2A} and hA_3 subtypes. The selectivity of MRE 2029-F20 for the human A_{2B} over A_1, A_{2A} and A_3 receptors was evaluated in radioligand binding assays by using [^{3}H] DPCPX, [^{3}H]ZM 241385 and [^{3}H]MRE 3008-F20, respectively. MRE 2029-F20 displays low affinity for the human A_1 receptor (Ki = 245 ± 31 nM) and no significant affinity for the human A_{2A} and A_3 subtypes (Ki >1,000 nM). This indicates that MRE 2029-F20 is 88- and more than 300-fold selective for the A_{2B} over A_1, A_{2A} and A_3 subtypes, respectively, which means a range of selectivity similar to [^{3}H]MRS 1754 (selectivity of 210-, 260- and 290-fold for A_{2B} over A_1, A_{2A} and A_3 subtypes, respectively) and slightly better than [^{3}H]OSIP339391 (selectivity of 70-fold for A_{2B} over A_1, A_{2A} and A_3 subtypes, respectively).

[^{3}H]MRE 2029-F20 bound specifically to the hA_{2B} receptor stably transfected in human embryonic kidney (HEK) 293 cells with K_D of 2.8 nM and B_{max} of 450 fmol/mg of protein. Saturation experiments of [^{3}H]MRE 2029-F20 binding in human neutrophils and lymphocytes detected a single high-affinity binding site with K_D values of 2.4 and 2.7 nM, respectively, and B_{max} values of 79 and 54 fmol/mg of protein, respectively, in agreement with real-time reverse transcription polymerase chain reaction studies showing the presence of A_{2B} mRNA. The rank order of potency of typical adenosine ligands with recombinant hA_{2B} receptors was consistent with that typically found for interactions with the A_{2B} subtype and was also similar in peripheral blood cells. NECA stimulated cAMP accumulation in both hA_{2B}HEK 293 and native cells, whereas phospholipase C activation was observed in recombinant receptors and endogenous subtypes expressed in neutrophils but not in lymphocytes. MRE 2029-F20 was revealed to be a potent antagonist in counteracting the agonist effect in both signal transduction pathways. In conclusion, [^{3}H] MRE 2029-F20 was revealed to be a selective and high-affinity radioligand for the hA_{2B} adenosine subtype and may be used to quantify A_{2B} endogenous receptors such as those present in neutrophils and lymphocytes that represent inflammatory cells potentially involved in the exacerbation of asthma and other inflammatory processes in which A_{2B} receptors are thought to be involved [29]. This was the first paper reporting both binding and functional studies in recombinant and native receptors. [^{3}H]MRE 2029-F20 has been used also to evaluate the effects of novel and recognised compounds at human recombinant A_{2B} adenosine receptors expressed in Chinese hamster ovary, in HEK 293 and at endogenous A_{2B} receptors in human mast cells (HMC-1). Saturation binding experiments performed using [^{3}H]MRE 2029-F20 revealed a single class of binding sites in hA_{2B}CHO, hA_{2B}HEK 293 and HMC-1 cells with K_D of 1.65, 2.83, 2.62 nM and B_{max} of 36, 475 and 128 fmol/mg protein, respectively. Moreover, the compounds tested were able to decrease NECA-stimulated and also forskolin-stimulated cAMP production, revealing them to be novel antagonists with an inverse agonist activity in recombinant and native human A_{2B} receptors [48].

Conclusions

In recent years remarkable attention has been paid to A_{2B} receptors due to their supposed role in important pathological states such as asthma, diarrhoeal processes, diabetic retinopathy, vasodilation, neoangiogenesis and nociception. For many years their study has been strongly limited by the lack of good and selective pharmacological probes and many efforts have been focused by chemists and biologists on the synthesis and testing of new selective ligands. The data presented in this review clearly underline the significant progress made in the field of A_{2B} receptor antagonists. Novel high-affinity and selective antagonist radioligands have been recently synthesized that will allow in the future the characterization of this adenosine subtype in endogenous systems (Table 1).

Table 1 Binding characteristics of the recently developed antagonist radioligands to A_{2B} recombinant receptors. Specific binding is expressed at the K_D value of each radioligand.

Radioligand	K_D (nM)	B_{max} (fmol/mg of protein)	Specific binding (%)	hA_1 Ki (nM)	hA_{2A} Ki (nM)	hA_3 Ki (nM)
[^{3}H]-MRS 1754[a]	1.13 ± 0.12	10,900 ± 600	70	403 ± 194	503 ± 10.8	570 ± 184
[^{3}H]-OSIP339391[b]	0.17 ± 0.05	20,102 ± 1,503	80	37 ± 7	328 ± 75	450 ± 55
[^{3}H]-MRE 2029-F20[c]	2.8 ± 0.2	450 ± 42	70	248 ± 30	>1,000	>1,000

[a] See ref. [41]
[b] See ref. [42]
[c] See ref. [43]

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