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Adenosine in the inflamed gut: a Janus faced compound

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Running header: Adenosine in the inflamed gut

Abstract

The purine ribonucleoside adenosine (Ado) has been recognized for its regulatory functions in situations of cellular stress like ischemia, hypoxia and inflammation. The importance of extracellular Ado as a modulator in the immune system is a theme of great appreciation and the focus of recent increasing interest in the field of gastrointestinal inflammation. In this review, the different aspects of Ado signaling during inflammatory responses in the gut are discussed, considering the contribution of the four known Ado receptors (ARs; A_1, A_{2A}, A_{2B}, and A_3), their mechanisms and expression patterns. Activation of these receptors in epithelial cells as well as in immune cells recruited to the inflamed intestinal mucosa determines the overall effect, ranging from a protective, anti-inflammatory modulation to a strong pro-inflammatory induction. Here we present the current advances in agonists and antagonists development and their potential therapeutic application studied in animal models of intestinal inflammation. In addition, alternative complementary approaches to manipulate such a complex signaling system are discussed, for example, the use of AR allosteric modulators or interference with Ado metabolism. Special features of the gut environment are taken into account: the contribution of diet components; the involvement of Ado in intestinal infections; the interactions with the gut microbiome, particularly, the recent exciting finding that an intestinal bacterium can directly produce extracellular Ado in response to host defense mechanisms in an inflammation scenario. Understanding each component of this dynamic system will broaden the possibilities for applying Ado signaling as a therapeutic target in gut inflammation.

Keywords: adenosine, adenosine receptor (AR), AR agonist, AR antagonist, allosteric modulators, anti-inflammatory, pro-inflammatory, colitis, gut microbiome, intestinal inflammation, inflammatory bowel disease (IBD), structure-activity relationships (SAR)

Introduction

Adenosine (Ado) **1** is an endogenous nucleoside formed by the purine base adenine attached to a ribose sugar. Besides being one of the building blocks of RNA, Ado is a very versatile signaling molecule, with a broad range of biological activities in many different tissues and cell types. Because of its action, similar to a local hormone, but with fundamental differences in the mechanisms of formation, Ado has been designated as a "retaliatory metabolite", with mostly protective functions and a direct link to ATP metabolism and energy status [[1]]. The levels of extracellular Ado, assumed to be around 1 μM in physiological conditions, can increase up to 100-fold in situations of cellular stress, such as ischemia, trauma, hypoxia or inflammation [[2]]. The main source of extracellular Ado is the catabolism of ATP released from stressed cells. Extracellular ATP is degraded to Ado by the action of ecto-nucleotidases like CD39 (ENTPD1 or apyrase, which converts ATP to ADP and AMP) and CD73 (ecto-5'-nucleotidase, which converts AMP to adenosine). The presence of Ado in the extracellular space can be limited by conversion to inosine catalyzed by adenosine deaminase (ADA), or by intracellular phosphorylation to AMP by adenosine kinase [2]. Membrane nucleoside transporters of two types are also important regulators of extracellular Ado levels: the active, sodium-dependent concentrative nucleoside transporters (CNT) and the passive, diffusion-driven equilibrative nucleoside transporters (ENT) [[3]].

There are four cell surface receptors known to respond to Ado, namely A_1, A_{2A}, A_{2B} and A_3 adenosine receptor (AR). They contain seven transmembrane domains and are intracellularly coupled to GTP-binding proteins. A_1 and A_3 receptors couple to and activate $G_{i/o}$ proteins that inhibit adenylate cyclase, leading to decreased cyclic AMP (cAMP) levels, while A_{2A} and A_{2B} receptors couple to G_s proteins, stimulating cAMP accumulation [[4]]. cAMP, in turn, is a very important second messenger involved in numerous cellular functions, like regulation of gene expression via protein kinase A (PKA) and the cAMP response element binding (CREB) protein. Other pathways engaged by ARs include phospholipase C (PLC) and Ca^{2+} signaling, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB or Akt) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated gene regulation [[5]]. Ado can regulate all these different processes in a cell type-, time- and concentration-dependent fashion. ARs are distributed on nearly every cell type [4] and the effects of Ado signaling have been demonstrated for many different tissues and organs. Importantly, up-regulation of all four AR subtypes during inflammation has been reported [[6], [7], [8]]. A scheme showing the main components of the Ado signaling network is depicted in Figure 1.

In this review, we sought to discuss the multiple faces of Ado, reflected in its intricate roles in gut inflammation. We present experimental evidences of the different effects of Ado in the context of the inflamed

gut and discuss the state of the art on Ado ligands development, some of them functional in *in vivo* studies of intestinal inflammation. Finally, we debate alternative strategies and other aspects we consider important to be taken into account for future developments aiming at Ado-signaling as a therapeutic target.

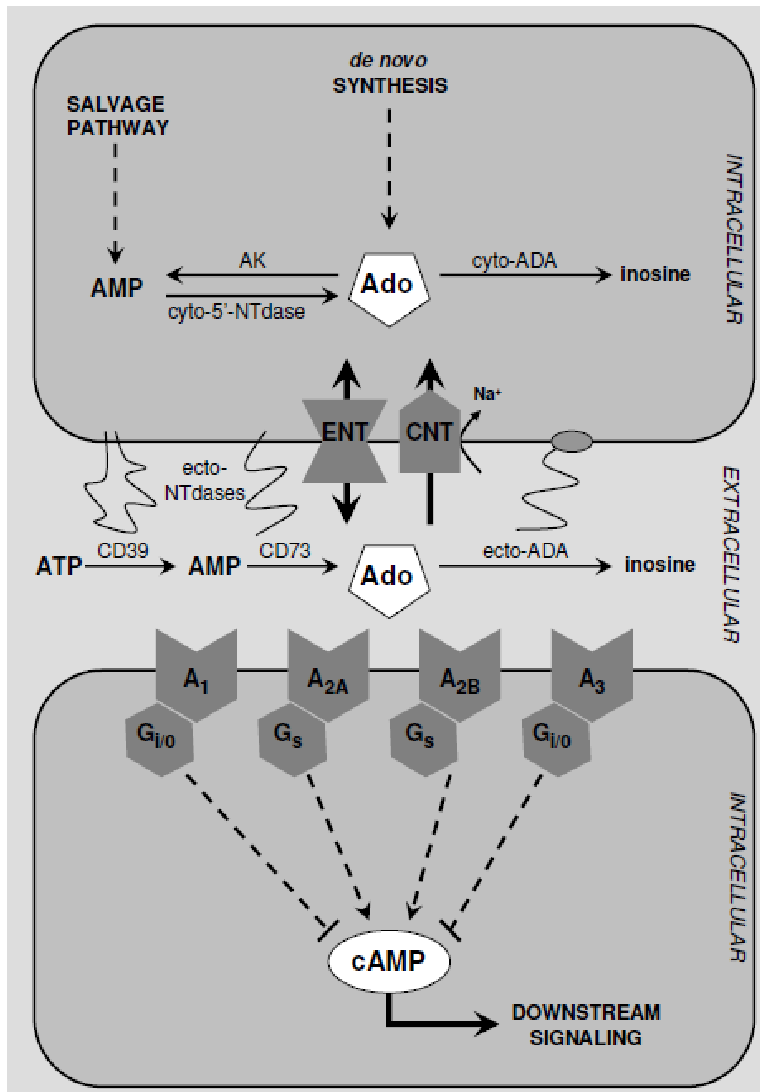

Figure 1: Adenosine metabolism, transport and signaling. Intracellular Ado can be synthesized *de novo* or via the salvage pathway, including dephosphorylation of AMP by cytoplasmic (cyto-) nucleotidase (NTdase). Two families of nucleoside transporters mediate Ado exchange through the plasma membrane: the equilibrative nucleoside transporter (ENT) allows bi-directional flux by diffusion, whereas concentrative nucleoside transporter (CNT) uses Na^+ coupling to bring Ado into the cell against the concentration gradient. Extracellular Ado is formed mainly from ATP degradation by surface ecto- NTdases like CD39 and CD73. Levels of extracellular Ado are further regulated by the activity of ecto-ADA, which converts Ado to inosine, or by cellular uptake, followed by degradation to inosine by cytoplasmic (cyto-)ADA, or phosphorylation to AMP by Ado kinase (AK). Biological activity of extracellular Ado in the target cells is mediated by four receptors (A_1

A_{2A}, A_{2B} and A_3) coupled to inhibitory- or stimulatory- G-proteins ($G_{i/o}$ or G_s). G-proteins are important signal transducers that can modulate for instance cAMP levels in the cell, interfering with many downstream pathways and respective cellular processes.

General effects of adenosine in intestinal inflammation

The role of Ado during inflammation in the gut is a result of its different effects upon intestinal epithelium and immune cells present in the mucosa. Here it is important to highlight that each cell-type usually expresses more than one AR subtype, and that there are many variables contributing to the final effect of Ado signaling. In intestinal epithelial cells, A_{2B} is the predominant Ado receptor expressed [[9]]. Immune cells, in turn, express A_{2A} AR, which has been extensively linked to the anti-inflammatory properties of Ado [[10], [11], [12], [13]]. Ado changes cAMP levels with EC_{50} 0.3 μM for A_1 and A_3 AR and 0.7 μM for A_{2A} AR, which means they are responsive to physiological concentrations of extracellular Ado. The potency of A_{2B} AR, however, is much lower (EC_{50} 24 μM) and it can be activated mainly in pathophysiological conditions [[14]]. In neutrophils, for example, at low Ado levels, activation of A_1 AR promotes chemotaxis and tissue infiltration. At higher concentrations present at the site of inflammation, Ado is known to inhibit effector functions via A_{2A} AR, attenuating tissue damage [[15]]. Neutrophil adherence to endothelium is also regulated by these two receptors in a way that A_1 AR is stimulating and A_{2A} AR is suppressing the process [[16]]. In dendritic cells (DC), expression pattern of different AR subtypes changes during maturation. In immature DC, Ado signaling, mainly through A_1 AR, promotes chemotaxis. After lipopolysaccharide (LPS)-induced maturation, A_{2A} AR is responding to Ado, resulting in reduced production of pro-inflammatory interleukin (IL)-12 [11].

Further interactions between different Ado receptor subtypes are also important to be considered. Studies in human peripheral blood mononuclear cells (PBMC) demonstrated that Ado-induced downregulation of pro-inflammatory cytokines is reversed by A_{2A} AR blockade, but enhanced by inhibition of A_1 or A_3 AR. Agonists for A_1 and A_3 AR, in turn, could abolish the effect on cytokine levels when A_{2A} was activated by a selective agonist in a similar way as the A_{2A} AR antagonist [[17]]. Such interactions may result from the opposite effect of A_1 and A_3 versus A_2 ARs in intracellular cAMP concentration and reflect the differential modulation of signal transduction pathways by each receptor subtype. In this context, the different characteristics of potency presented by the different receptors define the importance of Ado concentration and its time-dynamics during inflammation as crucial players in the overall effect of Ado signaling. Also dealing with the paradoxal effect of Ado in immune responses, a recent study indicated that, despite the suppressive effect upon T-cell activation, a

pre-exposure to Ado leads to desensitization of cAMP signaling, resulting in enhanced T-cell responses [18]. A similar mechanism had also been described for dendritic cells, Ado pre-exposed cells presenting subsequently higher activation by pathogen-associated molecular pattern (PAMP) and also higher capacity of stimulating T-cells [19]. With its conflicting effects, adenosine resembles the two-faced Roman deity Janus. Here we collect some examples on the different effects of Ado in inflammatory pathways in the gut, considering the complex scenario involving cytokine signaling and cross-talk.

Evidences of anti-inflammatory effects. The protective effects of Ado in inflammation and consequent tissue damage were known, but poorly understood until a study with A_{2A} AR knockout mice showing striking inflammatory phenotype in the absence of the receptor [20]. Those results drew attention to the therapeutic potential of modulating A_{2A} AR signaling to control inflammation. Furthermore, there are a number of studies describing inhibitory effect of Ado on pro-inflammatory cytokine production by different immune cells and the involvement of different Ado receptor subtypes. For example, Ado can reduce IL-12 and tumor necrosis factor (TNF)-α production by murine macrophages, an effect partially mediated by A_{2A} AR [21]. Conversely, in two independent and controversial reports, A_{2B} and A_3 ARs had also been implicated in suppression of TNF-α production by murine macrophages [22, 23]. A_{2A} AR is able to mediate the inhibition of IL-12 and simultaneous induction of IL-10 production by human monocytes [24]. In turn, A_{2B} AR activation in LPS-stimulated mouse macrophages is responsible for increased anti-inflammatory IL-10 production, by mediating the relief of a posttranscriptional repression mechanism [25]. Direct anti-inflammatory effects in human intestinal epithelium were also observed, using HT-29 cells as model. Reduction of MAPK phosphorylation and IL-8 expression and secretion in response to pro-inflammatory stimuli (e.g. TNF-α, IL-1, LPS) were observed when the cells were pre-incubated with Ado, showing a negative-regulation of the inflammatory process [26].

Other clear examples of anti-inflammatory effects of Ado are found related to hypoxic conditions. Hypoxia is intimately related to inflammation, as has been reported that a deficient supply of oxygen can induce inflammatory responses and conversely, inflamed tissue often develop hypoxic features [27]. Oxygen-dependent changes in gene expression are mediated by the hypoxia-inducible factor (HIF) and it can regulate Ado signaling at several levels. HIF mediates repression of both transporters ENT-1 and ENT-2, resulting in decreased Ado uptake by endothelial and epithelial cells [28, 29]. The consequent increase in extracellular Ado half-life is implicated in enhanced vascular barrier function, decreased epithelial permeability and neutrophil infiltration, attenuating hypoxia-induced mucosal inflammation *in vitro* and *in vivo* [28, 29]. The involvement of Ado in the interface between hypoxic and inflammatory responses was also found in RNA microarray studies in hypoxic

intestinal epithelium showing HIF-induced expression of CD39 and CD73, which was correlated to decreased permeability *in vivo* [30]. For endothelial cells, it was reported that ATP released from activated PMN can promote barrier function preferentially in posthypoxic cells. In this condition, the cells also presented increased expression of CD39 and CD73, responsible for the conversion of PMN-derived ATP into Ado. Moreover, hypoxia up-regulated the expression of A_{2B} AR, which was found to be the receptor mediating the effects observed in endothelial cells [31].

In accordance with an anti-inflammatory role for Ado signaling in inflammatory-hypoxia, HIF-1 has been shown to be induced with inflammation and to have a protective role in a murine model of colitis. Severity of clinical symptoms were inversely correlated with HIF-1 expression levels in mutant mice and CD73 was identified among the target genes up-regulated by HIF-1 in the colon [32]. Additionally, interesting evidence came from the investigation of HIF-1-mediated induction of the neuronal guidance molecule netrin-1. It was demonstrated that intestinal epithelial cells under hypoxic conditions present a higher expression of netrin-1, which in turn is able to inhibit trans-epithelial PMN migration *in vitro*, an effect dependent of A_{2B}AR engagement. Also *in vivo*, activation of A_{2B} AR by netrin-1 was demonstrated to have protective effects in hypoxia-induced colonic inflammation [33].

T-cells are other important targets for the anti-inflammatory effects of Ado. In murine $CD4^+$ T-cells, signaling through T-cell receptor (TCR) increases A_{2A} AR transcription and the consequent accumulation of cAMP, which in turn reduce TCR-mediated interferon (IFN)-γ production [34]. Also connected to T-cell activation are the effects of Ado in dendritic cells and regulatory T-cells (Treg). As discussed above, the expression of A_{2A} AR in mature DC has anti-inflammatory effects. Moreover, A_{2B} AR was reported to play an important role in Ado-induced inhibition of pro-inflammatory cytokines, as well in enhanced IL-10 production and reduced levels of major histocompatibility complex (MHC) II and CD86 expression by DC, resulting in less effective T-cell activation [35]. Indeed, adenosine deaminase activity is an important mechanism of dendritic cells to maintain their activation in the high-Ado environment in inflamed tissues [36]. In Tregs, the generation of Ado by expression and activity of CD39 and CD73 is essential to maintain their anergic state and suppression of T-cell responses [13, 37].

Also for A_1 AR an anti-inflammatory role had been demonstrated in murine macrophages and mesothelial cells. The preconditioning with A_1 AR agonists reduced sera levels of TNF-α and IL-6 in response to intraperitonial bacterial inoculation. This effect could be reversed not only by A_1 AR antagonist, but also by A_{2A} AR antagonist. Interestingly, activation of A_1 AR was shown to induce A_{2A} AR expression. These

observations, together with the absence of a preconditioning effect in A_{2A} AR knockout mice, indicated that A_1 AR agonist can reduce inflammation by induction of A_{2A} AR [38]. A_3 AR had also been implicated in anti-inflammatory effects of Ado. Injecting a selective agonist in endotoxemic mice, the circulating concentrations of TNF-α and IL-12 were reduced, whereas IL-10 levels were increased [39].

Evidences of pro-inflammatory effects. On the other hand, increases in pro-inflammatory mediators had also been reported as effects of Ado in different cell types, including intestinal epithelial cells, mainly involving signaling via A_{2B} AR. In T84 cells, A_{2B} AR activation stimulates luminal secretion of IL-6 in similar levels as induced by pro-inflammatory agents like TNF-α or *Salmonella typhimurium* colonization. Interesting to note is that the concentration of IL-6 released is able to induce intracellular Ca^{2+} influx and subsequent degranulation in neutrophils (which are a source of 5' AMP, converted to Ado by intestinal ecto-nucleotidase), resulting in a pro-inflammatory signaling loop [40]. Expression of IL-6 is also induced by A_{2B} AR activation in the epithelial-like cell line U373 MG [41]. Another study found indications of a pro-inflammatory role for A_{2B} AR, observing that under stimulation with stable Ado analog 5'-*N*-ethylcarboxamidoadenosine (NECA), the production of IL-6 by macrophages *ex vivo*, and also IL-6 levels in blood plasma *in vivo*, were enhanced. This effect could be reversed by genetic ablation of the receptor, as well as by selective A_{2B} AR antagonists. In contrast, the anti-inflammatory effect of NECA in this model, suppression of TNF-α release after LPS-activation, was not inhibited in the absence of A_{2B} activation, indicating this effect is mediated by a different receptor [42]. Accordingly, A_{2B} AR has also been implicated in stimulation of pro-inflammatory responses in mast cells. Indeed, in murine mast cells, genetic ablation of A_{2B} AR, as well as pharmacological inhibition, impaired Ado-induced degranulation and secretion of IL-6, IL-13 and vascular endothelial growth factor (VEGF) [43]. Stimulation of angiogenic factors secretion, like VEGF and IL-8, links Ado signaling to angiogenesis, another important feature of chronic inflammation [44]. Selective activation of A_{2B} AR induced IL-8 and VEGF in human mast cells, whereas the A_3 AR was responsible for enhanced angiopoietin-2 expression. The ability of mast cells to induce capillary formation *in vitro* was optimal when both receptors were activated, denoting a cooperative function in promotion of angiogenesis [45]. Moreover, a complex regulation of dendritic cells maturation has been attributed to A_{2B} AR, its activation during monocyte differentiation leading to the formation of a phenotypically distinct DC subtype. Besides the anti-inflammatory effects observed (in accordance to other studies as discussed above), DC generated in the presence of Ado displayed increased expression of pro-inflammatory factors such as IL-6 and cyclooxygenase-2, as well as increased secretion of VEGF and IL-8, therefore being more angiogenic compared to the conventional DC [46].

Effects in enteric neurotransmission and gut motility. Intestinal inflammation is very often associated with disruption in gut motile functions, in which Ado is known to exert modulatory effects. The four Ado receptor subtypes are differentially expressed in neural and non-neural areas of the human intestinal mucosa and present a differential spatial distribution in the intestine [47]. In healthy mouse ileum, the A_1 AR was implicated in the inhibitory effect of Ado in neuron-mediated contractile responses to electrical field stimulation, but Ado was unable to affect direct cholinergic muscle stimulation by carbachol. Under inflammation, however, this purinergic regulation seems to be impaired [48]. Also in healthy rat colon, A_1 and A_{2A} ARs are reported to mediate Ado regulatory effects in electrically-induced contraction, but not affecting carbachol-induced response [49]. However, in healthy human colon, a series of experiments also tested Ado effects on electrically-induced or carbachol-induced contractions using agonists and antagonists of A_1 and A_{2A} ARs. Here the results indicated that Ado inhibits colonic motility acting via A_1 AR in smooth muscles and via A_{2A} AR in neurons [50]. In addition to the regulation via A_1 AR, the activation of A_3 AR was shown to abolish in the guinea pig colon the contractile response to histamine [51]. It has been reported that A_3 knockout mice present altered intestinal motility and that A_3 AR are expected to have a constipating effect in the colon [52]. A_{2B} AR is also involved in the regulation of colonic motility, as demonstrated by delayed colonic emptying, increased stool retention and inhibited relaxation in $A_{2B}^{-/-}$ mice [53].

Studies targeting adenosine receptors

The study of Ado receptors as therapeutic targets, in particular the A_{2A} and A_{2B} AR, is currently receiving a great attention in the field of inflammatory diseases, as has been discussed by recently published reviews [54, 55, 56, 57, 58]. The great majority of results in clinical development, however, are not directed to intestinal inflammation. The use of Quantitative Structure-Activity Relationships (QSAR) in the development of AR agonists or antagonists is rapidly developing due to a fast growing number of compounds with assessed biological activities but is still not very common despite a large number of successful applications [59]. QSAR or docking approaches have emerged as promising tools for the prediction of Ado agonistic or antagonistic activities of chemical compounds before any synthetic attempt is made. The results of these QSAR applications can be seen in many new and highly active compounds reported in recent years. The intricacy of Ado signaling in intestinal inflammation not surprisingly reflects in the results obtained by manipulating such pathway in *in vivo* studies. Here we focused on some promising compounds developed and their potential clinical application, with emphasis for inflammatory diseases in the gut.

Targeting A_1 adenosine receptors

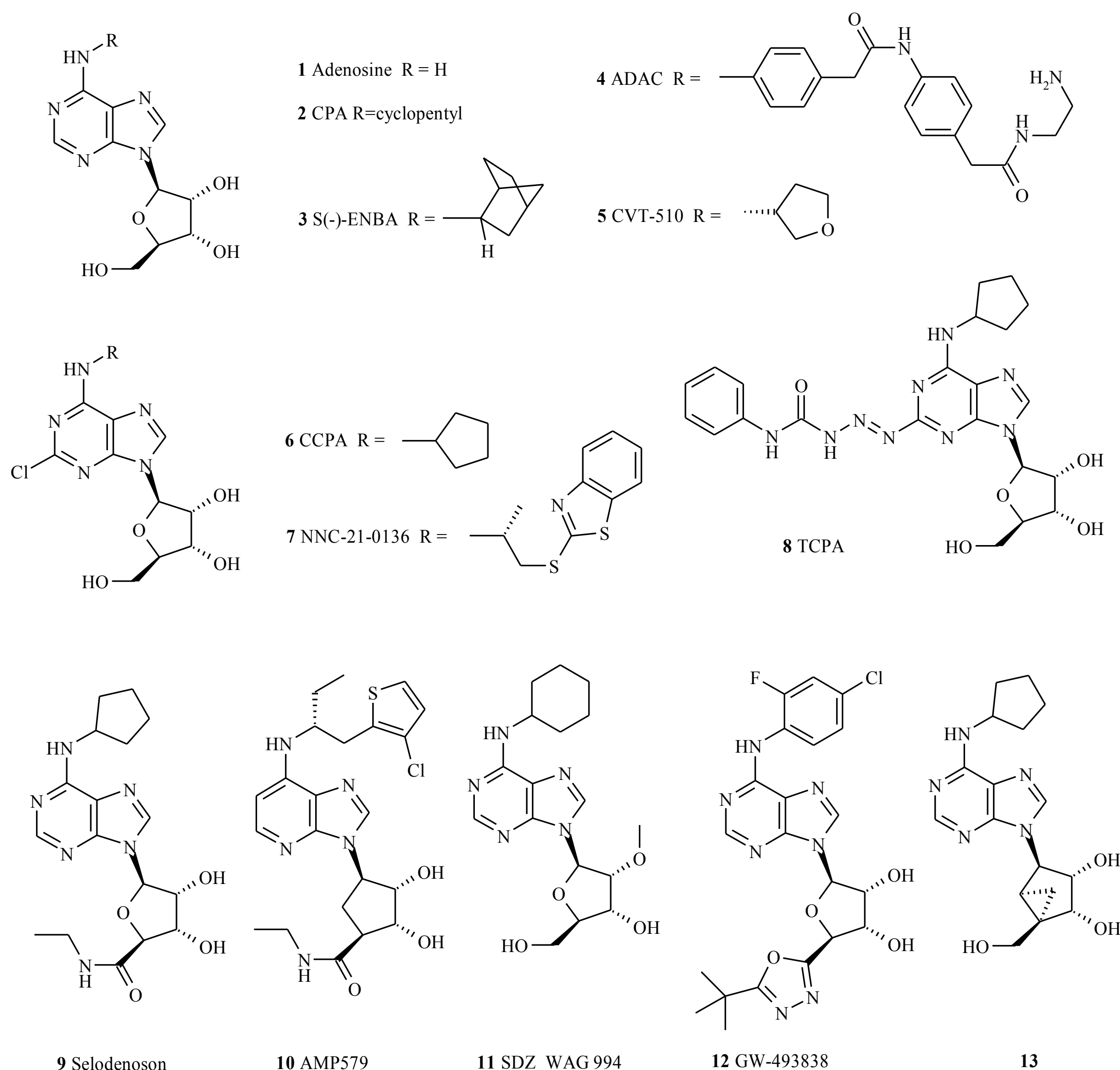

Figure 2: Compounds reported to be agonists of A_1 AR

The role of A_1 AR ligands in specific models of intestinal inflammatory diseases have not been well explored so far, maybe because of the great impact of A_{2A} and A_{2B} receptors in this field. However, effects in inflammatory features of intestinal ischemia/reperfusion injury or in systemic inflammation have been described. All synthetic A_1 AR agonists reported so far show structural similarities with its natural lead adenosine **1** (Figure 2) [60]. A hydrogen atom at N^6 is essential for high affinity to A_1 AR. N^6-Cycloalkyl monosubstituted adenosine analogues are highly potent and selective A_1 AR agonists and examples are N^6-cyclopentyl-adenosine (CPA) **2**, its (1*S*)-trans-2-hydroxy-derivative GR79236 and *S*(-)-ENBA **3** [61].

The adenosine amine congener (ADAC) **4** has been used as starting point for many agonists and radioligands [62]. The adenosine analogue CVT-510 **5** (=Tecadenoson) showed high selectivity lacking negative dromotropic, vasodilator, and hypotensive side effects and has entered clinical trials [63]. CCPA **6**, the 2-chloro-derivative of CPA, has a somewhat higher affinity to A_1 AR than CPA. Administration of either Ado or CCPA to rats prior to induction of ischemia/reperfusion (IR) injury resulted in attenuation of inflammatory neutrophil infiltration and oxidative stress in the ileum. The treatment restored the contractile responses to KCl but only in the presence of millimolar levels of carbachol [64]. Another chloro-derivative, NNC-21-0136 **7**, was reported by Knutsen *et al*. while searching for novel A_1 agonists with potent central nervous system effects. The compound displayed diminished influence on the cardiovascular system in mice due to the low intraperitoneal doses required [65]. Replacing chlorine at the C2-position with aminocarbonyltriazene-1-yl groups led to the highly potent TCPA **8** which had $K_i(A_1)$ = 2.8 nM and was highly selective compared to A_3 AR [66]. The *N*-ethylcarboxamido derivative of CPA, Selodenoson **9** (= DTI-0009 = RG 14202), is a very potent and selective A_1 AR agonist. Selodenoson has good oral bioavailability, a sufficient half-life for chronic applications and is largely eliminated by renal secretion, also activating renal A_1 ARs [67]. The agonist AMP579 **10**, with an 3H-imidazo[4,5-b]pyridin core and a tetrathydrofuran of the ribose replaced by a cyclopentane ring, showed $K_i(A_1)$ = 2 nM and $K_i(A_{2A})$ = 56 nM, with a favourable pharmacokinetic profile. It was assessed in clinical studies but failed after phase II [68]. Another analogue of CPA is SDZ WAG 994 **11** bearing an ethyl ether at the ribosyl moiety [69]. SDZ WAG 994 entered clinical trial phase I but was discontinued afterwards. The 4-chloro-2-fluorophenyl N^6-substituted adenosine analogue GW-493838 **12** is an A_1 AR agonist [70]. The compound was tested in clinical trials for neuropathic pain, however, no significant improvements were seen compared to the placebo group when treated with GW-493838 [71]. Several more highly potent compounds are in the pipeline for clinical applications, e. g. Inotek developed INO-8875, the 5'-nitrate of CPA, with $K_i(A_1)$ <1 nM and more than 10,000-fold selectivity vs. A_{2A} AR [72]. Replacing the tetrahydrofuran oxygen in the ribose moiety of CPA with carbon and fusing a cyclopropane ring to this cyclopentane gave compound **13** with very high affinity, $K_i(A_1)$ = 5.1 nM, and good selectivity against other ARs ($K_i(A_{2A})$ = 6,800 nM, $K_i(A_3)$ = 170 nM) [73].

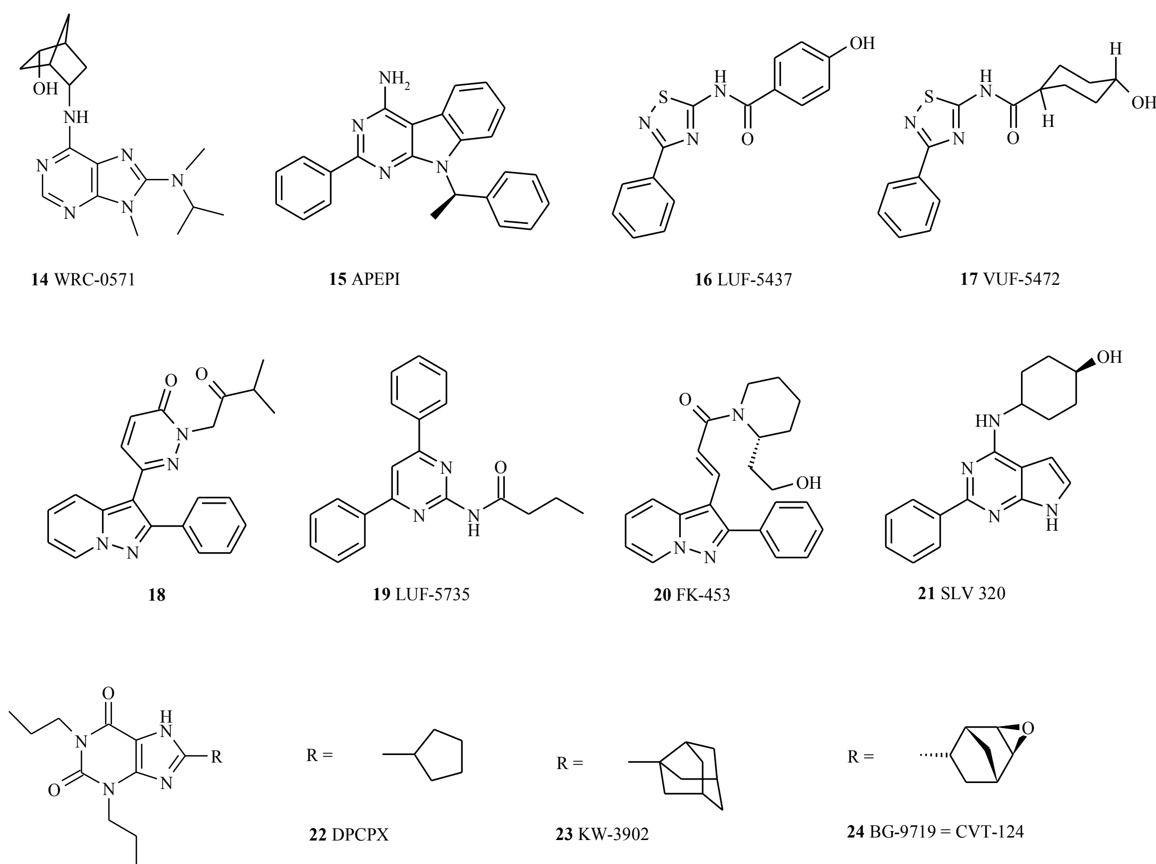

Figure 3: A variety of A_1 AR antagonists

The ribose moiety appears to be essential for agonistic activity at the adenosine receptors and adenine derivatives and analogues lacking the ribose moiety have been shown to act as antagonists. Searching for a potent and highly selective A_1 AR antagonist by structure activity studies of N^6-endonorbornyl-9-methyladenine derivatives, WRC-0571 **14** (Figure 3) was found. WRC-0571 is 62-fold selective for the human A_1 vs. A_{2A} AR and 4670-fold selective for the A_1 vs. human A_3 AR [74]. Compounds from a variety of different classes of heterocyclic compounds have been described as antagonists, including xanthines, adenines, 7-deazaadenines, 7-deaza-8-azapurines, triazolo[1,5-*a*]quinoxalines, and pyrazolo- [1,5-*a*]pyridines. Some of these compounds are chiral compounds like the (*R*)-7-(1-methylbenzyl)-2-phenylpyrimido[4,5-*b*]indole-4-amine (APEPI) **15** which is a potent and selective antagonist of the human A_1 AR [75]. Starting from much simpler thiazole and thiadiazole derivatives the thiadiazole LUF-5437 **16** was discovered as a highly active A_1 AR antagonist. Hydrogenation of the phenyl ring led to VUF5472 **17** which is less potent but more selective for the A_1 adenosine receptor [76]. Another class of compounds consists of pyrazolo[1,5-a]pyridine derivatives from which compound **18** was developed possessing high potency and selectivity for the A_1 AR [77]. Finally, from a large number of pyrimidine

derivatives designed with the help of molecular modeling, LUF5735 **19** was identified as a highly active substance [78].

The importance of chirality can be seen for FK453 **20** where the R-isomer is a potent and selective A_1 AR antagonists but the S-isomer is not [79]. The A_1 AR antagonist SLV320 **21** has $K_i(A_1)$ = 1 nM and more than 200-fold compared to the other human ARs [80]. Structure-activity analysis revealed that 1,3-dipropyl substitutions enhance potency compared to theophylline. These compounds have been further developed to 8-cyclopentyl-l,3-dipropylxanthine (DPCPX) **22** as an antagonist for A_1 AR having K_i = 0.45 nM for the rat A_1 AR and 330 nM for the human A_2 AR, giving a more than 700-fold A_1-selectivity [81]. Pre-treatment with this antagonist impaired the beneficial effect of Ado in attenuating inflammation and contraction inhibition in a rat model of ischemia/reperfusion (IR) injury [64]. In contrast, in another study focused on the motility effects of IR, the injection of DPCPX actually reversed the delay in colonic propulsion induced by IR [82]. A compound related to DPCPX is 1,3-dipropyl-8-(3-noradamantyl)xanthine (KW-3902) **23** which was found to be the most potent A_1 AR antagonist [83]. BG9719 = CVT-124 **24** possesses stronger potency and higher A_1-selectivity as the *S*-enantiomer, with Ki = 0.45 nM for the human A_1 AR and 2400-fold subtype selectivity, than the *R*-enantiomer [84].

Targeting A_{2A} adenosine receptors

Activation of A_{2A} AR is generally accepted to have anti-inflammatory properties after activation on immune cells. Its role in colitis was studied using a murine model where inflammation is induced by adoptive transfer of pathogenic T-helper (Th) cells. In this model, co-transfer of regulatory Th cells was able to prevent disease development. The authors observed that regulatory cells from A_{2A} AR knockout mice were not able to suppress T-cell-mediated colitis and that conversely, A_{2A} AR deficient pathogenic Th cells were not inhibited by WT regulatory cells. Moreover, it was shown that A_{2A} AR agonist ATL-202 (*N*-ethyl-2-adenosine-5'-uronamide) modulates cytokine production in both regulatory and pathogenic Th cells, reducing the production of pro-inflammatory cytokines while leaving anti-inflammatory cytokines unchanged [85].

CGS 21680C **25** (Figure 4) has high *in vitro* affinity for A_2 AR (IC_{50} = 13 nM) and 140-fold selectivity and was unable to bind to 17 other putative neurotransmitter/neuromodulator sites, indicating its selectivity as an adenosine receptor ligand [86]. CGS 21680 showed anti-inflammatory activity by reducing secretion of IL-2 and TNF-α by Th-1 and T-cytotoxic (Tc)-1 cells *in vitro* [87]. However, when tested in dextran sulfate sodium (DSS)-induced model of colitis in mice, intraperitonial administration of the compound was unable to improve disease

parameters like bodyweight and colon length and was ineffective as well in preventing the increased levels of macrophage inflammatory proteins (MIP) and IL-1β [88]. As the involvement of Ado signaling in regulation of motility had been demonstrated in healthy intestine, some studies aimed to investigate this involvement during inflammatory conditions. Here, CGS 21680 (0.01 μM) was able to reduce contractile responses to electrical stimulation on rat colon. The effect was significantly enhanced in inflamed tissue, when colitis was induced in the rats by 2,4-dinitrobenzenesulfonic acid (DNBS) administration. Possible causes for the higher activity of A_{2A} AR in colitic rats are increased receptor density and increased recruitment caused by inflammation-induced changes in endogenous Ado levels [89]. Interestingly, in this model of rat colitis, expression of A_{2A} AR and CD73 is higher in inflamed colon than in healthy tissue [49]. In contrast, the decrease in acetylcholine-evoked contractile functions resultant from 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced inflammation in rat ileum/jejunum preparations, was reversed by CGS 21680 (1μM) [90]. The closely related APEC **26** has been developed to a highly specific radioligand compound binding to the A_{2A} AR [91].

The 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-pur-in-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester (ATL146e) **27** was over 50 times more potent than CGS21680 for the human A_{2A} AR [92]. ATL-146e exerted anti-inflammatory effects in three different models of inflammatory bowel disease. The treatment reduced inflammatory tissue damage in acute rabbit colitis and was able to reduce immune cell infiltration in colonic mucosa in the chronic model of rabbit colitis. In the mouse model of spontaneous ileitis, activation of A_{2A} AR signaling also improved histological scores and reduced the production of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-4). Adoptively transferred ileitis induced by injection of T-cells from spontaneous ileitic mice in immunodeficient acceptors was also ameliorated by administration of ATL146e. These results encourage an application of A_{2A} AR agonists for the treatment of inflammatory bowel disease [93]. ATL-146e **27** has been further improved yielding ATL-313 **28** with higher selectivity [94]. ATL-313 also showed to be protective against mucosal inflammation during *Clostridium difficile* toxin A-induced murine ileal enteritis, where it was able to reduce mucosal edema, disruption and neutrophil infiltration [95].

During a search for the activation of the human A_3 AR by a wide range of N^6-substituted adenosine derivatives N^6-[2-(3.5-dimethoxyphenyl)-2-(2-methylphenylethyl)]adenosine (DPMA) **29** was found. Interestingly, it was demonstrated that DPMA is both a potent agonist for the A_{2A} AR (K_i = 4 nM) and a moderately potent antagonist for the human A_3 AR (K_i = 106 nM) [96]. Because compound UK-371104 **30** gave disappointing results in preclinical trials, it was further improved to UK-432097 **31** showing K_i of 0.5 nM for the

A_{2A} AR with K_i (A_1) = 300 nM and K_i (A_3) = 68 nM. Very recently the crystal structure of UK-432097 bound to the human A_{2A} AR has been reported [97]. However, UK-432097 failed in clinical trials to treat chronic obstructive pulmonary disorder (COPD) by inhalation, although it may have an application in inflammatory bowel disease (IBD) [98]. Compound GW328267X **32** has been developed and found to act as a potent and selective A_{2A} AR agonist and as a competitive A_3 AR antagonist. Furthermore, several anti-inflammatory effects of this new A_{2A} AR agonist have been demonstrated [99].

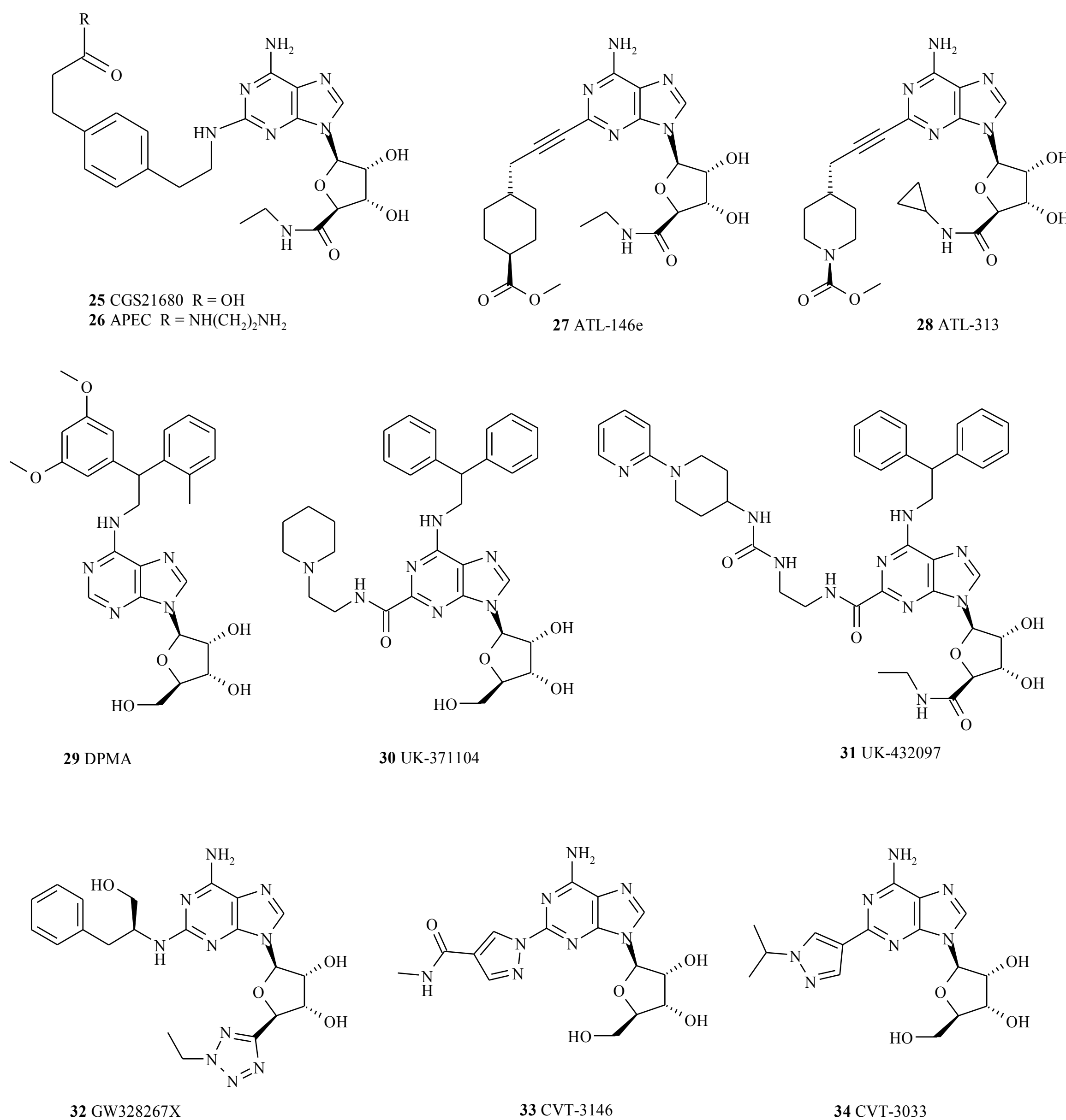

Figure 4: A_{2A} AR agonists

It has been shown that lipophilic substitution at C-2 of adenosine gives potent and selective A_{2A} AR agonists [100]. Testing 2-(*N*-1-pyrazolyl)-adenosine derivatives led to the identification of CVT-3146 **33** and CVT-3033 **34** which are selective for the A_{2A} over the A_1 and A_{2B} ARs having low affinity to A_3 AR [101].

Compound CVT-3146 went into clinical trials and, finally, on 10 April 2008, CV Therapeutics, Inc. of Palo Alto, California, USA, received the approval from the US Food and Drug Administration for the use of regadenoson (CVT-3146) as a pharmacological stress agent in radionuclide myocardial perfusion imaging in patients unable to undergo adequate exercise stress. This is the first approval of a synthetic Ado receptor ligand, which will be marketed as Lexiscan® [[102]].

One of the oldest, best known and most widely consumed antagonists of adenosine receptors is caffeine **35** (Figure 5). It interacts rather non-specifically with all four known ARs [[103]]. A_{2A} AR antagonists of several structural types have been described. Historically they were xanthines, starting from caffeine, followed by non-xanthines, but still they were non-selective for the A_{2A} AR compared to the A_1 AR. A very informative review appeared in 2007 offering an overview over structure-activity relationships of many A_{2A} AR antagonist [[104]]. During the search for selective antagonists, 8-styrylxanthines have also been evaluated for their activities as A_{2A} AR antagonists [[105]]. From a series of 1,3,7-trialkyl-8-styrylxanthines CSC **36** was identified having K_i =54 nM for the A_{2A} AR and A_1/A_{2A} selectivity of 518 [[106]]. The pharmacokinetic profiles of six A_{2A} AR antagonists have been evaluated *in vivo* in rats. It was found that 3,7-dimethyl-1-propargylxanthine (DMPX) and KW-6002 **37** had the best oral bioavailability and overall, KW-6002 was the best based on its bioavailability and half life [[107]]. Out of a series of 1,2,3-triazolo[4,5-e]1,2,4triazolo[1,5-c]pyrimidines, compound **38** (SCH 58261) was identified as potent and selective adenosine A_{2A} AR antagonist in binding assays (K_i = 2.3 nM, A_1/A_{2A} K_i ratio = 52.6) [[108]]. Out of this study SCH 442416 **39** has been developed, first as specific positron-emission tomography (PET)-dye and later as antagonist for clinical studies [[109]]. Screening a number of pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine derivatives for A_{2A} AR specificity revealed that N^7 substitution decreases the A_1 AR affinity but increases A_{2A} AR selectivity. From several derivatives based on pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines, compound **40** was identified having very good affinity (K_i = 0.94 nM) and high selectivity (A_1/A_{2A} = 787, A_3/A_{2A} > 10 000). This made **40** one of the first really selective A_{2A} AR antagonist [[110]]. Study of a large series of C9- and C2-substituted pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines led finally to highly active compounds, e. g. **41** or **42** [[111]].

The compound ZM241385 **43** has been synthesized as ^{125}iodo-compound and characterized by radioligand binding in membranes expressing A_{2A} AR. Membranes bound ^{125}I-ZM241385 with high affinity and no specific binding was detectable in membranes expressing functional A_1, A_{2B}, or A_3 AR, indicating high selectivity [[112]]. *In vitro* studies confirmed the high selectivity of ZM 241385 and could not detect additional

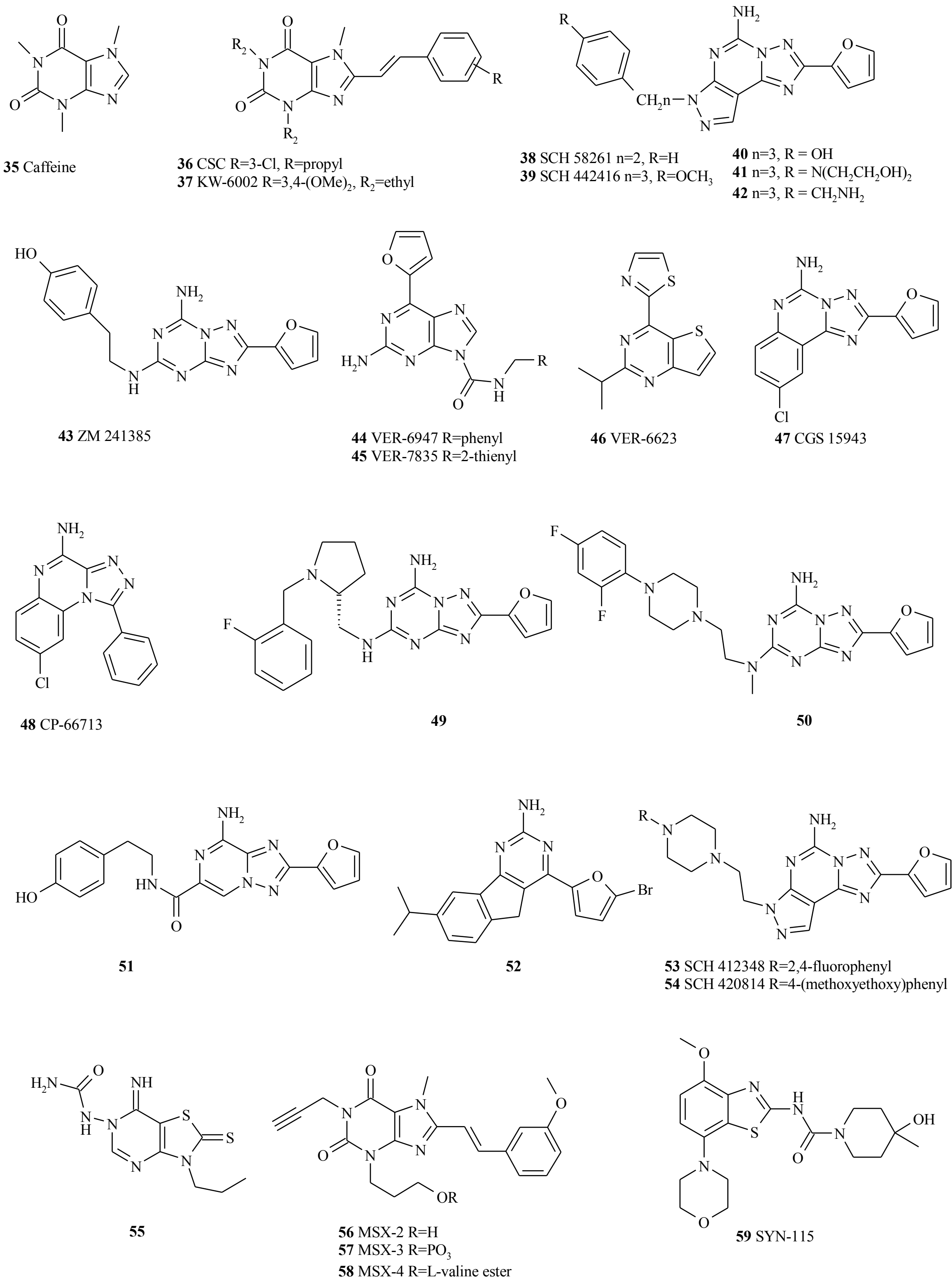

Figure 5: A_{2A} AR antagonists

pharmacological effects [113]. The compound was shown to increase electrically-induced contraction in rat colon, with higher efficiency in inflamed tissue, corroborating the involvement of A_{2A} AR in mediating Ado-induced motility disturbance in colitic mice [89].

The (-)-(R,S)-enantiomer of the antimalarial drug mefloquine was found to be a potent adenosine A_{2A} AR antagonist. Despite the compound was ineffective in *in vivo* animal models it was used as lead for a screening of more than 2,000 chemically diverse compounds. In addition to VER-6947 **44** and VER-7835 **45,** the thieno[3,2-dy]pyrimidine VER-6623 **46** (K_i = 1.4 nM) was found [114]. During the search for benzodiazepine receptor modulators, the potent adenosine antagonist CGS 15943 **47** based on [1,2,4]-triazoloquinazoline [115] and later the [1,2,4]-triazoloquinoxaline CP 66,713 **48** [116] were discovered, but both with still low selectivity. One of the first antagonists with moderate receptor selectivity was the xanthine KW-6002 **37** [105] which became now an investigational adenosine A_{2A} AR antagonist for the treatment of Parkinson's disease. Several other bicyclic systems have been described with good potency and selectivity, e. g. the [1,2,4]triazolo[1,5-a][1,3,5]triazine derivatives **49** [117], **50** [118] or **51** [119]. In a high throughput screening 4-aryl-5H-indeno[1,2-d]pyrimidin-2-ylamine derivatives were identified as potent A_{2A} AR antagonists. An optimization of the activity led to compound **52** with best characteristics having K_i (A_{2A}) = 0.8 nM and A_1/A_{2A} selectivity of 103 [120].

Testing a series of arylpiperazine derivatives of pyrazolo[4,3-*e*-]-1,2,4-triazolo[1,5-*c*]pyrimidines the 2,4-fluoro-aryl-derivative SCH 412348 **53** turned out to have the highest A_{2A}/A_1 selectivity. Replacing 2,4-difluorophenyl by 4-(methoxyethoxy)phenyl gave SCH 420814 **54** (Preladenant) and immensely increased the solubility in water, allowing oral administration of the drug [121]. SCH 420814 and SCH 412348 are potent competitive antagonists of the human A_{2A} AR (K_i = 1.1 and 0.6 nM, respectively) and have more than 1000-fold selectivity over all other adenosine receptors, making these compounds the most selective A_{2A} AR antagonists reported to date. Although some of these antagonists went already to clinical phase trials, the reports on novel compounds continue. Very recently novel bicyclic thiazolopyrimidine compounds synthesized as adenosine A_{2A} AR antagonists led to compound **55** possessing the strongest binding affinity (K_i = 0.0038 nM) and selectivity (737-fold) for the A_{2A} AR versus A_1 AR. It is interesting to note here that replacing the n-propyl group of **55** by ethyl reversed the selectivity of the compound making it an antagonist 500 fold selective for A_1 versus A_{2A} AR [122].

In an attempt to use prodrugs as A_{2A} AR antagonists MSX-3 **57** was found which is cleaved by phosphatases to MSX-2 **56** [123]. MSX-2 is a highly potent antagonist and the prodrugs MSX-3 **57** and MSX-4 **58** [124] have increased tissue selectivity due to the activation of MSX-2 on site. Although **57** showed excellent properties with very high affinity and selectivity, its application may be severely limited. More detailed studies revealed that the compound changes under light irradiation to almost inactive dimers. Current clinical trials are assessing the highly potent antagonists ST-1535 [125] and SYN-115 **59** [126].

Targeting A_{2B} adenosine receptors

The lack of potent and selective A_{2B} AR ligands prevented for many years a profound exploration of the therapeutic perspective of drugs interacting with this receptor. Progress in the discovery of highly potent and selective A_{2B} AR agonists in turn, is hampered by the lack of an appropriate radioligand and many research groups are involved in the discovery of such useful A_{2B} AR agonist radioligands. A recent review reported the most significant advancements in the synthesis of new compounds [[127]] and in the principal structure activity relationships studies [[128]]. Beukers and coworkers published a remarkable review on structure-affinity relationships of A_{2B} AR ligands [[129]]. A few agonists with high affinity have been reported. A non-adenosine derivative is LUF5835 **60** (Figure 6) [[130]] but it does not display high selectivity versus the other AR subtypes. Further development of LUF5935 led to the agonist BAY60-6583 **61** having an even better binding profile, with $EC_{50}(A_{2B})$ = 3 nM and higher than 3000-fold selectivity versus the other ARs [[131]]. In a model of intestinal ischemia/reperfusion, A_{2B} AR knockout mice presented enhanced inflammation after IR event, while treatment with BAY60-6583 protected wild-type mice from intestinal injury [[132]]. The adenosine derivative **62**, has $EC_{50}(A_{2B})$ = 82 nM, $K_i(A_1)$ = 1050 nM, $K_i(A_{2A})$ = 1550 nM, $K_i(A_3)$>5000 nM and is presently the only highly potent and selective A_{2B} AR agonist [[133]].

One of the compounds filling this gap of the urgently needed radioligands is OSIP339391 **63**, a pyrrolopyrimidine A_{2B} AR antagonist with $K_i(A_{2B})$=0.5 nM and a selectivity greater than 70-fold for A_{2B} AR over other human adenosine receptors [[134]]. The closely related compounds MRS1754 **64** [[135]] and MRS1706 **65** are highly potent and selective antagonists of human A_{2B} AR [[136]]. MRS1754 has been shown to reduce plasma levels of inflammatory mediators like IL-6 and MIP-2 and increase survival, enhancing macrophage function and bacterial clearance in septic mice [[137]]. Another related compound, MRE 2029-F20 **66**, possesses $K_i(A_{2B})$=5.5 nM and >180 fold selectivity) [[138], [139]]. Further optimization of this lead structure resulted in the highly active *N*-ethylnicotinamide ATL-801 **67** [[140], [141]] and a patent concerning compounds similar to ATL-801 has recently been filed [[142]]. The use of ATL-801 in the diet of colitic mice (induced either by DSS or piroxicam in IL-$10^{-/-}$ mice) resulted in significant lower severity of the disease, with reduced clinical symptoms and histological scores. Effects were observed in terms of suppression of pro-inflammatory cytokines, neutrophil infiltration and epithelial hyperplasia [[143]]. The protective effect of impairing A_{2B} AR signaling against induced colitis was confirmed in A_{2B} AR knockout mice [[144]].

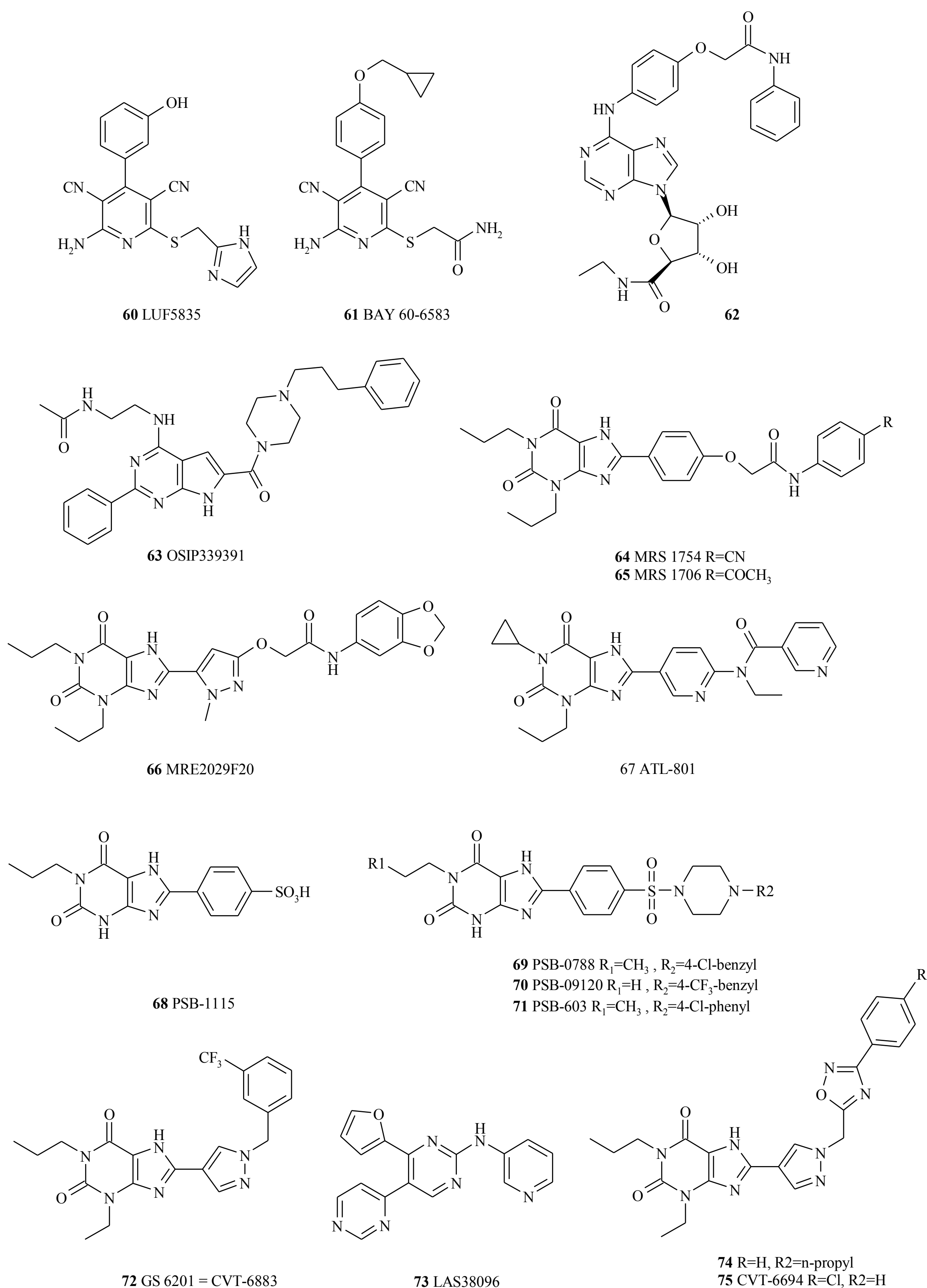

Figure 6: A_{2B} AR agonists and antagonists

SAR-studies on 1-, 3- and 8-substituted xanthine derivatives resulted in the discovery of another A_{2B} AR antagonist, the sulphoric acid PSB-1115 **68** [[145]]. Opposed to the results with the antagonist ATL-801, a similar study found that PSB1115-treated mice present an increase in DSS-induced colitis severity, which was also the case in Aa2br $^{-/-}$ mice. Here, IL-10 deficiency caused by the absence of A_{2B} AR activity appears as the cause of exacerbated inflammation, implicating IL-10 modulation by adenosine on epithelial cells as a central protective effect [[146]]. Accordingly, *in vivo* this antagonist caused a significant increase in hypoxia-induced vascular leakage in the colon, in contrast to the reversing effect by A_{2B}AR agonist **61**, supporting an anti-inflammatory role for this receptor. However, regarding the inflammation-induced contractile disturbance, PSB-1115 was protective in rat gastrointestinal preparations [90].

The specific A_{2B} AR antagonists MRS 1754 **64** and PSB-603 **71** reduced duodenal bicarbonate secretion in rats induced by ATP [[147]]. Adenosine deaminase-deficient mice showed less pulmonary inflammation when treated with GS-6201 (=CVT-6883) **72** due to a significant reduction in pro-inflammatory cytokines and chemokines [[148]]. In addition to the ones mentioned above, there are other promising candidates for clinical trials: PSB-0788 **69** ($K_i(A_{2B})$=0.39 nM, selectivity >850 fold) and PSB-09120 **70** ($K_i(A_{2B})$=0.157 nM, selectivity >140 fold) [142], LAS38096 **73** ($K_i(A_{2B})$=17 nM, selectivity >60 fold) [[149]], the 1,2,4-oxadiazol **74** ($K_i(A_{2B})$=1 nM, and selectivity >370-fold) [[150]] and CVT-6694 **75** ($K_i(A_{2B})$=7 nM, and selectivity >700-fold) [[151]].

Targeting A_3 adenosine receptors

5'-Uronamides and N^6-benzyl derivatives of adenosine were examined for affinity in radioligand binding assays in the rat brain A_3 AR. 5'-*N*-Methyluronamides and N^6-(3-substituted-benzyl)adenosines are optimal in potency and selectivity for A_3 AR, e. g. IB-MECA (=CF101) **76** (Figure 7) and Cl-IB-MECA **77** [[152]]. IB-MECA presented protective role in DSS-induced and IL-10$^{-/-}$ spontaneous colitis in mice. Administration of the agonist by gavage significantly reduced the levels of IL-1, IL-6, IL-12, MIP-1, MIP-2 and myeloperoxydase (MPO) levels in colon biopsies and improved disease symptoms [[153]]. In TNBS-induced colitis, IB-MECA was also reported to ameliorate inflammation, improving clinical and histological parameters. In this model, a microarray technique revealed that the A_3 AR agonist was able to prevent the dysregulation of gene expression induced by TNBS, preventing, for example, the upregulation of chemokine, cytokine and inflammatory genes [[154]]. However, A_3 AR knockout mice in contrast showed an improvement in disease parameters like histological scores, body weight changes, colonic infiltration and MPO activity, raising the hypothesis that the protective effect of IB-MECA results from high-dosage and unspecific receptor stimulation [52].

However, although these analogues show selectivity against the rat receptors, there is poor correlation with the human receptor due to low receptor sequence homology [155]. During the search for alternatives, 4‘-thio analogues of Cl-IB-MECA **77** turned out to be potent agonists for the human A_3 AR. Among them LJ568 **78** showed the highest binding affinity (K_i = 0.28 nM) and was highly selective for human A_3 AR against A_1 and A_{2A} ARs by 4800- and 36000-fold, respectively [156]. Another development is CP-608039 **79** which displayed full agonist activity at the human A_3 AR and greater than 1000-fold functional selectivity over the A_1, A_{2A}, and A_{2B} ARs. Furthermore, **79** showed no antagonist activity for any of the receptors [157]. CP-532903 **80** is a highly potent A_3 AR agonist with more than 210-fold selectivity [158].

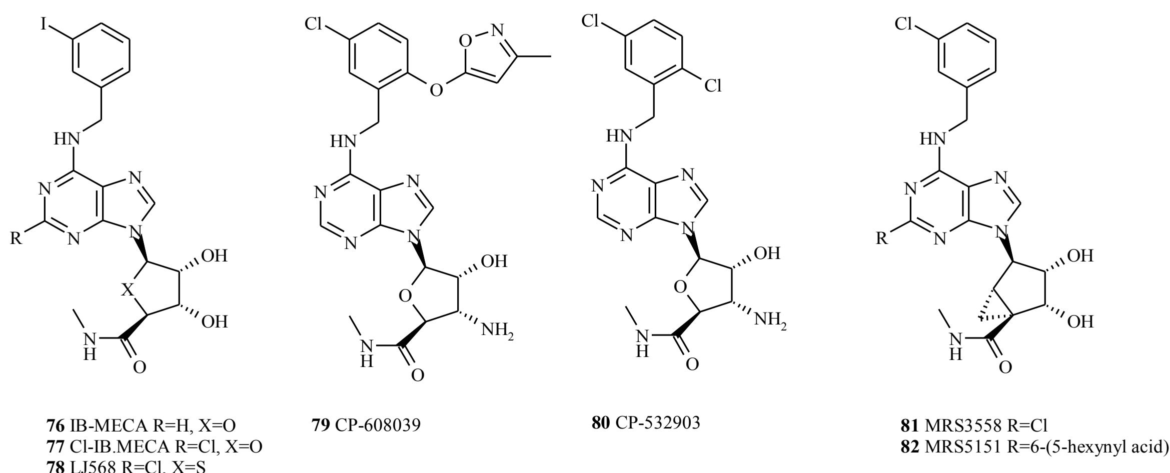

Figure 7: A_3 AR agonists

From testing a series of ring-constrained (*N*)-methanocarba-5’-uronamide 2,N^6-disubstituted adenine nucleosides compounds MRS3558 (=CF502) **81** and MRS5151 **82** [159] had been identified having both high sensitivity (K_i(human A_3) = 0.29 nM) and selectivity (>895-fold) [160, 161]. Truncation of the 5’-uronamide and replacement of chlorine by bromine in MRS3558 at the phenyl moiety changed the agonist to an antagonist and led to the A_3 AR antagonist MRS5147 **91,** with K_i(hA_3) = 0.73 nM and 2410-fold selectivity versus the A_1 AR [162], and the corresponding iodine analogue MRS5127 recently used as radioligand [163].

During the search for highly active A_3 AR antagonists, structure–activity relationships (SAR) of fused 1,2,4-triazolo[1,5-c]pyrimidine led to the highly potent OT-7999 **83** (Figure 8), now in preclinical trials [164]. When the water solubility of 2-phenylimidazopurin-5-ones was observed, these compounds were developed to PSB-10 **84**, a highly potent and selective human A_3 AR antagonist [165]. The phenyl analogue of PSB-10, PSB-11 **85**, has been shown to be a highly selective A_3 AR radioligand [166]. 2-(4-Morpholinoanilino)-N^6-cyclohexyladenine is known for inducing cell dedifferentiation but was also reported as a moderately potent antagonist for the A_3 AR. Optimization of the compound led to MRS 3777, **86** with a >200-fold selectivity [167].

Its 6-(3-chlorophenyl) derivative possesses K_i = 7.94 nM for the human A_3 AR and selectivity of 5200-fold [[168]]. The surprisingly simple thiadiazole **87** achieved high affinity for human A_3 AR (K_i = 0.79 nM) [[169]] and the highly potent and selective 1,4-dihydropyridine MRS1334 **88** possesses K_i = 2.69 nM [[170]]. MRS 1523 **89** has been found to be less active but very specific for the A_3 AR with K_i values to human A_1, A_2, and A_3 ARs of 15,600, 2,050, and 19 nM, respectively. The pyrazolo[4,3-e] 1,2,4-triazolo[1,5-c] pyrimidine MRE 3008-F20 **90** showed high A_3 AR affinity and has been developed to a radioligand of the human A_3 AR [[171]].

As has been mentioned before, DPMA **29**, a potent agonist for the A_{2A} AR, was demonstrated to be a moderately potent antagonist for the human A_3 AR as well. Dual acting A_1/A_3 agonists like DPMA, and also (N^6-3-chlorobenzyl)- **92**, N^6-(*S*-1-phenylethyl)- **93**, and 2-chloro-N^6-(*R*-phenylisopropyl)-adenosine **94** might be useful for cardioprotection and other applications [96]. N^6-[(1S,2R)-2-Phenyl-1-cyclopropyl]adenosine **95** was 1100-fold more potent in binding to human A_3 AR (K_i = 0.63 nM) than to the rat receptor. A broad screening of phytochemicals disclosed some flavonoids as A_3 AR antagonists. Optimization using structure-activity relationship led to MRS1067 **96** which was the most potent compound of this series [[172]]. Highly active compounds were found in rather diverse lead structures. All the following compounds display high selectivity towards the A_3 AR: the triazolo-quinazoline MRS1220 **97** (K_i = 0.65 nM) [[173]], the triazolo-quinoxaline **98** (K_i = 0.6 nM) [[174]] and KF26777 **99**, which is a cyclised derivative of xanthine with extremely low $K_i(A_3)$ = 0.20 nM and high selectivity [[175]]. Compound **100** however, based on triazolo-pyrazolo-pyrimidine, the classical core for adenosine receptor antagonists, and being fully soluble in water achieved the lowest K_i (0.01 nM) of all tested compounds for the human A_3 AR, with high selectivity over the other adenosine receptors [[176]]. A spiro-4',5'-uronamide derivative of adenosine **101** turned out to be an excellent human A_3 AR antagonist (K_i = 26 nM) [[177]]. A group at Novartis studied 5-heterocycle-substituted aminothiazole for their ability to act as adenosine receptor antagonists for both the A_{2B} and A_3 ARs. Out of them, compound **102** showed high affinity and selectivity and good absorption, distribution, metabolism and excretion properties in the rat. This result encourages testing of the new compound for a therapeutic role for a dual A_{2B}/A_3 antagonist in allergic diseases [[178]].

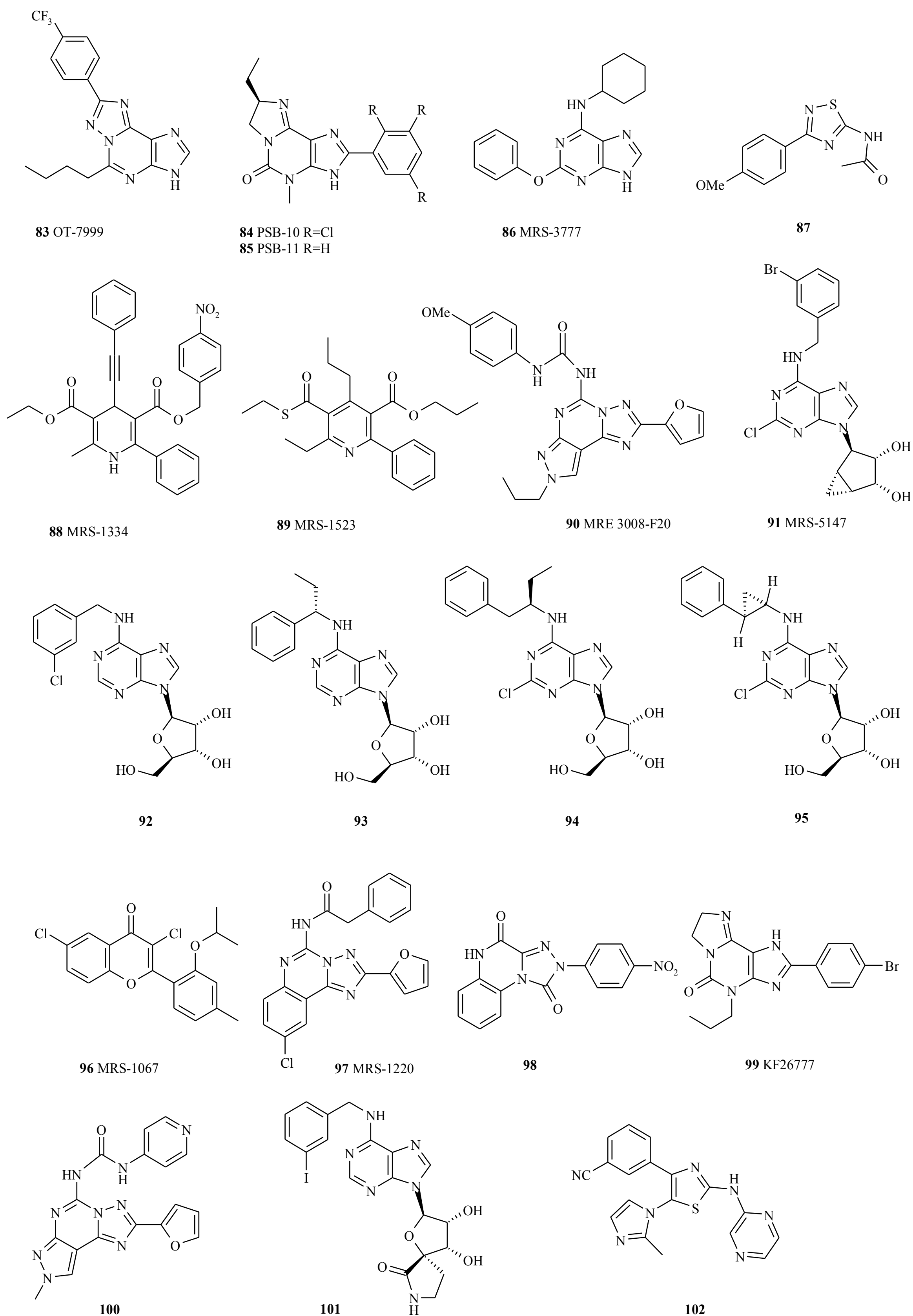

Figure 8: A_3 AR antagonists

Allosteric modulators of adenosine receptors

There are several agonists of adenosine which gave excellent results in preclinical trials, but only some of them made it to clinical trials. However, none of them survived there for long. There are many reasons for the failure of individual compounds but one of the main reasons is side effects. They were mostly caused by the lack of specificity for a single adenosine receptor subtype and, most importantly, for specific tissues. Only in recent years an alternative approach has been suggested which does not act directly at the adenosine binding site but changes the conformation of the receptor by interaction with an additional binding site [[179], [180]]. Compounds doing this are called allosteric modulators to distinguish them from compounds acting at the primary binding (orthosteric) site of the receptor accommodating the endogenous agonist. As a consequence of this definition an allosteric modulator does not show activity on its own but essentially requires the presence of either an orthosteric agonist or antagonist. A very comprehensive recent review on allosteric modulation on G protein-coupled receptors can be found in [[181]].

Although allosteric action mechanisms were known for more than a century [[182]] it was Monod and coworkers, in 1965, who developed the theoretical concept for this mechanism [[183]]. In the case of adenosine receptors they have a number of advantages. They enhance the activity of the endogenous agonist and this means that they hardly can be overdosed because once the allosteric sites are completely occupied by the endogenous agonist no further effect is observed. Another advantage is that the tissue itself controls their action because only where the tissue produces the endogenous agonist its physiological effects are enhanced. In addition, allosteric modulators show a high degree of subtype selectivity. Allosteric modulators have been reported for all classes of adenosine receptors although the majority of the studies were directed towards the A_1 AR [[184]]. Some of the earliest developed allosteric modulators of adenosine receptors are a number of 2-amino-3-benzoylthiophenes interacting with the A_1 AR [[185]]. Although an allosteric modulator is supposed to not interfere with the orthosteric site, in reality some allosteric modulators interact also with the orthosteric site of adenosine receptors. Optimization of substituents on the heterocyclic core of 2-amino-3-benzoylthiophenes shifted the original antagonistic activity for A_1 AR towards an allosteric enhancement, changing the ratio between the two activities to more than 1000-fold [[186]]. Of these compounds PD 81,723 **103** (Figure 9) showed the highest activity and soon became the standard most novel allosteric A_1 AR modulators were tested against. Such an attempt of standardization is highly appreciated because different researchers use diverse assays making the comparison difficult. A detailed evaluation of the activities of PD 81,723 revealed that the allosteric effects observed were specific for the A_1 AR and also specific for the agonist, leaving the antagonist binding unaffected [[187]].

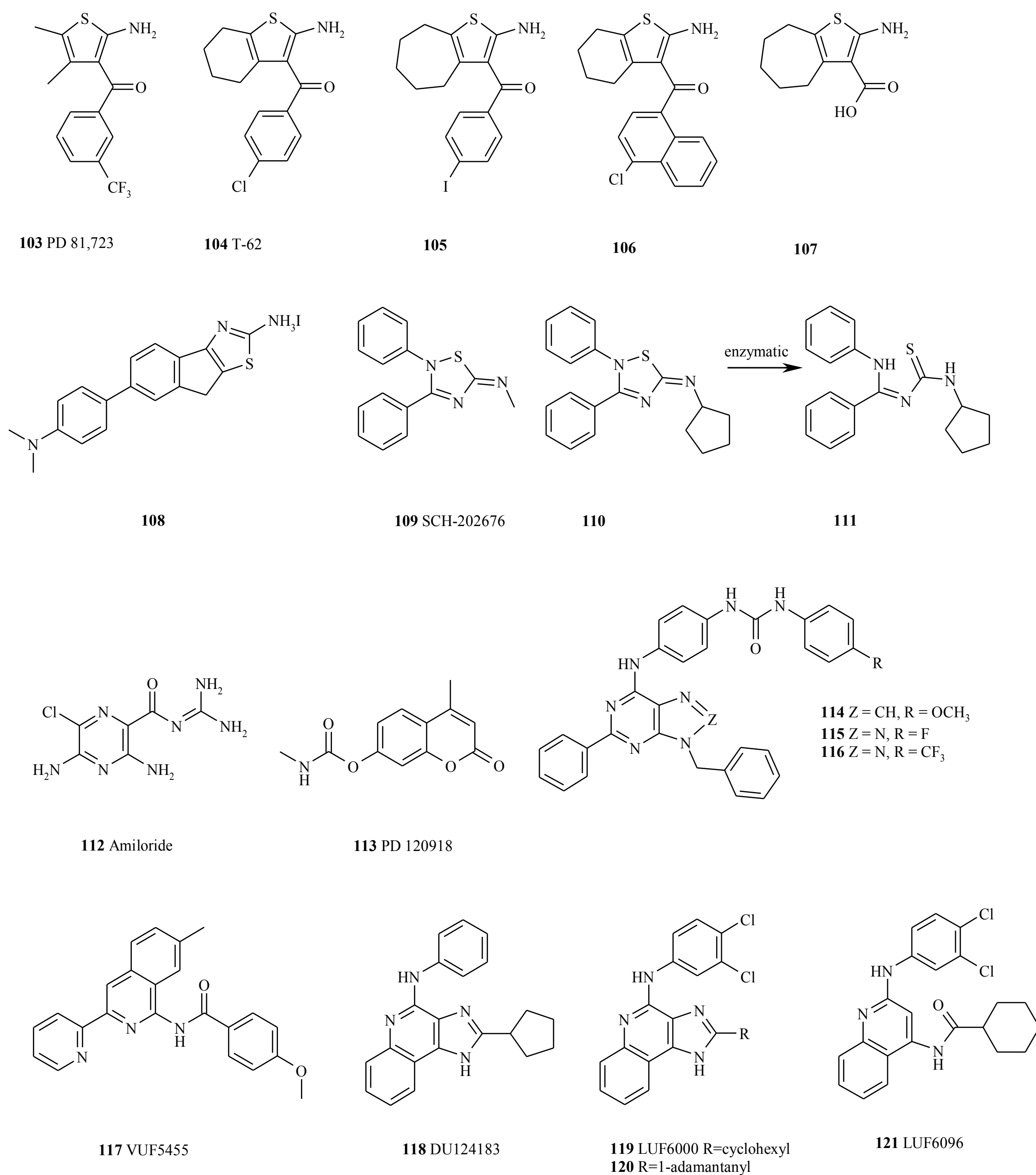

Figure 9: Allosteric modulators of AR agonists

Among a large number of PD 81,723 derivatives tested, T-62 **104** showed 123% activity compared to PD 81,723. Even at 0.1 μM, **104** and its bromo-analogue were more active than PD 81,723 [188]. Structure-activity studies revealed that bulky groups at the 5-position of the thiophene ring promoted antagonistic properties while alkyl chains at the 4-position on the heterocyclic ring, or lipophilic substituents on the 3-benzoyl nucleus, promoted allosteric activity [189]. Testing of another series of PD 81,723 derivatives identified **106** [190] and the iodo-derivative **105** [191] as compounds being more potent than PD 81,723. Systematic variation of the substituents at the PD 81,723 backbone and structure-activity analysis of the new compounds revealed that the 2-

amino group at the thiophene ring is essential for activity as allosteric modulator. Even more drastic changes of the PD 81,723 skeleton led to compounds with higher allosteric enhancing activity scores. A study of 2-aminothiophene-3-carboxylates identified **107** as more potent and efficacious than PD 81,723 [192]. Despite the impressive evidences of the potential of PD 81,723 and derivatives as drugs, these compounds have some general drawbacks. As aromatic amines they pose a carcinogenic risk and are also chemically unstable [193]. Chordia and coworkers discovered some 2-aminothiazoles as a new class of agonist allosteric enhancers of adenosine A_1 AR [194]. Systematic variation of these compounds led to the characterization of compounds which possess higher maximal allosteric enhancer activities than PD 81,723. While PD 81,723 had an EC_{50} >10 μM in binding studies to A_1, A_{2A}, or A_3 adenosine ARs, compound **108** had activity confined to A_1 AR with EC_{50} = 0.9 μM [195]. Surprisingly, IJzerman and coworkers could not detect any effect on the binding of an agonist to A_1 AR, although working on either identical or similar products, tested as free bases. Only if the reasons for these different observations are satisfyingly explained, the value of this type of compound can be assessed.

Moving towards clinical applications by *in vivo* studies, it was observed in rats that the antagonistic actions of the allosteric enhancer PD 81,723 on the A_1 AR cancelled the allosteric actions [196]. On the other hand, preclinical development of **104** (T-62) for the treatment of chronic pain has shown that it reduced hypersensitivity during inflammation in rats [197, 198].

As most studies have been done on modulators for A_1 AR, there is a considerable lack of allosteric modulators for A_{2A} and A_{2B} ARs [187]. Compound *N*-(2,3-diphenyl[1,2,4]thiadiazole-5(2*H*)-ylidene)methanamine, SCH-202676 **109**, has been called a pan-modulator because it acts both as agonist and antagonist by binding to adenosine receptors but it also affects ligand binding to additional receptors like human μ, δ and κ opioid, M_1 and M_2 muscarinic, α and β adrenergic and D_1 and D_2 dopaminergic receptors [199]. A more detailed study revealed that SCH-202676 differentially modulated A_1, A_{2A}, and A_3 ARs by selectively slowing or accelerating antagonist dissociation for A_1 AR and A_{2A} AR, respectively, and by accelerating agonist dissociation for A_3 AR [200]. The group of IJzerman synthesized a number of 2,3,5-trisubstituted [1,2,4]thiadiazole derivatives as analogues of SCH-202676 and identified **110**, differing by the substituent at the *N*-imino function. Studying the pharmacokinetics of this compound the authors could show that, upon incubation, **110** was reductively cleaved to the corresponding thiourea derivative **111**. This pointed to the notion that [1,2,4]thiadiazoles can be considered rather as highly reactive sulfhydryl modifying agents than as allosteric modulators. This finding also explains their astonishing overall lack of selectivity [201].

Up to now no allosteric enhancer specific for the A_{2A} AR has been developed. However, the diuretic amiloride **112** and analogues were demonstrated to be allosteric inhibitors for the A_{2A} AR. However, they did not show any effect on the dissociation rate of the agonist [[202]]. Later it has been shown that amiloride analogues are not interacting only with the A_{2A} AR but are also allosteric inhibitors of antagonist binding to A_1 and A_3 receptor subtypes [[203]]. Another allosteric ligand able to modulate A_{2A} AR activity is the chromene derivative PD 120918 **113**, which enhances agonist binding to the rat striatal A_{2A} AR [[204]]. Giorgi and coworkers synthesized a number of 2-phenyl-8-benzyladenines and 8-azaadenines and identified them as allosteric modulators of the A_{2A} AR. The compounds increased the binding of both agonist and antagonist to the orthosteric site of the receptor. Among the adenine derivatives, electron-donating groups on the phenyl ring of the urea moiety gave the best results and compound **114** was the most effective of the series. Just the opposite was observed for the 8-azaadenine where the highest efficacy was obtained by electron-withdrawing substituents in the same position of the phenyl ring. Here, derivatives **115** and **116** proved to be the most potent ones. The authors demonstrated the ability of **114** to significantly enhance the vasorelaxing effect induced by an agonist in endothelium-intact rat aortic rings. The selectivity assay of these compounds was also quite encouraging because compounds **114**-**116** showed only slight inhibitory properties towards A_1 and A_3 AR subtypes [[205]].

Several 3-(2-pyridinyl)isoquinoline derivatives were allosteric modulators to the A_3 AR, however, many also showed orthosteric binding affinity to this receptor. 4-Methoxy-*N*-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide **117** (=VUF5455) was a good agonistic allosteric modulator of the A_3 AR, being at the same time a rather weak orthosteric ligand (K_i = 1680 nM) [[206]]. Certain 1*H*-imidazo-[4,5-c]quinolines were identified as nonxanthine Ado receptor antagonists [[207]] and later also shown to be allosteric modulators for the A_3 AR. Out of these compounds, 2-cyclopentyl-4-phenylamino-1*H*-imidazo[4,5-c]quinoline (DU124183) selectively enhanced agonist binding and function at A_3 AR. In contrast to VUF5455 **117**, DU124183 also increased the maximum efficacy of A_3 AR agonists by approximately 30%. Similar to the other compounds, DU124183 **118** also possesses some orthosteric activity binding with moderate affinity (K_i = 820 nM) to human A_3 AR [[208]]. Further optimization of DU124183 revealed that substitution at the 2-position was necessary for allosteric enhancement. The 2-cyclohexyl-4-(3,4-dichlorophenyl)amino derivative LUF6000 **119** enhanced the maximum efficacy of the agonist Cl-IB-MECA **77** by 45%. Moreover, LUF6000 decreased the agonist dissociation rate without influencing agonist potency [[209]]. A rather surprising finding was that LUF6000 could convert an antagonist into an agonist opening completely new possibilities for the application of allosteric modulators [[210]]. The 1-adamantanyl-derivative **120** of LUF6000 showed considerable allosteric potentiation

with 210% activity compared to the control, and minimal orthosteric binding at the receptor [211]. Another optimization of LUF6000 led to the novel compound LUF6096 **121** which was able to allosterically enhance agonist binding, in addition showing very low orthosteric affinity for any of the adenosine receptors [212].

Interfering with adenosine degradation/formation

An alternative complementary approach to modulate Ado receptors activation is the manipulation of Ado levels by interfering with the enzymes involved in its metabolism. Such approaches, like the allosteric modulators, have the advantage of acting locally in situations of intense endogenous Ado release, like inflamed tissues. They have been recently highlighted for the treatment of cardiovascular disorders as site- and event-specific drugs that may overcome limitations of orthosteric receptor activators, like receptor desensitization or downregulation [213].

The conversion of adenosine to inosine by adenosine deaminase (ADA) is one important reaction limiting Ado biodisponibility and ADA inhibition results in the accumulation of adenosine. The activity of purine deaminases (including ADA) in blood and colonic tissue was observed to correlate inversely with inflammation in rat experimental colitis [214], while the levels of circulating adenosine is also reduced with inflammation, possibly due to an increased demand in the inflamed mucosa [215]. Therefore, some studies in animal models aimed to the modulation of this enzyme activity to determine the effects on intestinal inflammation.

When the commercially available ADA inhibitor, pentostatin (2-deoxycorformycin), currently indicated for use in hairy cell leukemia [216], was administered to IL-10$^{-/-}$ mice, inflammation was reduced by more than 50% and serum amyloid A levels were nearly normalized. At the same time, lymphocyte expansions in the colon and mesenteric lymph node dropped by up to 90%. Pro-inflammatory factors, e. g. IL-1β, IFN-γ, IL-6, TNF and C-X-C motif chemokine ligand (CXCL)-10, were reduced, while the regulatory forkhead box P3 (FoxP3) and transforming growth factor (TGF)-β were unchanged. Pentostatin effectively treated colitis by impairing T_{eff} cell expansion and reducing pro-inflammatory cytokine production [217]. Experiments in DNBS-induced colitic mice using the novel ADA inhibitor 4-amino-2-(2-hydroxy-1-decyl)pyrazole[3,4-*d*]pyrimidine (APP) confirmed the protective effects of ADA [218]. Moreover, it was reported that ADA is upregulated in inflamed tissue and the beneficial effects of ADA inhibitors are impaired by blockade of A_{2A} and A_3 ARs, but not A_1 and A_{2B} AR [219]. The use of ADA inhibitor in a model of murine enteritis induced by *C. difficile* toxin-A, where ileal ADA activity is enhanced, was also able to attenuate inflammation [220]. General mechanisms involved in the effects of

ADA inhibition include reduction in pro-inflammatory cytokine production, oxidative damage and lymphocyte depletion. Inhibition of NF-kB pathway in ileal tissue was observed, probably a result of the increased Ado concentration in the inflamed sites [220]. It is important to note that the product of ADA, inosine, also presents anti-inflammatory activity in monocytes, neutrophils, as well as in epithelial cells *in vitro* and shows beneficial effects in animal models of colitis [[221], [222]].

There is another aspect involved in the interference with ADA which goes beyond simply blocking adenosine dagradation. Although ADA is mainly a cytosolic enzyme, it exists also as an ecto-enzyme able to bind to specific cell surface proteins where CD26 and adenosine receptors A_{2B} and A_1 have been identified as specific ligand proteins. *In vitro* studies of DC and T-cell co-cultures showed that ecto-ADA expressed by DC interacts with CD26 in T-cells, providing a co-stimulatory signal that induces proliferation and pro-inflammatory cytokine production [[223]]. Recently it has been demonstrated that purified ADA behave like a positive allosteric modulator when binding to human A_1 adenosine receptors. The agonist affinity is clearly enhanced and the receptor functionality and sensitivity to adenosine is increased. This effect is caused by protein-protein interaction between the enzyme and the receptor and it is independent of ADA enzymatic activity. Furthermore, it is also independent of the nature of the ligand and an increase in the affinity for antagonist binding was also reported [[224]].

Another enzyme involved in adenosine metabolism is adenosine kinase, which catalyzes the phosphorylation of adenosine to form AMP, limiting cytosolic Ado concentration. When this reaction is inhibited, an increase in intracellular Ado results in enhanced Ado export, thus raising extracellular Ado levels. In fact, the adenosine kinase inhibitor GP515 was reported to reduce TNF-α production by human PBMC [[225]] and it has been investigated for its effects in experimental murine colitis. A significant improvement in clinical and histological scores was observed in colitic mice receiving the inhibitor i. p., parallel to decreased INF-γ production and splenocyte activation [[226]]. Adenosine kinase activity has other important implications for inflammatory responses. Intracellular AMP generated by Ado-phosphorylation activates AMP-activated protein kinase (AMPK), which can regulate downstream pathways linked to inflammation, for instance coregulation of HIF-1 [[227]]. AMPK is also involved in inhibition of NF-κB signaling, with significant influence on mechanisms of inflammation suppression, as recently reviewed elsewhere [[228]].

On the other side, the nucleotidases that convert ATP to AMP (e. g. CD39) and subsequently generate Ado from AMP (e. g. CD73) are also interesting targets for the modulation of Ado-mediated effects in inflammation. It has been shown that CD73 and CD39 are important for the Ado-mediated inhibition of PMN

accumulation after hypoxia-induced inflammation [229]. RNAi studies demonstrated that depletion of CD73 in endothelial cells leads to enhanced pro-inflammatory responses [230]. TNBS-induced colitis in mice resulted in an induction of CD73 transcripts in colonic mucosa and this enzyme presented a protective role in inflammation, as indicated by the increased severity of the disease in CD73 knockout mice. This study pointed to the induction of IL-10 via IFN-αA production as the mechanism by which CD73 dampened inflammation [231]. In an attempt to increase the tissue selectivity of the agonist, prodrugs were designed which are activated on site by nucleotidases. A screen of prodrugs of A_{2A} AR agonists activated by ecto-5'-nucleotidase identified 2-cyclohexylethylthio-adenosine-5'-monophosphate as the best compromise between the requirements of the ecto-nucleotidase and the A_{2A} AR [232].

It has been demonstrated that genetic deletion of CD39 resulted in more severe inflammation in mice with DSS-induced colitis, an effect that could be prevented by restoring enzymatic activity. This observation indicated a protective role for CD39 and was reinforced by single-nucleotide polymorphism analysis demonstrating that a tag for low expression of CD39 was associated with increased susceptibility to Crohn's disease [233]. Paradoxically, an increase in CD39 expression in inflamed colonic tissue was reported in another study in DSS-induced colitic mice. In this case, a decrease in ATP levels impairs purinergic sympathetic regulation, a possible cause of altered blood flow in IBD [234].

Practical examples of the importance of Ado regulation in inflammatory diseases and their treatment are found in some currently used immunosuppressant drugs. For instance, Methotrexate (Mtx) is commonly applied for induction of remission and maintenance therapy in IBD patients [235]. Its mechanism of action has been investigated and there are several evidences that induction of Ado release has a central role in anti-inflammatory properties of Mtx [236]. Mtx inhibits 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase, leading to AICAR accumulation. AICAR in turn can cause inhibition of adenosine deaminase and AMP deaminase, resulting in increased levels of Ado [237]. Further anti-inflammatory properties of AICAR were reported in TNBS-induced murine colitis, possibly through the activation of AMPK [238].

The activity of ecto-5'-nucleotidase CD73 and activation of Ado receptors are also implicated in the anti-inflammatory effect of Mtx in animal models [239, 240]. Importantly, Mtx treatment has been reported to modulate Ado levels *in vivo* in humans [241]. Sulfasalazine, also used in the treatment of IBD and other inflammatory disorders, shares with Mtx the ability to induce Ado accumulation in inflamed tissue [242]. Tacrolimus, or FK506, and Cyclosporin A, other drugs indicated to treat some cases of IBD, have been shown to inhibit adenosine uptake and adenosine kinase activity in endothelial cells [243] and T-cells [244].

Discussion

The lessons we can learn from the great amount of data regarding the roles of Ado in the intestine during health and inflammation are many and crucial for current and future developments in therapeutic targeting of Ado receptors and related pathways. This is valid for most processes regulated by Ado as has been recently reviewed by Nánási and coworkers [Error: Reference source not found]. Furthermore, additional aspects are peculiar of the gut and must be taken into account. These are the parallel interaction events taking place between the immune system and the intestinal mucosa, including the complex gut microbial community and biologically active compounds delivered by food (Figure 10).

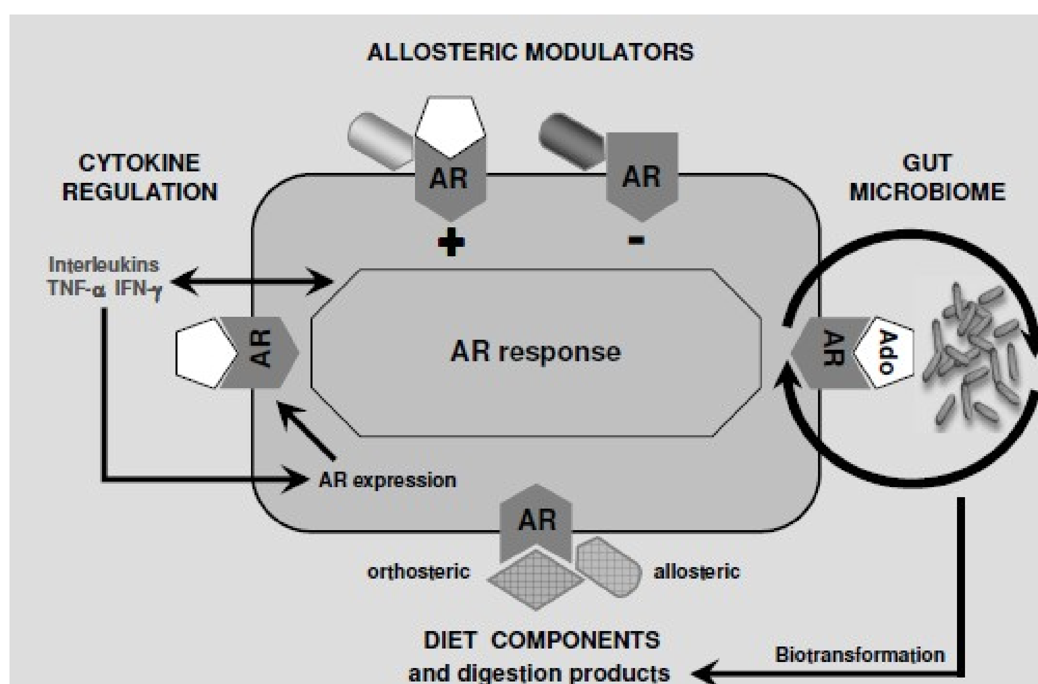

Figure 10: Different aspects interfering with Ado signaling in the gut. The development of new drugs targeting Ado signaling as therapy for intestinal inflammatory disorders can be improved if exploring different interacting aspects. For example: positive or negative allosteric modulators of Ado receptors (AR) to be employed also in combination with existent or newly discovered agonists/antagonists; mutual regulatory processes between Ado-system and cytokines like interleukins, tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ); presence in the gut of dietary components that can act as allosteric or orthosteric modulators of AR; and the interaction with the rich and diverse gut microbiome, either directly (production of extracellular Ado by the bacteria, for instance) or indirectly, via the bacterial processing of diet components into potential modulators.

Clinical application will improve with further progress in pharmacology. Although immense progress has been made in our understanding of the complex ways in which adenosine modulates a number of physiological and pathophysiological processes through the interaction with four subtypes of adenosine receptors, clinical evaluation of some adenosine receptor ligands remains problematic. Major problems include side effects due to the wide distribution of adenosine receptors, short half-life of compounds, lack of tissue specificity, and sometimes receptor desensitization or low receptor density, leading to reduced effects in the targeted tissue [[245]]. These problems stress the strong need for more selective ligands. In 2010 the x-ray structure of the human A_{2A} AR bound to the antagonist ZM 241385 has been reported. The elucidation of the 3D-structure of the protein in interaction with the antagonist identified a number of amino acids crucial for ligand interactions [[246]] and will definitely facilitate the search for highly active and extremely specific antagonists [[247]]. Compounds which combine two activities specifically may open new clinical applications. Furthermore, the biological evaluation of a number of compounds revealed their function both as allosteric modulators and as orthosteric antagonists of ARs [[248]] and it has been shown that allosteric modulators can reverse the action of a drug from an agonist to an antagonist. Further progress in this field will broaden our toolbox for treating the inflamed gut.

Cross-talk between cytokines and adenosine signaling must be considered. Cytokines can differentially regulate Ado receptor expression in the gut and immune cells. For example, it has been demonstrated that the expression of A_{2B} AR in T84 cells is upregulated by TNF-α, highly present in chronic inflammatory diseases [7] whereas IFN-γ, another important mediator in chronic inflammation, is implicated in downregulation of adenosine-mediated signaling, not affecting receptor expression, but by direct inhibition of adenylate cyclase [[249]]. However, in murine macrophages, IFN-γ presented a direct enhancing effect in A_{2B} AR expression, which in turn mediated the inhibition of IFN-γ -induced pro-inflammatory responses, suggesting a negative-feedback loop for controlling macrophage activity [[250]]. The expression of A_{2A} AR is upregulated by the Th1 pro-inflammatory cytokines TNF-α and IL-1β in human polymorphonuclear neutrophils [8] and in human monocytic cells, where these two cytokines enhanced the effect of A_{2A} AR agonist in suppression of IL-12 and stimulation of IL-10 production [[251]]. The responses were instead attenuated by IFN-γ, which reduced A_{2A} AR expression by these cells [251]. Similarly, TNF-α and IL-1β were reported to increase expression of A_{2A} and A_{2B} ARs in human microvascular endothelial cells, in contrast to INF-γ, which was able to upregulate only A_{2B} AR expression and presented a suppressor effect upon A_{2A} AR levels. This differential regulation is also clear at functional level: the increase in cAMP levels in response to A_{2A} AR activation is higher in TNF-α treated cells.

The cAMP response to non-selective Ado receptor stimulation in IFN-γ treated cells in turn, is mediated by A_{2B} AR [252]. Cytokines can also act in the level of Ado formation, as reported for IFN-β, which presented anti-inflammatory properties by induction of CD73 in endothelial cells [253].

Food is a source of active compounds interfering with adenosine signaling. A special condition of the gut is the direct exposure to a large number of different natural compounds from many different sources introduced by the food and its digestion in the gut lumen. Since many years it is known that several of these molecules interact with our body, modulating a broad range of processes. Among them are also interactions with ARs and a study revealed that flavonoids in the diet may influence adenosine action [254]. Interactions with ARs have not only been reported for flavonoids but also for other natural products alkaloids or lactams [255]. Two groups reported recently that our food may also contain AR allosteric enhancers. One finding is that the food dye Brillant Black BN displays allosteric interactions [256]. Another report brings attention to 2-arachidonylglycerol, generated from lipids by lipases, which acts as a negative allosteric modulator of the human A_3 AR [257]. Müller and her group identified a lignane of the olivil type, with partial agonistic activity in the A_1 AR at low millimolar range, which is the first AR agonist not structurally related to adenosine [258]. These findings give us a first impression about the AR modulating activity of food and the potential of diet and functional food in maintaining gut health.

Bacteria are important players in the inflamed gut by modulating adenosine signaling. An outstanding characteristic of the gut is its huge biodiversity of often highly specialized bacteria. Intestinal bacteria play a central role in eliciting/regulating intestinal immune responses and cytokine levels, thus interfering in many aspects with Ado pathways. The importance of such interference is evident considering that 10^{13} to 10^{14} bacterial cells are present in the human gut, outnumbering the total of eukaryotic cells in the host about 10-fold. Collectively, the genome of distal gut microbes contains over 100 times more genes than the human genome [259] comprising an incredible diversity [260]. The gut microbiome has been functionally linked to a wide range of processes related to nutrition and immunity, determining the health status of the host in conditions such as obesity, diabetes and inflammatory bowel disease [261, 262, 263]. Also important, the presence of intestinal pathogens and infectious diseases had been reported to interact with Ado pathways and several examples of mutual influence were investigated. Therefore, intestinal bacteria and infection are to be considered as players in the Ado signaling modulation scenario and here we discuss some examples of the roles they can perform.

Several diverse mechanisms involving Ado signaling can link distinct Ado receptor subtypes to an increase in the efficiency of bacterial infection. For example, the anti-inflammatory effects of A_{2A} AR activation

in Th cells in the gastric mucosa has been reported to cause increased colonization and persistence of *Helicobacter pylori*, an effect driven by expression of CD73 in regulatory Th cells [264, 265]. A_{2B} AR, in turn, was shown to be responsible for extracellular Ado-induced delay in intracellular *Chlamydia trachomatis* development in epithelial cells, also leading to persistent infection [266]. In intestinal epithelia, A_{2B} AR signaling stimulates fibronectin expression, enhancing adhesion and invasion of *Salmonella typhimurium* [267].

Gastrointestinal infections represent the most common cause of diarrhea, as a consequence of electrolyte imbalance and increase intestinal secretion. Ado has been implicated as a mediator of those effects through activation of A_{2B} AR and subsequent induction of ion secretion. The stimulation of Cl^- secretion in intestinal epithelial cells (T84) by PMN, for example, is a result of the conversion of PMN-derived 5'AMP to Ado by the nucleotidase CD73 expressed by epithelia [268]. Accordingly, a more recent study using a high-throughput gene expression analysis, identified higher expression of the A_{2B} AR gene (*Adora2b*) as one of the susceptibility factors in a model of infectious colitis induced by *Citrobacter rodentium*, pointing severe diarrhea as a cause of mortality in this model [269]. In enteropathogenic *Escherichia coli* (EPEC) infection (but not nonpathogenic *E. coli*) the killing of host cells results in ATP release, which is converted to Ado generating a secretory response. Moreover, EPEC is able to activate PLC and the consequent release of CD73 from T84 cell surface, responsible for the formation of Ado and the Cl^- secretion observed. The results indicate an important role for Ado in EPEC-induced diarrhea [270, 271]. Interestingly, the same group had shown later that host-derived adenosine have direct effect on EPEC cultures, stimulating growth, changing adherence patterns and regulating virulence factors expression [272].

Given the critical importance of extracellular Ado levels in the control of immune responses, it is reasonable that pathogens develop strategies to manipulate Ado signaling during infection and evade host defenses. A study on *Mycobacterium avium* infected macrophages, using cDNA expression array to address infection-induced changes in gene regulation, identified a sustained decrease in Ado receptor expression (2.5-fold). In this model, in the absence of T-cells mediators, macrophages were not able to eliminate *M. avium*, however the significance of the modulation of this particular gene in bacterial growth and survival was not specifically assessed [273]. Another clear example of such strategies is a work that identified the surface protein adenosine synthase A (AdsA) as a key virulence factor in *Staphylococcus aureus*. It was shown that the generation of Ado by the 5'-nucleotidase activity of AdsA causes an inhibition of *S. aureus* killing by neutrophils in the blood of infected mice. A strain with a mutation in *adsA* presented reduced survival in the blood and was unable to form kidney abscesses, in contrast to the wild-type strain. The phenotype could be

rescued by complementation with *adsA* expressing plasmid, and by exogenous Ado addition. The protein was shown to be expressed by several other Gram-positive pathogens and is also involved in enhanced survival of *Bacillus anthracis* in rat blood [274].

Recent results from our group [275] showed that *E. coli* respond to human β-defensin stress by releasing extracellular Ado, a process that may be of great importance for local immunomodulation in the gut. β-defensins are produced by intestinal epithelial cells and are upregulated during inflammation [276, 277]. Thus, the production of local Ado in this context may represent a protection mechanism where the bacteria downregulate local inflammation, inhibiting effector immune cells via A_{2A} AR, for instance. This hypothesis would be consistent with the beneficial effects reported for probiotic *E. coli* Nissle 1917 (EcN) in IBD [278]. Indeed, one of the effects described for EcN is the induction of β-defensin production by epithelial cells [279, 280]. On the other hand, as discussed above, activation of AR in gut epithelium by luminal bacterial-derived Ado could also trigger pro-inflammatory responses, in this case implicating the bacteria as an agent of inflammation. Ado is indeed regarded as an important immunomodulator in the pathophysiology of IBD [281, 282]. Array analysis in colonic mucosal biopsies or PBMC of IBD patients shows gene expression changes in many purine genes including *ADORA3, ADORA2A*, *ADORA2B*, *NT5E* (*CD73*), and *ADAR* [283].

Outlook

The progress in the elucidation of the complex regulation mediated by adenosine made over the last decades and the discovery of compounds of steadily increasing potency and selectivity opened many possibilities to treat forms of inflammatory bowel disease. However, the special situation of the gut with its myriads of highly diverse bacteria and a constant supply of bioactive compounds derived from our diet makes it a unique environment in our body. We have shown that several bacteria interact with the gut and the diet, complicating Ado regulation even further. But at the same time, this highly dynamic system offers also novel approaches, where Ado is produced on site, overcoming the problem of the short half-life of adenosine and offering the possibility of fine-tuning of Ado activity by specific allosteric modulators. This approach may be further supported by bacteria producing high amounts of Ado and a diet rich in allosteric modulators, e. g. certain flavonoids, lipids or lignans. We are confident that such a holistic approach will help in the much needed better control of inflammatory disorders in the gut, such as IBD.

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